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Marco Barchi · Massimo De Felici *Editors*

Germ Cell Development

Methods and Protocols

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Preface

In sexually reproducing organisms, germ cells (GSs) bear two major responsibilities: the maintenance of the species through the transmission of hereditary traits, and species evolution through the reshuffling of the genome. During early embryonal development, GCs develop from staminal GC precursors that give rise to primordial GCs (PGCs). The latter, by replicating and migrating, reach the primitive gonads where they will develop into male or female gamete precursors (spermatogonia or oogonia, respectively) depending on the assortment of the sex chromosomes complement (XY or XX, respectively) of the somatic gonadal cells. To reconstitute the diploid chromosome number at each fertilization, GCs undergo meiosis. The functional role of meiosis is to reduce the genome complement by half, which is accomplished by sequentially executing one round of DNA replication followed by two rounds of chromosome segregation. Between DNA replication and the first meiotic division in most organisms, paternal and maternal (homologous) chromosomes undergo homologous recombination. The latter is key for the reshuffling of the genome and proper segregation of homologous chromosomes at anaphase I. Importantly, recombination is initiated by a programmed wave of double strand breaks (DSBs) that occurs along chromosomes in specific regions called "hotspots." Precise positioning of hotspots is essential for successful recombination; therefore, alteration of the mechanisms determining hotspots may cause defects in pairing and synapsis of the homologous chromosomes, which may lead to sterility or to unbalanced segregation of chromosomes at anaphase I. In the latter case, when it is compatible with life (mostly it is the case of sex chromosomes missegregation), it causes in humans the onset of syndromes, such as Klinefelter (47, XXY), in which genetic-driven phenotypic manifestations are being studied.

Thus, GCs are unique in their ability to transfer genetic information across generations. As such, proper understanding of the mechanisms underpinning their origin, regulation, and differentiation is key to understanding alterations of these processes, which are paramount to the health of organisms and the survival of species. Until recent years, germ cell research was limited by the lack of in vitro models recapitulating male or female germ cell development. However, more recently, research in the field of stem cell biology has allowed an impressive acceleration in the expansion of new techniques for in vitro reconstruction of spermatogenesis and oogenesis, both in animal models and humans. In addition, the development of somatic cell reprogramming techniques makes it possible today to obtain stem cells from patient-derived somatic cells, providing a tool for molecular studies of human diseases, including the syndromes resulting from defects of developing germ cells.

These advances promise to provide new insight into the understanding of basic biological aspects of germ cell biology, as well as the opportunity for in vitro manipulation of germ cells for toxicology studies and for genetic intervention where ethically appropriate.

In this volume, we have collected well-established protocols for the isolation, purification, and establishment of in vitro GC systems at different stages of development in different organisms, including chickens, mice, rats, and humans. In addition, we describe cutting-edge analytic and informatic tools to study germ cell development at the single cell level and meiotic recombination. The volume is divided into four sections. The first section is devoted to methods for the isolation, purification, and transfection of PGCs, as well as methods for the purification of GCs from the fetal human testes and the adult testes of mice. Section II is

divided into two parts. The first part describes cytological methods for establishing in vitro systems for induction and culturing of PGCs from PGC-like cells and induction and editing of the mouse germline. The second part is dedicated to the isolation and culture of mammalian ovaries and oocytes. Section III describes a protocol for reprogramming somatic cells into pluripotent stem cells (iPS) from patients with Klinefelter syndrome. The last section focuses on descriptions of bioinformatic pipelines for studying GC development and recombination initiation and a tool for image analysis of chromosome structure and recombination events occurring in prophase I of meiosis.

The editors thank the many authors of these chapters for their willingness to share their protocols and expertise.

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Part I

Isolation and Purification of Primordial (and) Germ Cells

Chapter 1

Isolation and Purification of Viable PGCs from Mouse Embryos

Massimo De Felici

Abstract

In all organisms with sexual reproduction, sperm and oocytes derive from embryonic precursors termed primordial germ cells (PGCs) which pass on genetic information to subsequent generations. Studies aimed to unravel PGC development at molecular level in mammals can be traced at the early 1980s and were hampered by the difficulty in obtaining both sufficient quantities and purity of PGCs. For many laboratories, the isolation and purification methods of PGCs at different stages from embryos are the most shortcut and affordable tool to study many aspects of their development at cellular and molecular levels. In the present chapter, I focus on immunomagnetic cell sorting (MACS) and fluorescence-activated cell sorting (FACS) methods used in my laboratory for the purification of mouse PGCs from 10.5 to 12.5 dpc embryos before their differentiation in oogonia/oocytes in female and prospermatogonia in male.

Key words Primordial germ cells, MiniMACS, SSEA-1, CD-15, Gonadal ridges, AGM, FACS, OCT4, eGFP

1 Introduction

In the mouse embryo, it is now well established that PGC precursors are specified from a small group of proximal epiblast cells and become identifiable as alkaline phosphatase (AP)- and PRDM1-positive cells at the base of allantois in the extraembryonic mesoderm at around 7.5 days post coitum (dpc) (for a review, see Ref. [1]). It is not clear if at this time these cells are part of a heterogeneous population of pluripotent stem cells destinated to give rise to various lineage (for a review, see Ref. [2]). In any case, during gastrulation, PGCs can be traced within the developing hindgut endoderm at 7.75 dpc and into the aorta-gonad-mesonephros (AGM) region at 9.5–10.5 dpc. Between 11.5 and 12.5 dpc, PGCs become germline determined and initiate their sexual differentiation into prospermatogonia (male) and oogonia (female), instructed by the surrounding microenvironment of the gonadal

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niche. From about 13.5 dpc, prospermatogonia undergo a temporary GO mitotic arrest while oogonia enter the prophase of meiosis I as primary oocytes.

PGCs representing a key cell type for analysis of germ-soma differentiation as well as pluripotency at the molecular level. Moreover, in the last years, the possibility to generate primordial germ cells–like cells (PGCLCs) in vitro able to generate male and female gametes from pluripotent stem cells of various mammalian species including humans (for a review, see Ref. [3]) increased exponentially the interest about such unique cell type. Today single-cell genome and transcriptome sequencing methods enabling the simultaneous molecular analysis of hundreds, or thousands of cells, inside heterogeneous populations are considerably increasing our capability to analyze the PGC genome and transcriptome (for example, see Refs. [4–6]. Nevertheless, for many laboratories, the isolation and purification methods of PGCs at different stages from embryos remain the most shortcut and affordable tool to study many aspects of their development at cellular and molecular levels.

Historically, the isolation and purification of PGCs from mouse embryos between 8.5 and 12.5 dpc were attempted using cellular, molecular, and transgenic approaches in parallel with the progress of technologies as summarized in Table 1. At these stages, PGCs are characterized by motility capability and active proliferation; they are for the most part single or form small clusters and do not form firm contacts with the surrounding extracellular matrix molecules and surrounding somatic cells. Both female and male mouse PGCs express surface markers such as tissue-nonspecific alkaline phosphatase (TNAP), the carbohydrate epitopes bearing terminal Lewis X determinants (Stage-specific embryonic antigen-1 (SSEA-1) and the antigenic determinant of monoclonal antibody EMA-1, E-cadherin, and the c-Kit and CXCR4 receptors. In principle, antibodies against all these surface molecules can be used to purify PGCs at these stages. Moreover, PGCs express several genes typical of pluripotent stem cells such as *Pou5f1* (*Oct4*), *Sox2*, *Nanog* and of the germline such *Nanos3*, *Dazl* and *Ddx4* (*Vasa*). Therefore, PGCs can be purified also from embryos of transgenic mice carrying reporter genes under the control of such genes (for example, see Refs. [12, 13]).

In the present chapter, I focus on immunomagnetic cell sorting (iMACS) and fluorescence-activated cell sorting (FACS) methods used in my laboratory for the purification of mouse PGCs from 10.5 to 12.5 dpc embryos using anti-SSEA-1 (CD15) MicroBeads and the transgenic mouse line Oct4ΔPE: eGFP (OG2), respectively.

Table 1
Timetable of the methods used for the first time to isolate and purify mouse PGCs

Heath J. K. (1978) [7] (Cell 15, 299–306)	Manual dissection of gonadal ridges
De Felici M. and McLaren A. (1982) [8]	Percoll gradient
McCarrey J. R. et al. (1987) [9]	Immunopurification by FACS
Massimo De Felici and Maurizio Pesce (1995) [10]	Immunoaffinity adhesion
Pesce, M., and De Felici, M. (1995) [11]	Immunopurification by MACS
Abe K. et al. (1996) [12] (Dev. Biol. 189, 468–472)	Transgenic $TNAP^{\beta\text{-}geo}$ PGCs by FACS-gal
Szabò P. E. et al. (2002) [13]	Transgenic Oct4ΔPE:eGFP PGCs by FACS
Mayanagi T. et al. (2003) [14]	Nicodenz gradient

2 Materials and Instruments

2.1 Isolation of PGCs by MiniMACS

1. 10.5–12.5 dpc pregnant females of desired genetic background (*see* **Note 1**).
2. 1xPBS, sterile, for dissecting.
3. Hanks' Balanced Salt Solution (HBSS) or M2 (Sigma-Aldrich).
4. Trypsin/EDTA solution (0.025% trypsin and 0.01% EDTA) in PBS (Gibco).
5. DNase (1 mg/mL) solution (STEMCELL Technology).
6. Culture-tested fetal calf serum (FCS).
7. Poly-L-lysine-coated glass coverslips: soak coverslips in 200 μg/mL poly-L-lysine in distilled water for at least 1 h at r. t., wash three times in distilled water and air-dry.
8. Chromogenic solution for alkaline phosphatase (AP) detection: immediately before use dissolve 1 mg/mL fast red TR salt or fast blue BB salt (Sigma-Aldrich) in distilled water and add 40 μL/mL naphthol AS-BI or AS-MX alkaline solution (Sigma-Aldrich).
9. Fine scissors, dissection forceps and No. 5 watchmaker's forceps, sterile.
10. Eppendorf or equivalent single channel 10–100 μL pipette.
11. 18- and 23-gauge needle and 1 mL syringe for tissues dissection.
12. 5 mL test tubes and 1.5 and 2 mL Eppendorf tubes.
13. Stereomicroscope (the author uses a Wild M5a from Leica, maximum magnification 100×, equipped with both incident and transmitted light sources, the latter preferably with a movable mirror).

14. Anti-SSEA-1 (CD15) MicroBeads (130-094-530, Miltenyi Biotech).
15. MACS buffer: phosphate-buffered saline (DPBS) without Ca^{2}+ and Mg^{2}+, with 0.5% bovine serum albumin (BSA), and 2 mM EDTA (keep buffer cold at 2–8 °C and degas before use, as air bubbles could block the column).
16. MiniMACS™ Separator (Miltenyi Biotech).
17. MACS MultiStand (Miltenyi Biotech).
18. MACS MS separation columns (Miltenyi Biotech).
19. Class II Biological Hazard laminar flow hood (*see* **Note 2**).

2.2 Isolation of PGCs from OG2 Mice by FACS

Materials listed in the previous section from 2 to 11 are required also in this procedure.

1. 10.5–12.5 dpc pregnant CD1 females previously mated with OG2 homozygous males (*see* **Note 3**).
2. Hanks' Balanced Salt Solution (HBSS) or M2 (Sigma-Aldrich) plus 1 mg/mL BSA.
3. 5 mL FACS tubes compatible with flow cytometer.
4. Sorting flow cytometer (*see* **Note 4**).

3 Methods

3.1 Dissection of AGM and GRs

At 10.5 dpc, the region dissected away from the embryo (hindgut, dorsal mesentery, and gonadal ridges) corresponds to the region termed AGM (aorta, gonad, mesonephros) containing the most part of migratory PGCs (about 1000/embryo, while at 11.5 and 12.5 dpc PGCs are obtained from the gonadal ridges (GRs, about 3000/and 8000/embryo) ([15], and our unpublished data). Embryonic sex can be determined only by PCR of genomic DNA using specific primers for 10.5 and 11.5 dpc samples (*see* **Note 5**) or by assessing gonad morphology for 12.5 dpc (Fig. 1).

The procedure described in the following steps is routinely used in the author's laboratory, but it can be varied according to the operator's preference and experience.

Steps

1. Lay the freshly killed animal on its back and soak the abdomen with 70% alcohol. CD1 mice, usually employed in the author's laboratory, were housed and mated under conventional conditions (*see* **Note 1**). Positive plug test is considered 0.5-day *post coitum*. The developmental stage of embryos can be precisely

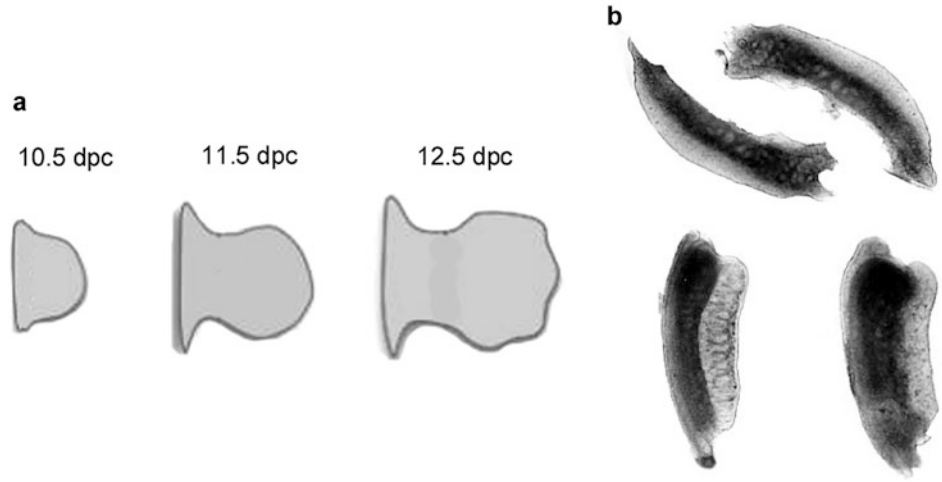

Fig. 1 (**a**) Morphology of limb buds of the mouse embryo between 10.5 and 12.5 dpc. (**b**) Sex indifferent 11.5 dpc gonadal ridges (upper), 12.5 dpc testis (right), 12.5 dpc ovary (left)

determined by observing the morphology of the hind limb bud (Fig. 1).

2. Pinch up the skin of the abdomen region between thumb and forefinger of both hands and pull it toward the head and tail until the abdomen is completely exposed and the fur is well out of the way.
3. Using forceps and fine scissors, cut the body wall, push the coil of gut out of the way, and locate the two horns of the uterus.
4. Remove the uterus intact by cutting across the cervix and the two uterotubal junctions and trim away the fat and mesentery with fine scissors.
5. Transfer the uterus in a petri dish in a volume of PBS sufficient to completely immerse the tissues and remove any residual fat and mesentery.
6. Transfer the uterus to a second dish of fresh medium and dissect the embryos with attached placenta by cutting the anti-mesometrial wall of the uterus with the tips of scissors. Remove the embryo's membranes by cutting at the junction of the placenta with watchmaker's forceps.
7. Cut with scissors the anterior half of the embryo just below the armpits and transfer the posterior half to a dish of fresh medium,
8. Under a stereomicroscope, turn back such posterior half and hold it with No. 5 watchmaker's forceps while making a cut along the ventral midline.
9. For 10.5 dpc embryos, using the tips of No. 5 watchmaker's forceps, remove viscera paying attention does not remove the

dorsal mesentery and the hindgut. Peel away these and associated GRs (gonad + mesonephros) from the dorsal region of the embryo and transfer them into HBSS or M2 (Fig. 2).

10. For 11.5 and 12.5 dpc embryos, scoop out liver and any remnants of intestine with No. 5 watchmaker's forceps and identify the GRs lying on the dorsal wall of the embryos on either side of the abdominal aorta. Slide the tips of the No. 5 watchmaker's forceps behind each GR and remove them from the embryo.
11. Transfer the GRs to HBSS or M2 and separate the gonads from mesonephroi using 25G needles.

3.2 Isolation of 10.5–12.5 dpc PGCs by MiniMACS

In this method, PGCs are separated from the other cell types by positive selection. This means that they are labeled with superparamagnetic microbeads conjugated to highly specific antibodies. During separation, the magnetically labeled PGCs are retained within a column, while unlabeled cells flow through. After a washing step, the column is removed from the magnetic field of the separator, and PGCs are eluted. Positive selection can be performed by direct or indirect magnetic labeling. The following procedure describes a direct labelling of PGCs by anti-SSEA-1 MicroBeads purchased from Miltenyi Biotechnology.

The simplicity and low cost make the MiniMACS separation procedure the method of choice for rapidly obtaining reasonable numbers of purified germ cells and somatic cells that can be used immediately for biochemical studies or be grown in suitable *in vitro* culture systems. In 2–3 h of work, one person should be able to isolate from 10 embryos of 10.5, 11.5, and 12.5 dpc (a reasonable yield from a pregnant CD1 female) about 5000–7000 (purity 70–80%), 10,000–15,000, and 40,000–50,000 PGCs (purity 85–95%), respectively (Fig. 3). The presence of supermagnetic microbeads coated with antibodies on the cell surface might be harmful for certain cell functions. However, we cultured PGC purified by MiniMACS for several days without any apparent deleterious effect (for example see Ref. [16]).

Steps

1. 10.5 dpc AGM (usually 10–20) or 11.5–12.5 gonads (usually 20–40) are transferred with 100 μL Eppendorf pipette in minimal amount of medium into a 1.5 mL Eppendorf tube containing 0.5 mL of trypsin-EDTA.
2. After about 5 min incubation at 37 °C carefully remove the solution and wash the tissues twice with 1 mL of HBSS or M2 + 5% FCS, allowing them to settle on the bottom of the tube. Leave the tissue in about 100 μL of medium and pipette up and down with the Eppendorf pipette for their complete

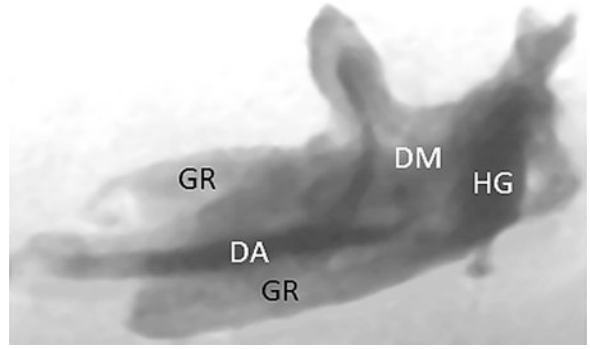

Fig. 2 Dissected AGM (aorta, gonad, mesonephros) from 10.5 dpc embryo

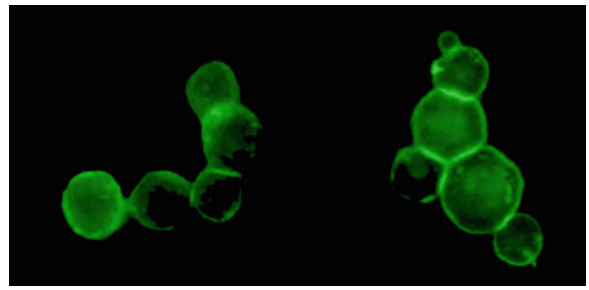

Fig. 3 Mouse 12.5 dpc PGCs stained with anti-SSEA-1 (CD15) FITC antibodies

dispersion (about 10 strokes are usually sufficient). Ensure a single cell suspension by passing through an 18- and 23-gauge needle.

3. Add 1 mL of MACS buffer containing 10 μg/mL DNase, centrifuge at 500 ×*g* for 10 min at 4 °C. Remove supernatant and resuspend the pellet in 80 μL of buffer with the Eppendorf pipette (*see* **Note 6**).
4. Add 20 μL anti-SSEA-1 MicroBeads and incubate with continuous agitation for 45–60 min at 4 °C.
5. Dilute the cells to a final volume of 1.5 mL with MACS buffer and transfer on top of an AS column previously washed, filled with the buffer, and placed in the magnetic field of a Mini-MACS separation unit (Fig. 4).
6. Collect the effluent as SSEA-1 negative cells (somatic cells) and wash the column twice with 1 mL buffer into 5 mL collecting tube.
7. Remove the column from the magnetic field and, using the plugger supplied with the column, flush out with 1.5 mL buffer the cells retained by the column (SSEA-1 positive PGCs) in a 2 mL Eppendorf tube.

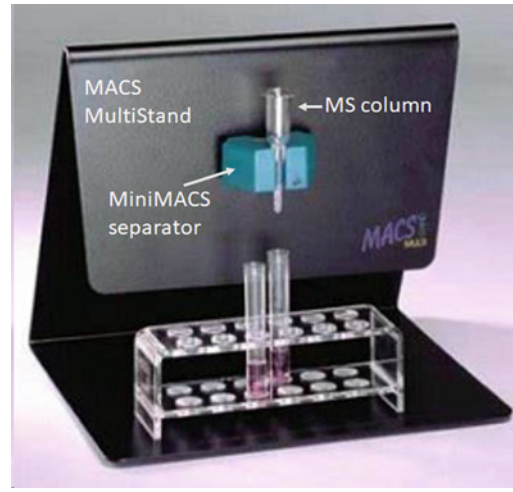

Fig. 4 A MiniMACS separation unit form Miltenyi Biotec

8. To assess PGC purity, a small sample of the cell suspension (20–30 μL) can be transferred onto a polylysinated coverglass and stained for AP.

3.3 Isolation of 10.5–12.5 dpc PGCs by FACS

At these developmental stages, FACS can be used to purify viable PGCs labeled with antibodies anti-SSEA-1 (CD15) or other surface markers (e.g., EMA-1, E-cadherin, Kit) conjugated with various fluorochromes commercially available from several companies. Alternatively, PGCs can be isolated from transgenic mice expressing reporter genes under the control of early PGCs genes (e.g., *Pou5f1*, *Prdm1*, and *Dppa3*). Here we describe PGC purification from OG2 transgenic mice (Oct4ΔPE:eGFP) expressing enhanced green fluorescent protein (eGFP) under the control of the *Poufl* (*Oct4*), promoter and distal enhancer [17] (Fig. 5a).

Steps

1. After **steps 1** and **2** of Subheading 3.2 *Isolation of PGCs by MiniMACS*, add 1 mL of HBSS plus 1 mg/mL BSA containing 10 μg/mL DNase, centrifuge at 500 ×*g* for 10 min at 4 °C. Remove supernatant, and resuspend the pellet in 0.5 mL of HBSS with the Eppendorf pipette (*see* **Note 6**).
2. Transfer the cell suspension into a FACS tube on ice and proceed to flow cytometer.
3. Cell debris and dead cells are excluded from the analysis based on scatter signals and propidium iodide fluorescence. Viable cells are gated based on forward scatter (FSC) vs. side scatter (SSC) profile. eGFP vs. FSC will delineate eGFP-positive PGC

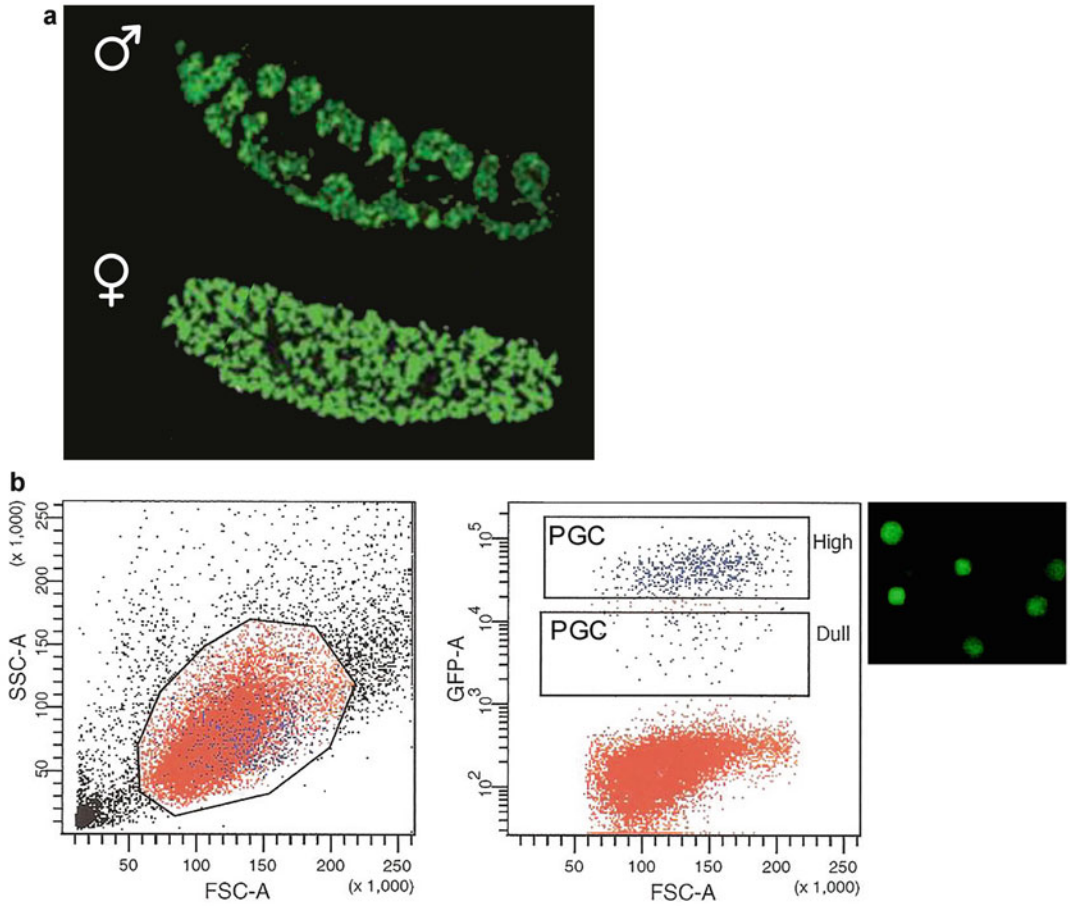

Fig. 5 (**a**) 12.5 dpc ovary and testis from OG2 mouse. Note a spotty pattern of fluorescence in the ovary, whereas a wavy pattern reflecting testicular cords formation is visible in the testis. (**b**) Representative FACS profile of disaggregated gonads isolated from 12.5 dpc CD1/OG2 embryos. Right: FSC-A/SSC-A dot plots of the cell suspension showing the cell population selected for fluorescence analysis. Left: GFP-A/FSC-A showing two eGFP positive PGC populations to be sorted displaying high or dull fluorescence; Inset: a group of PGCs positive for eGFP-OCT4

population of cells and the remaining eGFP negative cells as somatic cells (Fig. 5b), (*see* **Note 7**).

4. To assess PGC purity, a small sample of the cell suspension (20–30 μL) can be transferred onto a polylysinated coverglass and observed onto a fluorescence phase contrast microscope. The purity of sorted PGC population was usually >98%.
5. Sorted cells can be used immediately for biochemical studies or be grown in suitable *in vitro* culture systems.

4 Notes

1. Use the mouse strain of your choice, although we have fond that outbred strains such as MF1 and CD1 mice give larger numbers of PGCs than some inbred strains (e.g., BalbC). The procedure described here and the mean number of PGCs obtained are from CD1 embryos. Moreover, consider that PGC number can vary considerably among embryos.
2. The safety cabinet is necessary to carry out the whole procedure if sterility must be maintained.
3. The OG2 mice can be purchased from Jackson Laboratory, Cat # 003715. To ensure large litter sizes and large numbers of germ cells per gonad homozygous OG2 male can be mate with CD1 females. For the present protocol, usually, a minimum of two litters is pooled.
4. For the present procedure, cell sorting was performed on a FACSAria II (Becton Dickinson) interfaced with the FACS-DiVa 6.0 Software.
5. If sexing is required, each embryo is dissected into a 24-well plate containing warm DMEM+10% FCS and a tail tip is taken for PCR sexing. We used genomic PCR of mouse Ube1 [18] or *Xlr* and *Sly* [19] genes. Once results are available (2–3 h) embryos are pooled based on sex.
6. For optimal performance, it is important to obtain a single-cell suspension. Check a small sample of the cell suspension under a microscope and if necessary, pass the suspension through a 23-gauge needle once more or filter through a 40 μm cell strainer (BD Falcon). The percent of PGCs in the cell suspension estimated by APase staining (*see* **step 8**) was less than 0.1% and approximately 5% and 30–35% at 10.5, 11.5, and 12.5 dpc, respectively.
7. Although the most part of PGCs showed high eGFP, we and others observed a little population of weakly eGFP positive cells. At 10.5 and 11.5 dpc, these latter represented between 9% and 12% of the whole eGFP positive cells. Hypotheses about such minority PGC population are discussed in [20]. Although FACS is the method of choice for cell sorting when high cell purity is needed, it suffers, however, from (sometimes very) high losses, requires trained personnel, and is expensive to own and operate. The estimation of PGC recovery under the present conditions varied from 65% to 70%. This means that from 10 embryos of 10.5, 11.5, and 12.5 dpc heterozygotes CD1/OG2 females, about 5000, 15,000, and 35,000 PGCs can be obtained, respectively. Finally, PGCs

isolated by FACS from transgenic fluorescent proteins should not have any potential adverse effect caused by antibodies bound to their cell surface.

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Chapter 2

Purification and Transfection Methods of Chicken Primordial Germ Cells

Luiza Chojnacka-Puchta and Dorota Sawicka

Abstract

Primordial germ cells (PGCs) play a special role in the vertebrate life cycle since they are the precursors of germ cells through which the genome is passed to the next generations. PGCs are found in different locations and variable numbers in the chick embryo, as in other species, depending on the developmental stages. Here, we describe in detail a method based on the Percoll gradient, routinely used in our laboratory, allowing us to obtain from blood and gonad anlages significant numbers of viable PGCs which can be successfully cultured or efficiently genetically modified.

Key words Chicken primordial germ cells, Percoll density gradient, Electroporation, Lipofection, Immuno-cyto-staining

1 Introduction

Primordial germ cells (PGCs) play a special role in the vertebrate life cycle since they are the precursors of ova and spermatozoa through which the genome is passed to the next generations.

In birds the germline is established early in the embryo, likely by maternal specification [1]. In the chick embryo, PGC precursors are initially detected as a scattered pattern in the area of pellucida, the central region of the Eyal-Giladi and Kochav stage X embryo [2]. From this position, these cells are translocated to the anterior extraembryonic region called the germinal crescent, where they, now termed PGCs, are incorporated into the forming extraembryonic vascular network [stages 8–10 according to Hamburger and Hamilton (HH 8–10)] [3], and start to circulate within the bloodstream (stage HH 11). Reaching the gonadal anlage region, PGCs exit the blood vessels and colonize the gonads (stage HH 16). About 30 PGCs are found at X stage embryo in the area of pellucida, 200–250 in the germinal crescent, and more than 1000 are in the gonads after embryo incubation for 7 days (stages 26–29 HH)

Marco Barchi and Massimo De Felici (eds.), *Germ Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 2770, https://doi.org/10.1007/978-1-0716-3698-5_2,

[5]. Considering such dynamics localization, at the 10 HH stage, PGCs can be obtained from blastoderm, at 13–17 HH stages (2.5- to 3-day-old embryos) from blood and at stages 26–29 HH stages (5.5- to 6-day-old embryos) directly from the embryonic gonads. PGCs isolated from these stages have been successfully used for transfection [4, 5] or cryoconservation [6].

Purification methods for chick PGCs range from density gradients using Nycodenz [7], Ficoll [8], or Percoll [9] to immunomagnetic cell sorting (MACS) and fluorescence-activated cell sorting (FACS) [11]. Chick PGCs were purified from blood using ammonium chloride-potassium (ACK) lysis buffer to eliminate red blood cells, followed by in vitro culture [10]. Using such methods, the purity of PGCs overcame 90% at best.

Chick PGCs can be identified based on morphological criteria, i.e., large size and presence of a large amount of glycogen in the cytoplasm [12]. Glycogen granules can be evidenced also by periodic acid-Schiff (PAS) staining [13, 14]. Moreover, chick PGCs were stained for alkaline phosphatase activity, a classical PGC marker also in mammalian species [15], and for many immunological epitopes for surface glycoproteins. The best known and characterized is SSEA-1 (Stage-specific Embryonic Antigen-1) [16], which is effective only after stage 10 HH. Other immunological markers are EMA-1 (Epithelial Membrane Antigen) [17] expressed from the crescent stage [18] and MVH (Mouse Vasa Homolog) [19]. Interestingly, all these immunological markers are common to mammalian PGCs (*see* Chap. 1, by M. De Felici, in the present book)

Here we describe a simple and rapid method based on Percoll density gradient suitable to purify chicken PGC from blood and early embryonic gonads that can be successfully transfected using the electroporation/lipofection method for germline manipulation. Details are in Table 1.

Table 1
Average numbers of chicken embryos, PGCs isolated from blood and gonads, purity and efficiency of transfection methods

Type of PGCs	Number of embryos[a]	Number of total cells after isolation	Number of total after Percoll purification	Purity of PGCs (%)	Electroporation efficiency (%)	Lipofection efficiency (%)
bPGCs	60	11.6×10^6	1.76×10^6	64	75	50
gPGCs	30	4.53×10^6	7.56×10^5	N/A	73	39

Data obtained in our experiments

[a]The number of embryos used to isolate PGCs depends on the quality of embryos and operator skills. Some of them can be unfertilized or undeveloped

2 Materials

2.1 Reagents

1. Freshly fertilized eggs.
2. 0.2 M EDTA: Prepare 100 mL of 0.2 M EDTA by solving 7.44 g EDTA in 100 mL distillated water. Mix and adjust pH 7.4 with NaOH. This solution must be autoclaved. Prepare 10 mL of 0.1 mM EDTA by dilution of 5 μL 0.2 M EDTA in 10 mL 1× PBS (without calcium or magnesium) and mix.
3. 0.25% trypsin EDTA: Dilute 40 μL 0.25% trypsin EDTA solution in 960 μL OptiMEM I to obtain 0.01% trypsin; make fresh every time.
4. Antibiotic: Penicillin/streptomycin (10,000 U/mL).
5. OptiMEM I supplemented with 5% FBS. For 50 mL: add 2.5 mL FBS to 47.5 mL of OptiMEM I. Store at 4 °C.
6. Percoll 100% to prepare density solutions: 50%, 25%, and 12.5%. Make the steps by diluting the 100% Percoll, as shown in Table 2. All solutions are freshly made; phenol red is used to provide visual contrast for different phases.
7. Culture complex medium: OptiMEM I, 2% CS—chicken serum, 10% FBS—fetal bovine, 20 ng/mL bFGF—basic fibroblast growth factor, 9 ng/mL mLIF—murine leukemia inhibitory factor, 5 ng/mL hSCF—human stem cell factor, 1% antibiotic (and selective antibiotic).

2.2 Equipment

1. Egg incubator.
2. Cell tram air/oil pump with capillary holder and fine glass micropipette (90 × 135 mM).
3. Dissection microscope (a binocular stereoscopic dissection microscope is preferred).
4. Centrifugator with swinging-bucket rotor and buckets.
5. CO_2 incubator (5%).
6. Cell counter/hemocytometer.

Table 2
Steps to prepare Percoll solutions

Step	Percoll final concentration (%)	OptiMEM I with 1% FBS (mL)	100% Percoll (mL)
1.	50	10	10
2.	25	15	5
3.	12.5	17.5	2.5

The amounts of medium OptiMEM I with 5% FBS and Percoll are calculated for a final volume of 20 mL

7. 15 mL tubes, 1.5 mL Eppendorf tubes.
8. Inverted/fluorescent microscope.
9. 4-well plates, petri dishes.
10. Electroporator, 0.4 mL cuvettes.

3 Methods

Prepare a workplace, where cells will be isolated from chicken embryos by wiping the table with a disinfectant or 70% ethanol solution. Also wipe the elements of the stereoscope and cell tram air/oil pump. Perform all procedures using gloves and wear a lab coat.

3.1 Preparation Embryos to Isolate of PGCs

1. Incubation eggs.

Incubate freshly fertilized laid chicken eggs (egg or meat breed) at 37.8 °C and 60–62% relative humidity for 50–56 h to achieve chicken embryos at 14–16 HH and for 6 days to obtain chicken embryos at 28–29 HH (*see* **Note 1**).

3.2 Isolation of PGCs from Bloodstream (bPGCs)

1. Prepare 0.5 mL of 0.1 mM EDTA solution in 1 × PBS and supplement with 1% antibiotic. Prepare the cell tram air/oil pump and put the fine glass micropipette into a capillary holder. Fill the tip of the fine glass micropipette with the prepared solution. EDTA solution will prevent the drawn blood from clotting.
2. Take out chicken eggs from the incubator and wipe with a 70% solution of ethanol (*see* **Note 2**).
3. Gently transfer the contents of the egg to a sterile petri dish. Be careful not to damage the embryo. Transfer the petri dish under the stereoscope.
4. Take blood from the dorsal aorta of embryos (HH stages 14–16) using fine glass micropipette and suspend into EDTA solution to obtain suspension-contained bPGCs and blood counts (Fig. 1). Afterward, centrifuge cell suspension (400 × g, 5 min) and resuspend in medium (0.5 mL) Opti-MEM I supplemented with 5% FBS and 1% antibiotic.
5. Stain cells with 0.4% trypan blue (1:1) and count in a hemocytometer under an inverted microscope.

3.3 Isolation of PGCs from Gonads (gPGCs)

1. Prepare 1 × PBS containing 1% antibiotic at room temperature (RT).
2. Take chicken eggs from incubator and wipe with a 70% ethanol solution.

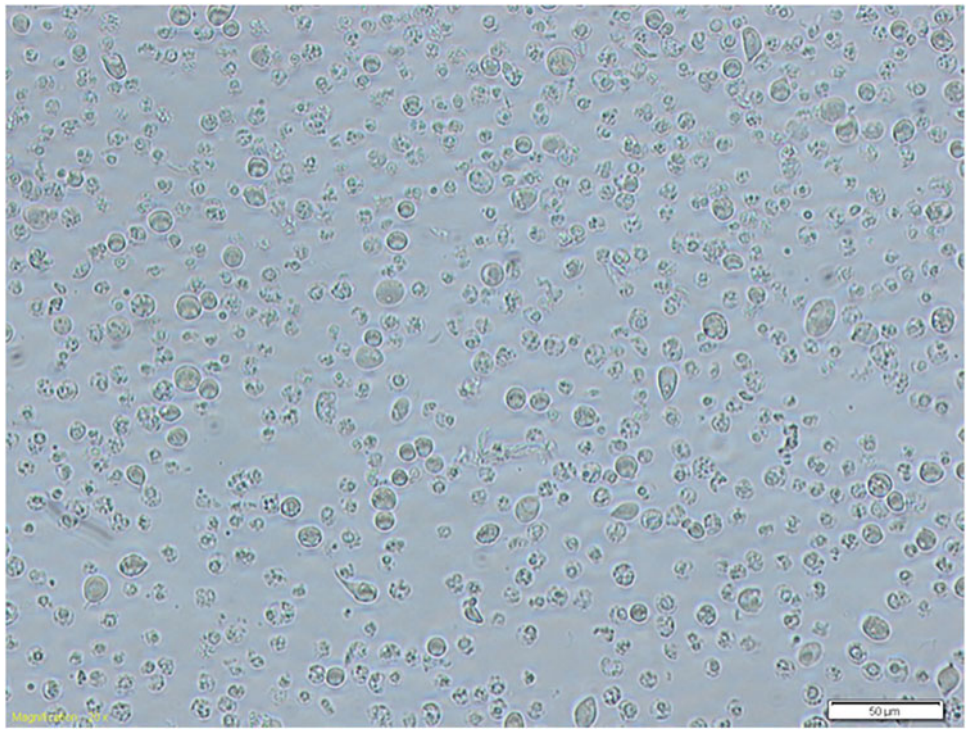

Fig. 1 Suspension of cells isolated from bloodstream of chicken embryos (HH stages 14–16) containing bPGCs and blood counts (20×)

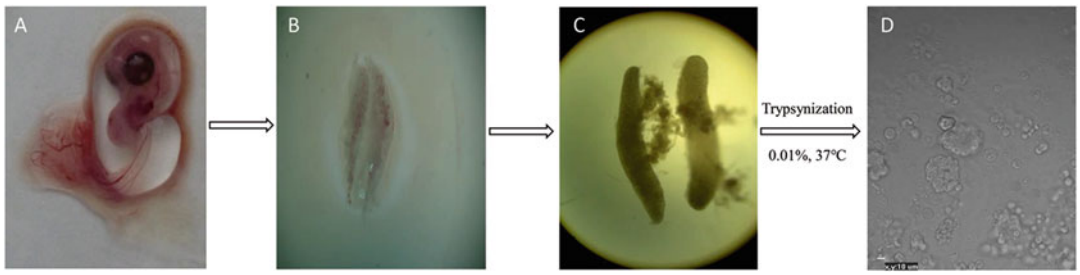

Fig. 2 Steps/scheme of isolation suspension of cells including gPGCs from gonads of 6-day-old chicken embryos. (**a**) chicken embryo on the 6-th day of incubation, (**b**) mesonephros with gonads (6.3×), (**c**) isolated gonads (40×), (**d**) suspension of cells containing gPGCs (20×)

3. Transfer the embryo (28–29 HH) (Fig. 2a) to a petri dish and purify the remains of the yolk by washing several times with 1 × PBS containing 1% antibiotic.
4. Transfer the embryo to a clean petri dish and isolate mesonephros under a stereoscope (Fig. 2b).
5. Cut along the inner edge of the mesonephros with a 0.4 mM blade to isolate gonads (Fig. 2c) and transfer gonads into the tube with 1 × PBS containing 1% antibiotic.

6. Mash the gonads with an insulin needle to increase the surface available for the enzyme.
7. Pipette vigorously and transfer all content to the new tube.
8. Centrifuge (400 × g, 5 min).
9. Add 1 mL of 0.01% trypsin and digest for 1 min at 37 °C in a heating block placed in a laminar airflow chamber. Inactivate the enzyme by adding 100 μL of FBS.
10. Centrifuge suspension of cells, including gPGCs (400 × g, 5 min), and resuspend in 0.5 mL medium OptiMEM I supplemented with 5% FBS and 1% antibiotic. The obtained cells are presented in Fig. 2d.
11. Stain cells with 0.4% trypan blue (1:1) and count in a hemocytometer under an inverted microscope.

3.4 Purification and Identification of PGCs

3.4.1 Percoll Purification

1. Prepare Percoll solutions: 50%, 25%, and 12.5% in medium OptiMEM I supplemented with 5% FBS. Form density gradient by layering 3 mL each of 50%, 25%, and 12.5% density of Percoll in a clear 15 mL conical centrifuge tube (*see* **Note 3**).
2. Suspend cells isolated from the bloodstream or gonads in 3 mL of OptiMEM I with 5% FBS.
3. Place suspension of cells (3 mL) on the top of the density gradient (Fig. 3) and centrifuge at 1.160 g for 20 min at 20 °C (*see* **Note 4**).
4. Gently collect bPGCs or gPGCs between 50% and 25% Percoll layers (*see* **Note 5**).
5. Wash PGCs twice in medium OptiMEM I (with 5% FBS, 1% antibiotic).
6. Resuspend the cell pellet in medium OptiMEM I (with 5% FBS, 1% antibiotic), and centrifuge at 400 × g for 5 min at RT.
7. Gently remove supernatant, cells are ready for further processing or other analysis. The purified PGCs isolated from the bloodstream or gonads are presented in Fig. 4.

3.4.2 Identification of PGCs

PAS Staining

1. Fix purified 10 μL suspension PGCs in 10 μL 4% glutaraldehyde for 5 min to dry.
2. Rinse twice in 1 × PBS.
3. Immerse cells in a 0.5% aqueous periodic acid solution for 5 min at room temperature.
4. Wash samples twice with 1 × PBS and incubate with Schiff's Reagent for 15 min at RT.
5. Wash samples twice with 1 × PBS and observed under a light microscope (Fig. 5).

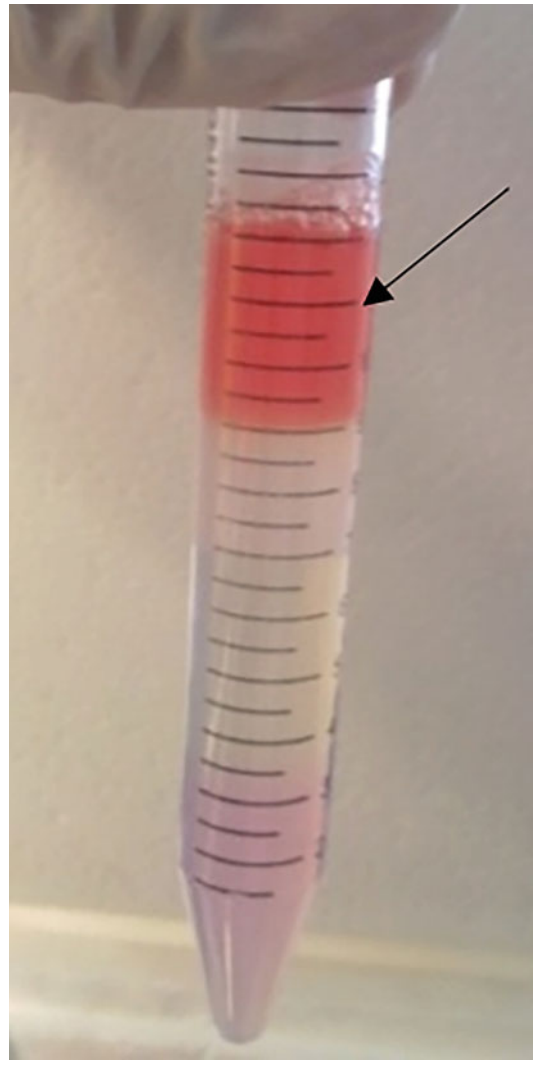

Fig. 3 Percoll gradient with a layer of cells isolated from the bloodstream of embryos at 14–16 HH contained suspension of bPGCs and blood counts (arrow). The bottom layer (50% Percoll solution) was stained by phenol red

Immunocytochemistry

1. Take 100 μL purified cells (approx. 4.5×10^5 cells) and add 1 mL 1 × PBS, centrifuge at 400 × g for 5 min, and resuspend in 1 × PBS to rinse culture medium.
2. The PGCs suspension fix in 1% paraformaldehyde for 15 min.
3. Wash in 1 × PBS and centrifuge at 400 × g for 5 min.
4. Block cells with 2.5% goat serum for 30 min at RT.
5. Centrifuge at 400 × g for 5 min.

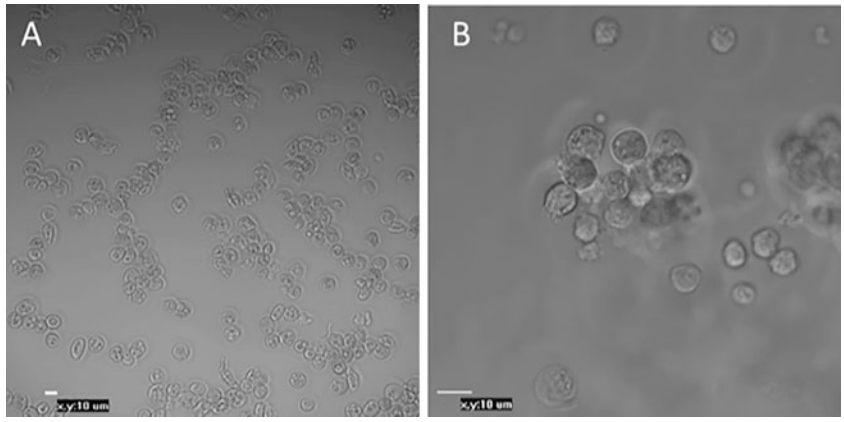

Fig. 4 The PGCs isolated from chicken embryos and purified via Percoll centrifugation density. PGCs are round cells ranging in diameter from 10 and 14 μM with a large nucleus and large, clearly visible vacuoles. (**a**) bPGCs (20×) and (**b**) gPGCs (40×)

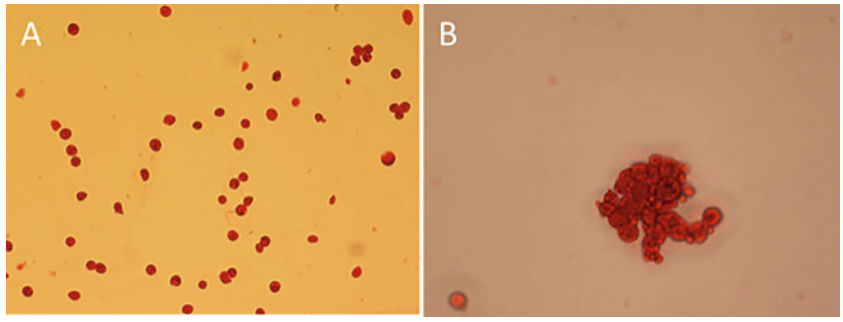

Fig. 5 The purified chicken PGCs stained with PAS. Positive results of staining (red- stained cells) indicate the presence of glycogen vesicles in the PGCs, which confirms their origin. (**a**) bPGCs (20×) and (**b**) gPGCs (40×)

6. Incubate cells with an antibody against a specific PGC marker (anti-stage-specific embryonic antigen 1 (SSEA-1)-FITC/PerCP) for 1 h according to the manufacturer's protocol (*see* **Note 6**).
7. Observe samples under fluorescent microscope at an excitation wavelength of 488 nM using a 515/30 filter or 682/33 (respectively for used fluorochromes) (Fig. 6).

3.5 Transfection Methods and Culture of PGCs

3.5.1 Electroporation

1. Centrifuge purified PGCs at 300 × g for 3 min and resuspend in medium without any additions (0.45 mL).
2. Add plasmid (20 μg) and transfer cells to 0.4 mM cuvette.
3. Perform electroporation under conditions: 200 V, 900 μF.
4. Transfer cells into the well of a 4-well plate and culture for 24 h (37 °C and 5%, CO_2).
5. After 24 h, replace the medium with a culture complex medium (*see* **Notes 7** and **8**).

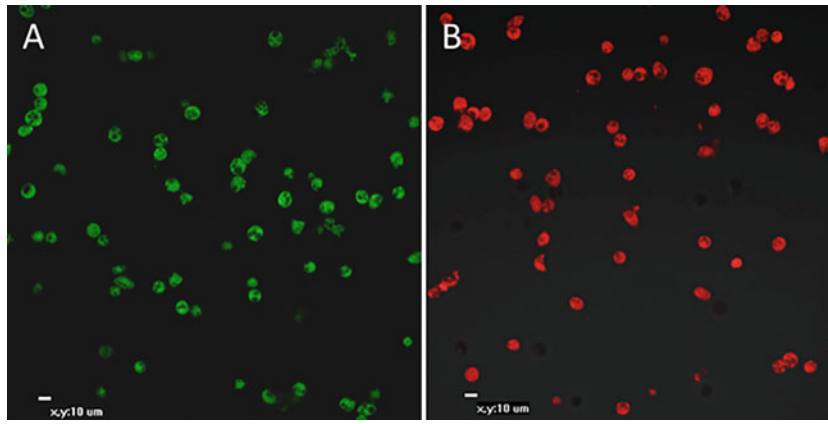

Fig. 6 The purified chicken PGCs subjected to immunofluorescence staining with antibodies for the specific cell surface antigen SSEA-1 after excitation wavelength of 488 nM. Staining of PGCs in green by anti-SSEA1-FITC (**a**) and red by anti-SSEA1-PerCp (**b**) confirmed their identity (20×)

3.5.2 Lipofection

1. Seed isolated and purified PGCs into 4-well plate.
2. All reagents: X-tremeGENE HP DNA Transfection Reagent, DNA and medium must be at RT (15–25 °C) (*see* **Note 9**).
3. Dilute DNA with medium OptiMEM I without any additions to a final concentration of 1 μg plasmid DNA/100 μL medium.
4. Transfer 100 μL of medium containing 1 μg DNA into a sterile tube.
5. Briefly vortex vial of Transfection Reagent and add 3 μL directly into the medium containing DNA from **step 3**. Mix gently.
6. Incubate for 15 min at RT.
7. Add transfection complex to PGCs in a dropwise manner. Gently shake the wells.
8. Incubate cells for 24 h.
9. Change medium to culture complex medium (*see* **Notes 7** and **8**).

4 Notes

1. Prepare the incubator with conditions: 37.8 °C and 60–62% relative humidity. At least 50 eggs for one isolation is needed. The most important thing is the temperature in the egg incubator. Plug in and turn on the incubator. Wait for the temperature to stabilize. Remember, the more eggs you have, the longer it will take to get to the temperature and stabilize. Because the warm air naturally rises to equalize the warm air in each part of the incubator, install a fan with air circulation

best. Also, we recommend using a hygrometer, a device that measures humidity in the air. To increase the humidity, put a container with distilled water into the incubator.

2. Place the eggs in a horizontal position and leave for 5–10 min, allowing the embryo to assume the appropriate position when it is released from the egg.
3. 100% Percoll stored at 4 °C should have pH 8.5–9.5. We find that Percoll solutions is best to prepare fresh each time, before density gradient centrifugation. They need to be used at room temperature. Use phenol red to provide visual contrast for different phases. Add drop-by-drop directly to each 3 mL of load density successively: 50%, 25% and 12.5% into clear 15 mL tube, avoiding bubble formation.
4. Carefully layer cells onto the top of the 12.5% layer immediately before centrifugation. Place the gradient into a swinging bucket rotor carefully and centrifuge.
5. Carefully remove the gradient and collect PGCs from above the red layer between 50 and 25 phases. To make collection easier, first, remove the top layer.
6. We used an antibody sc-21702 Santa Cruz Biotechnology, Santa Cruz, CA, USA.
7. Change medium every 3 days and culture cells prior to further use (injection or cryoconservation). We recommend using OptiMEM I medium with supplements: 2% CS, 10% FBS, 20 ng/mL bFGF, 9 ng/mL mLIF, 5 ng/mL hSCF, 1% antibiotic and selective antibiotic.
8. Check the effectiveness of electroporation PGCs based on viability if you use selective antibiotics or other markers. Add selective antibiotic after transfection to obtain stable transfectants. We used genetycin (G418) at a concentration of 50 μg/mL.
9. Perform lipofection of PGCs with the use of one of the commercially available lipofectants. In our laboratory we use the X-tremeGENE HP DNA Transfection Reagent (Roche) and lipofection protocol according to the manufacturer's instructions. We recommend the use of a 3:1 rate. This procedure does not require the removal of lipofectant (e.g., X-tremeGENE HP DNA Transfection Reagent or FUGENE 6 Transfection Reagent), which may be needed for the other reagents.

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Chapter 3

Isolation and In Vitro Propagation of Human Spermatogonial Stem Cells (SSCs)

Janmejay Hingu, Guillermo Galdon, Nicholas A. Deebel, and Hooman Sadri-Ardekani

Abstract

Preservation of human spermatogonial stem cells (SSCs) may be suitable for young male patients at risk of male infertility due to various causes, such as gonadotoxic treatment or genetic diseases. With optimal cryopreservation, cell viability can be retained to reestablish spermatogenesis in the future through autologous transplantation or in vitro differentiation of SSCs. This protocol outlines techniques to optimize the SSCs isolation and in vitro culture. With particular emphasis on the microscopic characteristics encountered, this protocol outlines a broader approach to processing tissues with varying morphologies among patients.

Key words Male infertility, Spermatogonial stem cell (SSC), Isolation, Propagation

1 Introduction

Infertility is the inability to conceive after 12 months of unprotected intercourse and remains a public health issue for both men and women with equal prevalence. While many etiologies contribute to an infertility diagnosis, the male factor is present in approximately 50% of infertile couples and is the sole causative factor in 20–30% of couples [1]. Azoospermia (absence of sperm on two consecutive centrifuged semen analyses) is the most severe form of male infertility, ultimately leaving couples with limited options for biological paternity in the setting of a negative microdissection testicular sperm extraction procedure (mTESE) [2]. Despite a negative sperm retrieval, testicular biopsies showing hypospermatogenesis, maturation arrest, or Sertoli cell only (SCO) may still present putative spermatogonial stem cells (SSCs). Similarly, prepubertal patients haven't started spermatogenesis and cannot provide sperm yet. When faced with gonadotoxic treatment for childhood malignancy, such as chemotherapy and radiation, they

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may become azoospermic [3–5]. Consequently, current research efforts have focused on SSC therapy to treat male factor infertility.

Spermatogenesis begins with the SSC, a diploid cell that maintains the ability for self-renewal and downstream differentiation into spermatozoa. Given its origin in spermatogenesis, using SSC for male infertility is a logical choice for patients with maturation arrest or initially diagnosed SCO with salvageable SSC on the biopsy [2, 6]. Investigative SSC therapies have included SSC transplantation and in vitro spermatogenesis. The formed differentiated germ cells could potentially be used for in vitro fertilization (IVF), either intracytoplasmic sperm injection (ICSI) or experimental round spermatid injection (ROSI) [7–10]. Given that SSC is a scarce population in azoospermic patients, in vitro propagation of SSC is a critical step toward the feasibility of stem cell-based fertility treatments.

This chapter aims to provide our institution's protocol and technical considerations for SSC culture in mammalians. Previous works from our lab have included SSC isolation, propagation, and partial differentiation to the round spermatid level in a unique culture system [10–12]. This was initially performed in adult men where SSC could be isolated and propagated for 28 weeks up to a concentration of 18,540-fold [11]. Subsequently, this application was expanded to prepubertal boys in which SSCs were cultured for up to 29 weeks [12]. Most recently, this culture system has been successfully applied to a mouse model and human patients with Klinefelter syndrome, given the frequency of azoospermia, negative mTESE, and the need for alternative therapies [8, 9, 13].

2 Materials

2.1 Reagents

1. Phosphate-buffered saline (PBS, 1×).
2. Minimal Essential Media 1×.
3. Fetal calf serum.
4. DNase (Roche) stock solution of 2 mg/mL. It is stored at −20 °C in 200 μL aliquots. Keep in ice before use.
5. Collagenase NB 4 (Serva) stock solution of 10 PZ U/mL. It is stored at −20 °C in 200 μL aliquots. Keep in ice before use.
6. Neutral Protease NB (Serva) stock solution of 10 DMC U/mL. It is stored at −20 °C in 100 μL aliquots. Keep in ice before use.
7. *Enzyme mix*: 5 mL 1× MEM DNase (8 μg/mL) + SERVA Collagenase 4 PZ U + neutral Protease* 0.2 DMC U + 20 μL extra DNase (2 mg/mL). If cells are intended for transplantation into humans, Neutral Protease NB GMP Grade is recommended.

8. Trypsin-EDTA 0.25% (Gibco).
9. 1 × MEM 10%FBS.
10. StemPro™ complete medium (Tables 1 and 2) (*see* **Note 1**).

2.2 Equipment

1. Laminar flow cabinet.
2. Shaking water bath with temperature control.
3. Water bath or bean bath with temperature control.
4. Centrifuge.
5. Microcentrifuge.
6. Pipette controller.
7. Serological pipettes (5, 10 mL).
8. Micropipettes.
9. Micropipette tips (2–1000 μL).
10. Conical tubes (10, 50 mL).
11. Microcentrifuge tubes (1.5 mL).
12. Nylon filter (77 μM, 55 μM).
13. Culture plates (size and surface will depend on the cells obtained).
14. Petri dishes.
15. Gloves.
16. Tweezers.
17. Surgical blade.
18. Aluminum foil.
19. Parafilm.
20. Ice.
21. Cell culture incubator.
22. Optical microscope.
23. Dissection microscope or stereoscope.
24. Hemocytometer or Bürker-Turk cell counting slide.

3 Methods

3.1 Before Isolation

1. Preheat water or bean bath to 37 °C. Fill with autoclaved water. Set culture media within to incubate before use.
2. Preheat the shaking water bath to 32 °C. Fill with autoclaved water.
3. Ensure sterile conditions are present inside the laminar flow cabinet.

Table 1
Chemicals used to supplement StemPro-34™ SFM

Chemicals/media	Storage	Stock conc.	Solvent	Amount for 500 mL	Final conc.
Solids					
Bovine albumin	4 °C	–	–	2.5 g	5 mg/mL
D(+) glucose	RT	–	–	3 g	6 mg/mL
Ascorbic acid	RT	–	–	8.8 mg	1×10^{-4} M
Apo-Transferrin	−20 °C	–	–	50 mg	100 µg/mL
Pyruvic acid (sodium pyruvate)	4 °C	–	–	15 mg	30 mg/mL
D-Biotin	4 °C	–	–	5 mg	10 µg/mL
Liquids					
2-betamercaptoethanol	RT	–	–	1.7 µL	5×10^{-5} M
DL-lactic acid	4 °C	–	–	500 µL	1 µL/mL
MEM non-essential amino acids	4 °C	–	–	5 mL	10 µL/mL
StemPro™-34 SFM Nutrient Supplement	−20 °C	–	–	13 mL	26 µL/mL
Insulin	−20 °C	12.5 mg/mL	10 mM HCl	1 mL	25 µg/mL
Sodium selenite	−20 °C	0.25 mg/mL	milliQ water	10 µL	30 nM
Putrescine	−20 °C	100 mg/mL	milliQ water	50 µL	60 µM
L-glutamine	−20 °C	200 mM	–	5 mL	2 mM
MEM gitamin solution	−20 °C	–	–	5 mL	10 µL/mL
b-Estradiol	−20 °C	0.6 mg/mL	100% EtOH	25 µL	30 ng/mL
Progesterone	−20 °C	0.6 mg/mL	100% EtOH	50 µL	60 ng/mL

RT stands at room temperature

Table 2
Growth factors added to supplemented StemPro™-34 SFM immediately before use to make StemPro™ complete

Growth factors	Storage (°C)	Stock conc.	Solvent	Amount for 50 mL	Final conc.
Epidermal growth factor (EGF)	−20	200 µg/mL	10 mM acetic acid/0.1% BSA	5 µL	20 ng/mL
Human basic fibroblast growth factor (H bFGF)	−20	10 µg/mL	PBS/0.5%BSA	50 µL	10 ng/mL
Leukemia inhibitor factor (LIF)	4	10 µg/mL	–	50 µL	10 ng/mL
Glial cell line-derived neurotrophic factor (GDNF)	−20	10 µg/mL	PBS/0.1% BSA	50 µL	10 ng/mL
Fetal calf serum (FCS)	−20	–	–	0.5 mL	1%
Penicillin/streptomycin (Pen/Strep)	−20	–	–	0.25 mL	0.5%

4. Ensure that the incubator has achieved optimal conditions for culture (37 °C, 5% CO_2) and shows no signs of contamination.
5. The sample of testis tissue can be received from the operating room freshly or already cryopreserved viable tissue. The isolation process may vary slightly depending on whether fresh or frozen testis tissue is used.
6. Prepare 1× MEM with DNase mix (20 µL of 2 mg/mL DNase per 5 mL 1× MEM). Each sample will require about 100 mL.
7. Prepare 1× MEM 10% FBS (*without DNase*) for cell culture.
8. Prepare StemPro™ Complete for cell culture (Tables 1 and 2) (*see* **Note 2**).

3.2 Isolation

1. Transfer approximately 20 mL of 1× MEM/DNase into three Petri dishes.
2. If the sample is fresh, deposit it on a petri dish with 1× MEM/-DNase and move on to **step 5**. If the tissue is frozen, allow it to adjust to room temperature for 2 min. Then continue thawing the cryovial with lukewarm running tap water and wipe off residual water with 70% ethanol (*see* **Note 3**).
3. As thawing continues, the tissue inside the cryotube will gradually transition from solid to liquid. When the tissue becomes liquid enough, but there is still a portion of solid pellet yet to be thawed, empty the cryovial contents into a petri dish containing 1× MEM/DNase for the first wash.
4. Transfer the tissue into a second fresh petri dish containing 1× MEM/DNase using a pair of tweezers. Next, move the tissue into a new petri dish (the third one) containing 1× MEM/-DNase (*see* **Note 4**).
5. Remove the tunica albuginea, if necessary, using a pair of tweezers and a surgical blade (*see* **Note 5**).
6. Separate the seminiferous tubules of the testis apart and remove connective tissue, if present, using tweezers under a dissection microscope (Fig. 1). Note the tubules' appearance (thick, thin, long, short, etc.) (*see* **Note 6**).

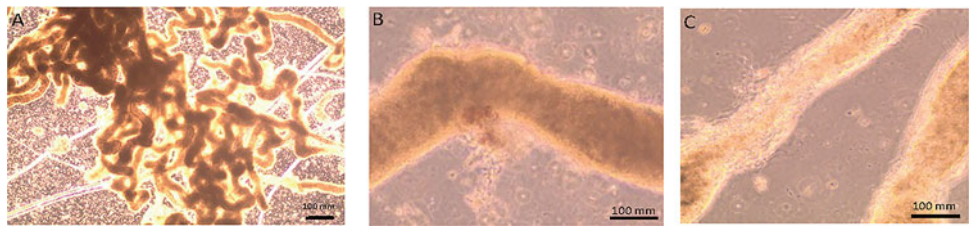

Fig. 1 Bright-field microscopic view of seminiferous tubules before the addition of enzyme mix (**a**, **b**) and subsequent incubation in which the lumens of the tubules appear opaque (**a**, **b**) and transparent (**c**)

7. Transfer the tissue suspended in 1× MEM/DNase to a 15 mL tube.

   If tube contents appear clear, allow tubules to settle at the bottom of the tube. If tube contents appear turbid, centrifuge (5 min at 65 g without brake), and remove the supernatant. Wash with 1× MEM/DNase until the supernatant is clear.

8. Remove the supernatant, if present, from the tube until only 5 mL are left. Then add 5 mL of Enzyme Mix, bringing the total volume to 10 mL.
9. Close the tube and seal the cap with parafilm. Submerge the tube completely in the shaking water bath and incubate for 30 min (32 °C, 120 cycles/min).
10. Remove the tube from the incubator and open it. *Without* pipetting up and down, take a 10 μL sample and place it on a microscope slide for viewing (Fig. 1). If present, note the thickness of the peritubular cell layer and any fenestrations. Incubate longer, if necessary, until the tubules appear fenestrated, with most extra tubular cells released but still full of intratubular cells inside.
11. Centrifuge the tube (5 min at 16 g) *without brake*. Remove the supernatant if present.
12. Wash with 5 mL 1× MEM/DNase. Centrifuge (5 min at 16 g), *without brake*, and discard the supernatant.
13. Add 5 mL of fresh Enzyme Mix.
14. Close the tube and seal the cap with parafilm. Submerge the tube completely in the shaking water bath and incubate for 30 min (32 °C, 120 cycles/min).
15. Remove the tube from the incubator and open it. Pipette gently up and down about ten times. Take 10 μL and place it on a microscope slide for viewing (Fig. 1). Note changes in the peritubular cell layer thickness and fenestrations since the first incubation (*see* **Note 7**).
16. Discard any clumps of tubules, if present.
17. Filter the entire suspension through a 70 μM nylon filter into a new 50 mL tube. Then, filter the rest again through a 40 μM nylon filter into a new 50 mL tube. Cells of the exact origin may be pooled together during filtration.
18. Centrifuge (5 min at 350 g without brake).
    (a) If the supernatant is clear, discard it.
    (b) If the supernatant is turbid, take 10 μL and place it on a microscope slide for viewing. If large cells are present, repeat centrifugation to avoid losing stem cells.

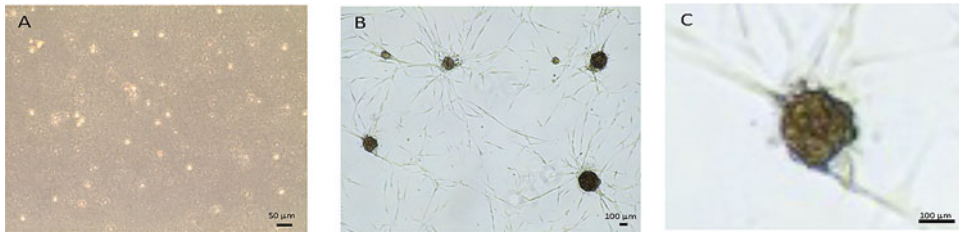

Fig. 2 Isolated testicular cells in culture. Seeded single cells after isolation (**a**), Germline Stem Cell cluster formation on top of somatic cells 3 weeks after cell culture (**b**, **c**)

19. Add 500 μL 1× MEM to the pellet and resuspend gently. Transfer to a microcentrifuge tube and spin down (5 min at 180 g, with brake).
20. Discard the supernatant and dissolve the pellet in ±500 μL 1× MEM/10% FCS (*without* DNase), depending on the size of the cell pellet.
21. Count the isolated cells using a Hemocytometer.
22. Culture cells in a 6-well plate with 1× MEM/10% FBS at a concentration of 5000–10,000 cells/cm^2. Incubate overnight at 37 °C, 5% CO_2.

3.3 Cell Culture

1. Keep the cells inside the incubate at 37 °C, 5% CO_2, replacing 1× MEM/10% FBS with StemPro complete medium (Tables 1 and 2).
2. Observe the cells in the culture regularly (Fig. 2). Note cell morphology, the confluence of attached cells, and cell debris or contamination (*see* **Notes 8** and **9**).
3. Refresh culture media 2–3 times a week. Use the media volume recommended by the manufacturer. Avoid losing floating cells by collecting spent media in a tube and centrifuging to form a pellet. Resuspend the pellet with new media and reintroduce the volume evenly among culture wells. Shake the plate gently to ensure the cells seeded spread evenly around the well (*see* **Note 10**).
4. When 80–90% confluency is achieved, cells should be trypsinized and passage into a new plate to continue propagation. If somatic cell overgrowth is suspected, differential plating can be considered 12–48 h after seeding.

3.4 Cell Cryopreservation

1. Passage cells with trypsin EDTA incubation and then count them.
2. Plan the way you want to freeze your cells depending on the study's goals and cell availability (*see* **Note 11**).

3. Centrifuge cells (5 min, 180 g, with break). Remove the supernatant and resuspend the pellet in 750 µL 1× MEM/20% FCS (Room Temperature).
4. Transfer the cell suspension into a cryovial.
5. Add 750 µL 1× MEM/20% FCS/16% DMSO while gently shaking the cryovial.
6. Transfer the cryovial into a cold Nalgene freezing box (Mr. Frosty). Maintain at −80 °C for around 12 h but no longer than 24 h.
7. Transfer the cryovial to a liquid N_2 tank.

4 Notes

1. The reagents shown in Table 1 are added to 500 mL StemPro™-34 serum-free medium to supplement the medium. Final concentrations are based on the 500 mL StemPro™-34 SFM, not the total volume.
2. After mixing reagents, filter the mixture with vacuum pressure over a 22 µM filter. Immediately before use, add growth factors shown in Table 2 to 50 mL supplemented StemPro™ SFM.
3. An aggressive change in the temperature of the cryovial may trigger an expansive reaction of the gas inside. This may break the cryovials, compromising the sample integrity or even becoming a safety hazard for the operator. Ensure the initial thawing is gradual and that appropriate safety guideline is followed. We also recommend briefly opening the cryovials to release air pressure as soon as the thawing process allows.
4. As previously reported, dimethyl sulfoxide DMSO is one of the most commonly used cryoprotectants for testicular tissue [14], but at room temperature, DMSO becomes cytotoxic. Three time-sensitive consecutive washes are done to minimize tissue exposure to DMSO at room temperature.
5. During this step, it is recommended to weigh the tissue to calculate the isolation efficacy (number of cells per mg tissue).
6. Seminiferous tubules from simple testicular biopsies are usually more laborious to separate than in mTESE samples. The further separated the tubules are, the more adherent (mucous-like) they appear and the more influential the enzymatic digestion will be.
7. The tubular cell layer should disintegrate at this point, releasing the stem cells. The thickness of the tubular cell layer can differ between patients. The incubation with enzymes can be continued for longer to achieve the desired characteristics of the peritubular cell layer. If cell clumps form, dissociate them with trypsin-EDTA.

8. Taking pictures of the cells in culture is encouraged.
9. If there is uncertainty about cell viability, Live/Dead staining (Invitrogen LIVE/DEAD™ Viability/Cytotoxicity Kit) followed by fluorescence microscopy can be performed before refreshing media.
10. Avoid keeping cultured cells without media for too long during the refresh process.
11. In our experience, we use 1.8 mL cryovials with up to 4,000,000 cells per cryovial. Higher cell concentrations may compromise cell viability after thawing.

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Chapter 4

Purification by STA-PUT Technique of Male Germ Cells from Single Mouse and RNA-Extraction for Transcriptomic Analysis

Chiara Naro, Claudio Sette, and Raffaele Geremia

Abstract

Transcriptomic analyses of germ cells at different stages of differentiation have shed light on the transcriptional and post-transcriptional mechanisms regulating gene expression that ensure the correct progression of spermatogenesis and male fertility. In this chapter, we describe a method to isolate meiotic and post-meiotic germ cells, based on gravimetric sedimentation, starting from a testicular germ cell suspension isolated from a single adult mouse. We also describe how to assess the purity and quality of the collected fractions of germ cells and how to optimize the extraction from these samples of RNA for subsequent RNA-sequencing experiment. In our experience, this protocol is suitable for germ cell isolation and transcriptomic analysis for mouse models with spermatogenic defects, overcoming the limits that reduced fertility poses to the obtaining of experimental animals.

Key words Male germ cells, Cell purification, STA-PUT, Gravimetric sedimentation, RNA extraction, Transcriptome analysis

1 Introduction

Spermatogenesis is the complex differentiation process taking place in the testes, leading to the generation of mature male gametes [1, 2]. During this process spermatogonial stem cells undergo profound genomic and morphological variations, which occur along three subsequential phases: (i) amplification of spermatogonia population, (ii) meiotic commitment and division into primary and secondary spermatocytes, (iii) differentiation from haploid round spermatids to mature spermatozoa (i.e. spermiogenesis) [1, 2]. Initiation and accomplishment of each of these phases of spermatogenesis is sustained by stage-specific patterns of gene expression, providing each phase of the necessary protein repertoire [3–7]. Understanding of the biological processes and of underlying molecular mechanisms driving spermatogenesis relies on the

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possibility to isolate and analyze male germ cells at the defined stage of differentiation. In this regard, the STA-PUT technique represents, since the late 1960s, a pivotal procedure in this field of study. This technique takes advantage of the fact that the individual types of testicular cells have different size, thus sedimenting at different velocities in a BSA gradient, independently from the shape. The physical characteristics of the method have been extensively described by Miller and Phillips using blood cells [8] and then used for the separation of mouse male germ cells in the laboratory of Dr. W.R. Bruce [9]. The effectiveness of the technique has been re-evaluated from a morphological point of view [10], re-examined also recently proposing different equipment [11], and widely used for the study of DNA synthesis and transcriptional and translational activity in the different phases of spermatogenesis and for a variety of biochemical and metabolic studies [12–16]. The recent advancement of transcriptomic approaches has provided remarkable insights into the transcriptional and post-transcriptional molecular mechanisms underlying the tightly and dynamically controlled gene programs promoting male germ cells differentiation [17, 18]. Most of these studies exploited RNA-sequencing (RNA-seq) from either bulk-testis, collected at different time-points during the first synchronous wave of murine spermatogenesis, or from male germ cells at different stage of differentiation, isolated by either centrifugal elutriation or Fluorescence-activated Cell Sorting (FACS) [3, 6, 7]. Both these approaches show limitations: i. presence of heterogeneous populations of somatic cells (i.e., Sertoli cells, Leydig cells) is a confounding element in the identification of germ-cell-specific gene expression patterns by RNA-seq of bulk-testes; ii. stage-specific male germ cell isolation by centrifugal elutriation requires injection of a high number of testicular cells obtained by pooling testes from multiple mice. This latter aspect is a critical limitation for transcriptomic studies aimed at the identification of gene expression alterations correlated to spermatogenic defects in mice with reduced reproductive potential, due to the small number of animals. In our laboratory, we showed that gravimetric decantation (STA-PUT) of testicular cellular suspension is a reliable method to collect homogenous population of meiotic (spermatocytes) and post-meiotic (round spermatids) male germ cells from a single mouse, from which it is possible to isolate a sufficient amount of RNA for reliable total-RNA seq analysis [4]. Moreover, we showed this method to be applicable and reliable also for the isolation of germ cells and subsequent transcriptomic analysis from a mouse model with relevant spermatogenic defects, with reduced testis size and impaired male germ cells differentiation, such as the *Sam68* knock-out model [19, 20], therefore proving its applicability to the late-generation transcriptomic analysis. Herein, we will describe a detailed protocol for the fractionation of meiotic spermatocytes and post-meiotic round spermatids from a single adult mouse and the isolation of good-quality RNA for further transcriptomic analysis.

2 Materials

2.1 Animals

The procedure described in this chapter refers to testicular germ cells suspension obtained from the digestion of testes of 12-week old mouse from the C57BL/6 strain (*see* **Note 1**).

2.2 BSA Gradients

1. 1.5 L EKRB (120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 11 mM). All components are diluted in water. This solution is used for the preparation of both the BSA gradients and the testicular germ cells suspension. Solution is filtered through a 0.22 μM filter and stored at 4 °C (*see* **Note 2**).
2. Bovine serum albumin (BSA) 3% in EKRB: Dissolve 12 g BSA in 400 mL of EKRB at room temperature using magnetic stirring. The solution is filtered through a 0.22 μM filter and stored at 4 °C. 250 mL and will be used as such to create the gradient. The excess will be used to prepare 250 mL of 1% BSA in EKRB, 60 mL 0.5% BSA in EKRB, and 15 mL 0.2% BSA in EKRB by the dilution of an appropriate amount of EKRB w/o BSA (*see* **Notes 3** and **4**).

2.3 STA-PUT Apparatus

1. Sedimentation chamber: The sedimentation chamber had a conical base carved out of a square plate with a side of 15 cm and a thickness of 4 cm. The cone had a diameter of 12 cm at the base and was 3 cm deep with a hole of 4 mm diameter at the apex. Above the conical base a 15 cm long tube with an internal diameter of 12 cm was glued to complete the chamber.
2. Three cylinders, necessary for the formation of the albumin gradient to be loaded into the sedimentation chamber, with the following characteristics:
   (a) Two cylinders measuring 7.5 cm internal diameter and 15 cm height on a square base with 10 cm sides to ensure stability. At the base of one cylinder (n.1) there is one outlet represented by a small conical plexiglass tube with an internal diameter of 2 mm; at the base of the second cylinder (n.2), there are two of such outlets placed at the two ends of the cylinder diameter.
   (b) The third cylinder measured 1.8 cm internal diameter and 15 cm height on a square base of 5 cm sides to ensure stability. At the base of the cylinder (n.3), there are two outlets as described for cylinder n.2 (*see* **Note 5**).
3. One baffle: A 1.2 cm diameter stainless steel hemisphere with three 2 mm pegs (*see* **Note 6**).
4. Silicone tubing 2 mm internal diameter.

5. Straight mini tubing connector to fix the tubing to the base hole of the STA-PUT.
6. One 10 mL syringe for sample injection, hold by a support clamp, attached through a bosshead to a laboratory stand.
7. Two 3-way stopcocks and luer lock adapters if necessary to connect tubing.
8. Three curved tubing clamps, placed on the connecting tubes between the cylinders and the syringe.
9. One tubing clip, Hoffman pattern, placed on the connecting tube between the syringe for sample injection and the STA-PUT chamber.
10. Two magnetic stirrers for cylinders n. 2 and 3.
11. Two magnetic stirring bars for cylinders n. 2 and 3.
12. A small laboratory jack to support the cylinder n.1 at the same level of cylinders 2 and 3.
13. A perforated support about 10 cm high for the STA-PUT (laboratory jack or polystyrene box).

2.4 Testicular Germ Cells Preparation

1. Shaking water bath set at 32 °C.
2. Refrigerated bench centrifuge.
3. Sterile blades.
4. 15 mL sterile polypropylene tubes.
5. Hemocytometer for cell counting.
6. Minimum Essential Medium (MEM).
7. Collagenase from Clostridium histolyticum (Sigma Aldrich, Cat # C7657; CAS 9001-12-1): 5 mg/mL in MEM, equal to a 20-fold stock solution (*see* **Note 7**).
8. DNase I from bovine pancreas (Sigma Aldrich, Cat # DN25; CAS 9003-98-9): 2 mg/mL dissolved in MEM, equal to a 40-fold stock solution (*see* **Note 7**).
9. Trypsin from bovine pancreas (Sigma Aldrich Cat # T9201; CAS 9002-07-7): 10 mg/mL in MEM, equal to a ten-fold stock solution (*see* **Note 7**).
10. Fetal bovin serum (FBS).

2.5 Fractions Collection and Analysis of Germ Cells Nuclear Morphology

1. Refrigerated bench centrifuge.
2. 15 and 50 mL sterile polypropylene tubes.
3. 1.5 mL sterile polypropylene tubes.
4. Hoechst 33342 10 mg/mL in water (ThermoFisher Scientific Cat #H3570).
5. Microscope slides.

2.6 RNA Purification

1. Refrigerated bench centrifuge.
2. miRNeasy Mini Kit with DNase (Qiagen).
3. DNAse-RNAse free 1.5 mL tubes and tips (*see* **Note 8**).

3 Methods

3.1 Assembly of the STA-PUT Apparatus

Prior or during the germ cells isolation procedure, place the apparatus in a cold room or in a large refrigerator, where all procedures will be performed. The setup of the apparatus is illustrated in Fig. 1.

1. Place Cylinders n. 2 and n. 3, with a stirring bar of the proper size inside, on two magnetic stirrers. Place the cylinder n. 1 on a laboratory jack of the same height as the stirrers. The three cylinders should be connected with silicone tubing and placed on a shelf approximately 30 cm above the base of the STA-PUT to allow for gradient flow during loading.
2. Place firmly the STA-PUT chamber, with the baffle inside (*see* **Note 9**), on the perforated support to allow the passage of the cell suspension and the gradient to and from the base hole of the chamber.

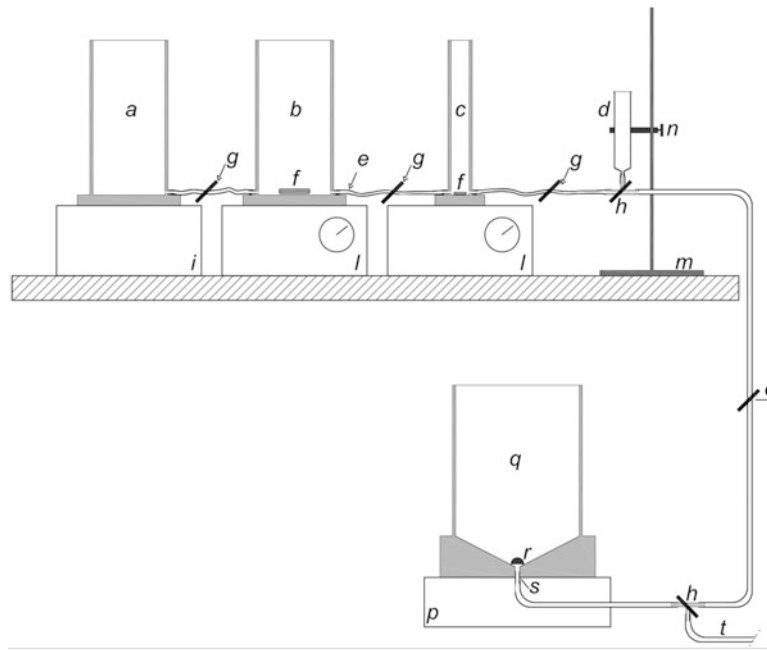


Fig. 1 The STA-PUT apparatus: (**a**) cylinder n.1 containing 3% BSA; (**b**) cylinder 2 containing 1%BSA; (**c**) cylinder 3 containing 5% BSA; (**d**) 10 mL syringe for EKRB and cell suspension loading; (**e**) silicone tubing; (**f**) magnetic stirring bars; (**g**) curved tubing clamps; (**h**) three-way stopcock; (**i**) small laboratory jack; (**l**) magnetic stirrer; (**m**) laboratory stand; (**n**) support clamp and boss-head; (**o**) tubing clips, Hoffman pattern; (**p**) perforated laboratory jack; (**q**) STA-PUT; (**r**) baffle; (**s**) straight mini tubing connector; (**t**) exit for the collection of fractions

3. Fix the 10 mL syringe for the EKRB overlay and cell suspension injection with a clamp on a laboratory stand at the same height as the cylinders and place a three-way stopcock under it.
4. Connect the three-way stopcock fixed to the syringe with the STA-PUT, using silicone tubing and interposing a second three-way stopcock (*see* **Note 10**). Close the tubing connecting the three cylinders and cylinder nr. 1 with the three-way stopcock under the syringe with curved tubing clamps. Close the tubing connecting the syringe with the STA-PUT chamber with a Hoffman pattern clip (*see* **Note 11**).

3.2 Isolation of Male Germ Cells from Single Mouse

1. Euthanize the animal according to the direction of the ethical commission of your country, quickly recover the testes from the epididymis, and place them on a Petri dish containing EKRB.
2. Release seminiferous tubules removing the albuginea with the help of scissors and a tweezers and transfer them 15 mL Falcon tube containing 5 mL of EKRB and add 0.05 mg/mL DNase I and 0.25 mg/mL Collagenase (*see* **Note 12**).
3. Place the tube in a water bath under constant shaking (~130 shakes/min), at 32 °C, for 10–15 min, until the seminiferous tubules appeared completely dispersed and freed of the interstitial tissue upon visual observation. Tubules will appear as long filaments floating in the medium (*see* **Note 13**).
4. Wash the tubules twice with EKRB, by letting the tubules sediment at unit gravity and pouring away the supernatant. Add 1 mL of EKRB and transfer the tubules to a Petri dish by pouring.
5. Gently cut with a sterile blade the released seminiferous tubules in 2–3 mm fragments and collect them in a 15 mL tube. Wash them once by sedimentation after the addition of 10 mL EKRB with 0.05 mg/mL DNase I.
6. Resuspend the tubules in 5 mL of EKRB and add 0.05 mg/mL DNase I and 1 mg/mL trypsin and place back the tube into the shaking bath at 32 °C for 30 min.
7. Stop the digestion by adding 5 mL of pre-warmed EKRB containing 0.05 mg/mL DNase I and fetal bovine serum or BSA to reach final concentrations of 10% or 0.4%, respectively (*see* **Note 14**).
8. Let the undigested tubule fragments to sediment under unit gravity and collect the supernatant containing the single germ cell suspension.
9. Centrifuge the germ cell suspension for 10 min at 1200 rpm at 4 °C, with very slow acceleration.

10. Remove the supernatant and resuspend the remaining compact cellular pellet in the small amount of residual supernatant by gentle shaking of the tube. Then, gradually add 12 mL of cold EKRB containing 0.2% BSA and 0.05 mg/mL DNAse I.
11. Count the cells of the suspension using a hemocytometer. Usually, from the digestion of the testes of 12-week-old mouse from the C57BL/6 strain a suspension of ~25–30 * 10^6 monodispersed cells is obtained.
12. Keep cell suspension in ice or in the cold room before proceeding with the STA-PUT sedimentation.

3.3 STA-PUT Sedimentation

1. During the germ cells isolation procedure, pour into the appropriate cylinders the BSA solutions necessary for the formation of the gradient. Take great care to eliminate air bubbles in the connecting silicone tubing.
   (a) Pour 250 mL of 3% BSA in EKRB into cylinder n. 1. Fill the connecting tube with cylinder no. 2, by raising the latter and releasing the clamp on the tube until the solution fills it and then tight it again.
   (b) Pour 250 mL of 1% BSA in EKRB into cylinder n. 2 and operate the same procedure described above to fill the connecting tube with cylinder n.3.
   (c) Pour 15 mL of 0.5% BSA in EKRB in cylinder n.3. Open the three-way stopcock toward the syringe and fill the connecting tube as described above. Tight the clamp when the fluid level in cylinder n. 3 will be at the same level as the others, if necessary correct filling levels by adding or removing solution. Pour out the eventual solution entering the syringe and open the three-way stopcock in the syringe versus STA-PUT direction.
   (d) Turn on the magnetic stirrers underneath cylinders n. 2 and 3 and remove the clamps between the cylinders and the syringe (*see* **Note 15**).
2. Fill the tubing connecting the syringe and start loading the STA-PUT. With both the three-way stopcocks, the one fixed to the syringe and the intermediate, positioned so that the flow is toward the STA-PUT and with the Hoffman clip partially open, pour into the syringe 24 mL of EKRB that will start flowing toward the STA-PUT chamber in two successive phases. Close the clip after the first 10 mL of EKRB and make sure that no air bubbles are left inside the tubing. If present, the air bubbles will be removed hitting the tub with little taps so that the air will move toward the syringe or the STA-PUT.

3. After pouring the second aliquot of EKRB, adjust the opening of the Hoffman clip to regulate the flow rate of the EKRB solution to approximately 3 mL/min. Just before the syringe empties, pour the 12 mL cellular suspension into the syringe. Once in the STA-PUT chamber cellular suspension will form a 1 mm layer (*see* **Note 16**).
4. At the end of the loading of the germ cells suspension, paying attention that no air enters the tubing, rotate the three-way stopcock below the syringe so that the flow is from the cylinders to the STA-PUT chamber and the gradient will start to be formed below the cell suspension.
5. Operate on the Hoffman clip to gradually accelerate the flow: up to 6 mL/min while filling the cone of the STA-PUT; reaching 10 mL/min while filling the cylinder (less then 1 mm of cylinder height per min).
6. When the cylinders n1, n2, and n3 are progressively empty, close the clamps placed on the tubes which connect them to the following section of the apparatus to prevent air bubbles from entering therein. At the end of the loading, approximately 1 h, close the Hoffman clip and start measuring the sedimentation time (*see* **Note 17**).

3.4 STA-PUT Fractionation and Analysis of Fractions

At the end of the appropriate sedimentation time, proceed with the collection of the gradient fractions corresponding to pachytene spermatocytes and round spermatids. An optimal separation of these cell types is achieved in approximately 2.5 h of sedimentation, including the loading time. By this time, spermatocytes and round spermatids will form slightly opaque bands, visible if observing the STA-PUT with a light on the opposite side of the chamber, and will have reached the approximate height of 2.5 and 1.3 mm, respectively, from the surface of the EKRB top layer. Three more bands can be observed: two slower migrating with respect to round spermatids, represented by spermatozoa and residual bodies the first, and by elongating spermatids the second, and one intermediate to spermatocytes and spermatids, represented by spermatogonia and primary spermatocytes. After draining of the gradient, characterization of germ cells within the collected fractions is performed by the evaluation of the cellular nuclear morphology by the simple and fast technique of Hoechst staining (*see* **Note 18**). Meiotic pachytene spermatocytes and round spermatid are distinguished on the basis of their size and nuclear morphology. Pachytene spermatocytes have larger nuclei (approximate diameter of 12 μM), containing dense clumps of chromatin (Fig. 2a is shown as a reference). Round spermatids have small nuclei (an approximate diameter of 8 μM), with one or two densely stained chromocenters in the middle (Fig. 2b is shown as a reference).

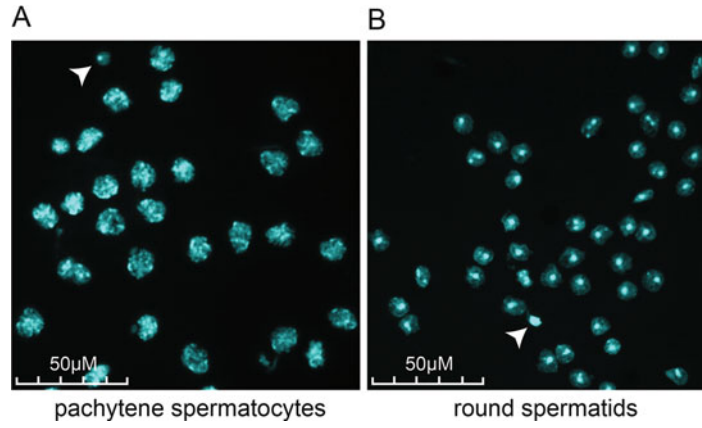


Fig. 2 Representative images of Hoechst nuclear staining of purified cellular populations by STA-PUT technique of meiotic pachytene spermatocytes (**a**) and post-meiotic round spermatids (**b**). Scale bar, 50 μM. Arrowheads indicate contaminating cells in the two collected fractions

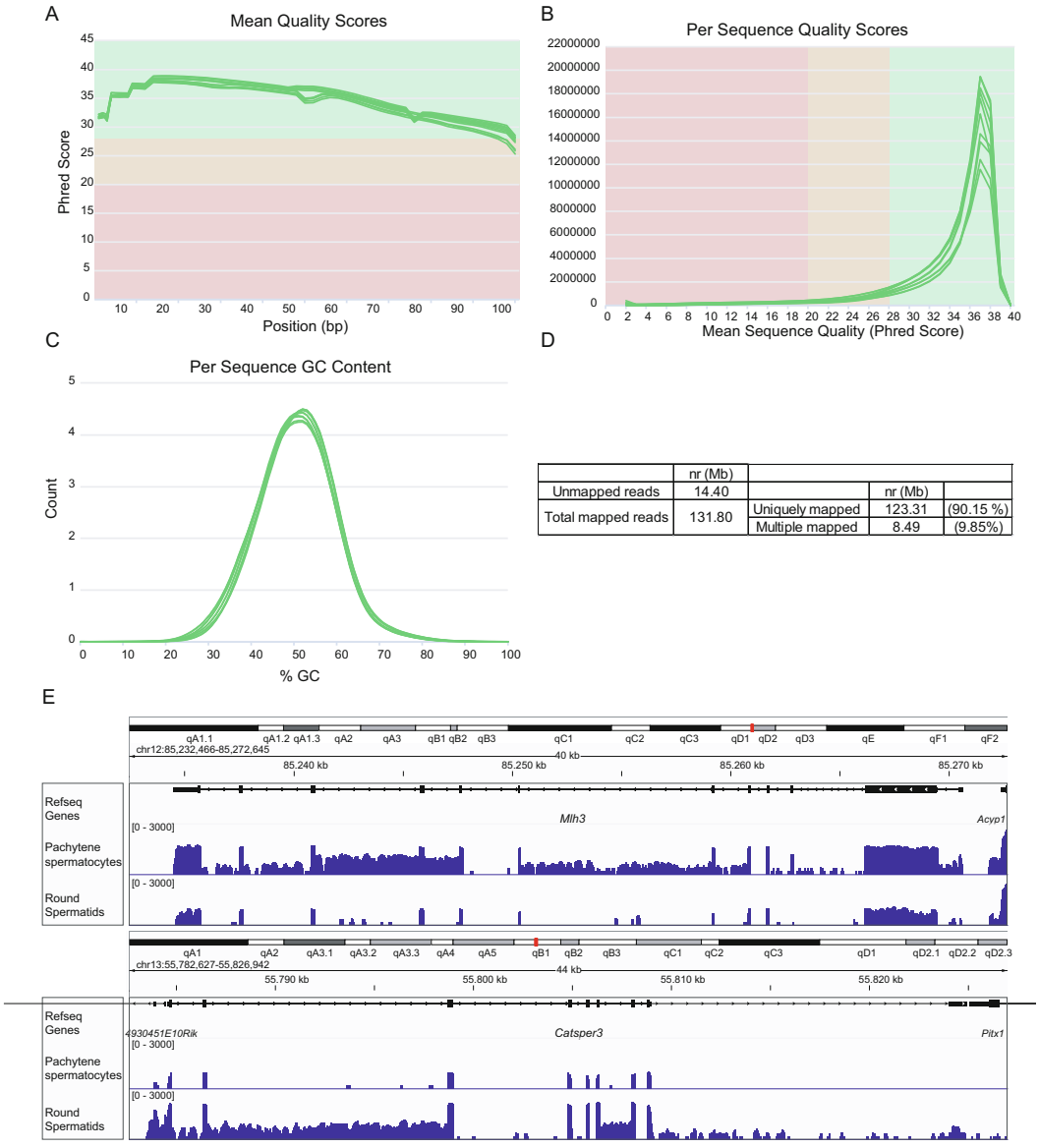
1. Drain the sedimentation chamber from the bottom, by opening the second three-way stopcock or detaching the tubing from below the syringe and adjust the flow rate to approximate 10 mL/min.
2. Discard the first 150 mL, equal to the content of the cone of the sedimentation chamber plus 3 mm above it.
3. Collect 12 mL fractions in 15 mL falcon tubes (the volume corresponding to a 1 mm thick gradient layer), and number them backward, starting from the last collected fraction containing the top of the gradient and proceeding toward the first and keep tubes refrigerated. Collectively approximate 30–32 fractions are collected.
4. Centrifuge the fraction collection tubes for 10 min, at 1500 rpm at 4 °C, in a swinging rotor. Pour off the supernatant gently, resuspend cells in remaining liquid, and add 0.5 mL EKRB and 0.05 mg/mL DNase I. Keep cells refrigerated, either on ice or in the cold room.
5. Proceed with the analysis of the nuclear morphology of the germ cells collected in the fractions close to those expected to contain the germ cells of interest, i.e., fractions from 12 to 16 for spermatids and from 24 to 28 for spermatocytes. For these selected fractions transfer 40 μL of cells into new 1.5 mL tubes and stain cells by adding 10 μL of 0.05 mg/mL Hoechst 33342 in EKRB to reach a final concentration of 0.01 mg/mL.
6. After 15 min of incubation with Hoechst 33342, spot cells on microscope slides and observe them using a fluorescence microscope (*see* **Note 19**).

7. Pool cellular fractions homogenous for cell morphology and degree of cell purity to obtain the two samples corresponding to the pachytene spermatocytes and round spermatids cellular populations. Selected fractions are pooled in 15 mL tube, and before centrifugation for 10 min at 1200 rpm at 4 °C, an aliquot is counted in hemocytometer. Usually, by loading a suspension of ~30 * 10^6 monodispersed cells, pools of 4 * 10^6 spermatids and 8 * 10^5 spermatocytes are obtained, with a level of purity ranging between 85% and 95% (*see* **Note 20**).
8. The cell pellet is resuspended in 1 mL of PBS, and the cell suspension is transferred to 1.5 mL tube to be further centrifuged for 10 min at 1200 rpm at 4 °C.
9. Discard the supernatant and directly proceed to further molecular biology analysis on the cellular pellet or snap-frozen it in nitrogen liquid until further analysis.

3.5 RNA Extraction for Transcriptomic Analysis

As a reference, by application of this protocol, we were able to extract at least 1.5 μg of RNA from sample of ~4 * 10^5 meiotic spermatocytes and of ~1 * 10^6 round spermatids. We showed the grade quality and the quantity of these RNA samples to allow reproducible high-throughput RNA-sequencing experiments, characterizing the gene expression and alternative splicing program supporting the meiotic transition of murine spermatogenesis [4] and dissecting the regulatory role of the RNA binding protein (RBP) Sam68 in this process [19]. RNA samples were converted in polyA plus RNA-seq libraries, sequenced using a 100 bp paired-end format on an Illumina HiSeq 2000 instrument. As shown by analysis performed with the MultiQC Tool [21], reads of all the sequenced samples showed high Phred Scores (an index for the base quality in the cDNA sequencing), along their entire length (Fig. 3a, b), with no bias in the GC content of the sequences (Fig. 3c). On average, 130 Mb of reads were sequenced for each sample, of which more than the 90% was uniquely mapped on the reference genome (Fig. 3d). Figure 3e shows, by The Integrative Genomics Viewer (IGV) visualization tool [22], the different distribution of the RNA-seq reads detected in pachytene spermatocytes and round spermatids for two representative genes specifically expressed in either of the two cell types: i.e., the homologous recombination protein Mlh3, highly expressed in spermatocytes, and the sperm-specific calcium channel CatSper3, highly expressed in spermatids [4].

1. For high-quality RNA extraction, as required for transcriptomic analysis, directly resuspend the cellular pellet in 700 μL of Qiazol by vortexing for 1 min and ensure that no cell clumps are visible.



	nr (Mb)			
Unmapped reads	14.40		nr (Mb)	
Total mapped reads	131.80	Uniquely mapped	123.31	(90.15 %)
		Multiple mapped	8.49	(9.85%)

Fig. 3 Graphs reporting results of the MultiQC tool for the quality analysis of RNA-sequencing of spermatocytes and spermatids samples from our study [4] and deposited in the GEO database under the accession number GSE95138. (**a**) Distribution of the Phred quality score along the length of the sequenced RNA-seq reads for every samples. (**b**) Distribution of the Phred quality score for all the reads of each sample. (**c**) Distribution of the GC content within the RNA-seq reads of all the samples. (**d**) Table illustrating the average mapping statics. % of uniquely mapped reads with respect to the total number of total mapped reads is indicated. (**e**) Visualization of the RNA-seq reads profile of *Mlh3* and *Catsper3* genes in pachytene spermatocytes and round spermatids. Exon-intron gene structure (black boxes and lines) and sequence reads (blue lines) are indicated. Graphs were drawn using the Integrative Genomics Viewer (IGV), visualization tool

2. Proceed to RNA extraction by incubating for 5 min at room temperature or store Qiazol lysed cells at −80 °C (*see* **Note 21**).
3. Add 140 μL chloroform (0.2 mL), shake 30 s, and incubate for 2–3 min at room temperature.
4. Centrifuge at 12,000 × g for 15 min at 4 °C.
5. Transfer 300 μL of the upper aqueous phase into a new tube and add 1.5 volume of ethanol 100% (450 μL) and mix thoroughly by inverting the tube several times.
6. Pipet 375 μL of the sample into the RNeasy Mini spin column in a 2 mL collection tube and centrifuge at 8000× g for 15 s at room temperature.
7. Discard the flow-through and repeat the same step described in 6 for the remaining sample.
8. Wash with 350 μL of the RW1 solution and centrifuge at 10,000 × g for 15 s at room temperature.
9. Add onto the membrane of the column a mix of 10 μL RNase-free DNaseI (Qiagen) with 70 μL RDD buffer and let incubate for 15 min at room temperature.
10. Add 350 μL RW1 and centrifuge at 10000 × g for 15 s at room temperature.
11. Wash twice with 500 μL RPE buffer and centrifuge: the first washing step at 10000 × g for 15 s and the second at 10000 × g for 2 min, both at room temperature, to dry the membrane (*see* **Note 22**).
12. Add 30 μL of RNase-free water to the column, let it stand on the bench at room temperature for 2 min, and elute RNA by centrifuging at 10,000 × g for 1 min at room temperature.
13. Keep the RNA samples on ice if you want to proceed immediately with quantity and quality analyses (e.g., quantification with Nanodrop, Bioanalyzer, Qbit) or store them at −80 °C until further use.

4 Notes

1. C57BL/6 mice were maintained on a normal 12 h light/dark cycle in the animal facility of the University of Rome Tor Vergata, according to the Guideline of the Italian Institute of Health (protocol n. 1088/2016-PR).
2. Filter the EKRB solution after having taken the volume of EKRB necessary to prepare the 3% BSA in EKRB.
3. Prepare the more diluted gradients of BSA in EKRB under a sterile, hood by mixing the previously filtered EKRB and 3% BSA gradient in the appropriate ratio.

4. EKRB and BSA gradient can be prepared a day in advance and stored at 4 °C.
5. The components of the STA-PUT apparatus used in this study were not industrially produced and are not commercially available. However, they are made of plexiglass, and they can be easily built in a plastic products processing laboratory. Our apparatus was made by Fedele82 snc. (via degli Apuli 53, 00185 Roma, Italy) and was a replica of a prototype made in the machine shop of the Ontario Cancer Institute, Toronto, Canada, for the biophysics department where Miller and Phillips have studied the sedimentation properties of eukaryotic cells in fetal calf serum gradients [8]. A Canadian firm supplies glass STA-PUT, and they can be contacted to eventually arrange dimensions (ProScience Inv. - Glass Shop Division, 770 Birchmount Road, Unit #25, Scarborough, Ontario M1K 5H3 CANADA. Email: glassshop@proscience.com).
6. The baffle can alternatively be obtained cutting the bottom of a piercable centrifuge tube to obtain the empty hemisphere with three 2 mm pegs and filling it with epoxy, including a weighting nut or lead shots.
7. After dissolving in MEM enzymes, filter them through a 0.22 μM filter and store in aliquots at −20 °C. Alternatively, enzymes can be made fresh each time. To ensure a high-quality tissue digestion frozen aliquots should not be older than 3 months and thaw-freeze cycle should be avoided.
8. Do not use autoclaved pipette tips or tubes, as autoclaving does not inactivate RNase and autoclaves might contain RNases. To avoid RNA degradation by contaminating RNases during extraction, pay attention to decontaminate pipette and the bench-work with RNase decontamination solutions, wear gloves, and do not touch anything with bare hands to avoid contamination by RNases from the skin.
9. Placing the baffle at the apex of the STA-PUT cone is very important during the loading of the germ cells to laterally divert the inflow of the cell suspension, avoid its mixing with the ERKB overlay, and ensure optimal stratification. It is also important to ensure proper layering of the incoming albumin solutions of increasing concentrations for gradient formation.
10. The three-way stopcock placed along the tubing connecting the syringe to the STA-PUT will be used to divert the gradient flow during unloading at the end of the cell separation procedure. Alternatively this stopcock can be avoided, in which case the tube will be detached from the three-way stopcock under the syringe and directly used to discharge the gradient.

11. The Hoffman pattern clip allows a better control of the flow rate during the loading of the cells and the gradient into the STA-PUT.
12. Addition of DNase I during all the steps of the isolation of germ cell from seminiferous tubules is necessary to avoid cell aggregation. Cell damaging during tissue manipulation and enzymatic treatments could cause the release of DNA molecules that will favor cells aggregation, thus preventing the obtaining of single cell suspensions.
13. Gentle hand-shaking of the tube for few-time could help dissociation at late phases and allow a closer monitoring of its progression.
14. Excessive fragmentation of tubules should be avoided, as it results in a loss of germ cells.
15. The chosen gradient is initially nonlinear, due to the small amount of 0.5% BSA, then becoming a linear 1–3% BSA. This design ensures that the concentration range of BSA between the cell layer (0.2%) and the beginning of the gradient (0.5%) is minimal and therefore the risk of streaming is reduced. Indeed, otherwise the difference between the BSA concentration in the injected cell suspension (0.2%) and the gradient (1%) would favor the aggregation of cells and their streaming through the gradient. This deleterious event would cause the single cell separation procedure to fail.
16. The amount of EKRB and of cell suspension, 24 and 12 mL respectively, will result in 2 mm overlay of buffer followed by 1 mm cell suspension once in the STA-PUT. The layer of EKRB is important to protect the underlying 1 m cell suspension layer from the strong vibrations that the liquid meniscus causes while rising along the internal plexiglass surface of the STA-PUT.
17. Do not disturb the apparatus during the sedimentation time and the subsequent phase of fractions collection, as any mechanical agitation could disturb the gradient and the germ cells sedimentation.
18. The nuclear staining procedure described here in Subheading 3.5 does not involve long-term fixation of cells on glass slides, but it is aimed at a fast and rapid observation. Alternatively, slides for longer-time observation and eventual storage might be obtained as described in [23]. Briefly, germ cells are spotted on slides coated with poly-L-lysine (which allow glass adhesion of cells in suspension), fixed for 10 min with 4% PFA, stained with Hoechst and then slides mounted with mounting medium.

19. Hoechst 33342 should be preferred to Hoechst 33258 when performing the staining on viable not fixed cells, given its higher cell permeability.
20. We proved this protocol to efficiently recover homogenous population of meiotic spermatocytes and round spermatids even when loading a smaller initial cellular population, as it may occur when applying the procedure to subfertile/infertile murine model. As a reference, when we loaded on the gradient a suspension of ~14 * 10^6 monodispersed male germ cells isolated from 12-week old *Sam68* knockout mice we obtained ~1.5 * 10^6 round spermatids and ~4.5 * 10^5 meiotic spermatocytes.
21. Guanidinium thiocyanate–phenol solutions from other brands are equally suitable. Resuspension of cellular samples in these solutions and storage at −80 °C preserves RNA integrity for several months.
22. Carryover of residual ethanol during RNA elution should be avoided, as it could interfere with subsequent analysis. To this end it is advised to change the collection tube in these two spin-drying phases, and when moving the column from the collection tube to elution tube, take care that the column nozzle does not contact the walls of the collection tube, where ethanol residual could be present.

Acknowledgments

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Chapter 5

Isolation of Mouse Germ Cells by FACS Using Hoechst 33342 and SYTO16 Double Staining

Mark E. Gill, Hubertus Kohler, and Antoine H. F. M. Peters

Abstract

In the adult mouse testis, germ cells of various developmental cell states co-exist. FACS isolation of cells stained with the DNA dye Hoechst 33342 has been used for many years to sub-divide these cells based on their DNA content. This approach provides an efficient way to obtain broad categories of male germ cells: pre-meiotic spermatogonia, meiotic spermatocytes and post-meiotic spermatids. The addition of a red filter for Hoechst staining enables further sub-division of spermatocytes depending on sub-stages of meiotic prophase. However, separation of different stage spermatids using Hoechst staining alone is not possible. We recently reported a methodology, combining Hoechst staining with a second DNA dye (SYTO16) that enables the further separation of these cells into three sub-populations: round, early elongating, and late elongating spermatids (Gill et al., Cytometry A 101:529–536, 2022). This method makes it possible to obtain rapidly and simply pure fractions of male germ cells from multiple developental stages from the same animal.

Key words Spermatogenesis, Spermatid, Spermatocyte, Germ cell, FACS, Mouse

1 Introduction

Generation of sperm via postnatal spermatogenesis enables lifelong fertility in male mammals. This process involves the periodic differentiation of a pool of adult stem cells to generate differentiating spermatogonia, a pool of mitotically active spermatogenic precursor cells [2]. These cells then commit to entry into meiosis, and in the mouse, they spend more than 1 week completing meiotic prophase [3]. Following the completion of the two meiotic divisions, haploid spermatids are formed. These cells spend a further week as round spermatids, defined by their nuclear morphology, before initiating a terminal phase of differentiation, during which the nucleus elongates and chromatin undergoes a dramatic re-configuration and compaction [4]. The final result of this compaction is the generation of mature spermatozoa, which exit the testis and enter the epididymis via the rete testis.

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Isolation of male germ cells in specific developmental stages from adult mouse testes has been a research goal for more than 50 years [5]. Early attempts took advantage of the highly different sizes of cells in different stages of spermatogenic differentiation, with meiotic spermatocytes being significantly larger than haploid spermatids (and spermatogonia being of intermediate size). Centrifugal elutriation allows efficient separation of meiotic from post-meiotic cells but requires specialized equipment and multiple animals for isolation [5–7]. Alternatively, isolation of germ cells using via BSA gradient separation (the so-called STA-PUT method; *see* Naro C. et al., Chap. 4, in this volume) can also separate these cellular populations but cannot further separate elongating spermatids of different stages from each other [8]. FACS (fluorescence-activated cell sorting) following staining of testicular cell suspensions with a DNA dye (such as Hoechst 33342) allows for purification of germ cells based on DNA content, such that diploid (spermatogonia), 4C (spermatocytes) and haploid (spermatid) populations can be separated [9]. Unlike elutriation or STA-PUT, FACS is not limited by the initial starting cell number but requires a longer time to sort the same number of cells. While staining of cells with Hoechst 33342 enables the separation of spermatids from spermatocytes, it does not allow easy and efficient separation of elongating spermatids from round spermatids. Taking advantage of the observation that the fluorescence from the DNA dye SYTO16 dramatically increases upon spermatid elongation [10], we recently developed a simple protocol for dual labeling of cells with Hoechst 33342 and SYTO16 that enables efficient separation of meiotic and post-meiotic cells from mouse testes into several highly pure sub-populations [1].

2 Materials

Most materials are made fresh just before sorting (unless noted).

2.1 Single Cell Suspension Generation

1. *DNase I stock solution*: 5 mg/mL DNAse I in ultra-pure water, stored at −20 °C.
2. Gey's balanced salt solution, GBSS.
3. *Collagenase solution*: 200 U/mL Collagenase I (*see* **Note 1**) and 5 μg/mL DNAse I in GBSS. Prepare 12 mL per mouse to be sorted.
4. *Trypsin solution*: Add Trypsin to a final concentration of 0.025% (Dilute stock solution 1:10) in Collagenase solution. Prepare 12 mL per mouse to be sorted.

2.2 Cell Staining

1. 1 mM SYTO16 in DMSO (ThermoFisher).
2. 10 mg/mL Hoechst 33342 in ultra-pure water.
3. 300 μM DRAQ7.
4. Fetal bovine serum, FBS.

2.3 FACS

1. BD FACS Aria III cell sorter.
2. Cell strainers (30 μM and 40 μM).

2.4 Microscopic Quality Control

1. 10-well diagnostic slides.
2. Phosphate buffered saline PBS.
3. *PFA-T*: 1% paraformaldehyde, 0.15% Triton X-100, 50 mM sodium borate, adjust pH to 9.2.
4. *Hypotonic buffer*: 30 mM Tris pH 8.2, 50 mM sucrose, 17 mM sodium citrate dehydrate.
5. *Blocking solution*: PBS + 1% Bovine serum albumin, BSA.
6. Mouse anti-γH2AX monoclonal antibody (Millipore 05-636).
7. Rabbit anti-SYCP3 polyclonal antibody (abcam ab15093).
8. Goat anti-TNP2 polyclonal antibody (Santa Cruz Biotechnology sc-21106).
9. Mouse anti-PRM1 monoclonal antibody (Briar Patch Biosciences MAb-Hup1N-150).
10. DAPI 10 mg/mL DAPI in ultra-pure water.
11. Donkey anti-mouse AlexaFluor 647.
12. Donkey anti-rabbit AlexaFluor 568.
13. Donkey anti-goat AlexaFluor 568.
14. VectaShield mounting media.

3 Methods

All procedures are carried out at room temperature unless otherwise noted.

3.1 Single Cell Suspension Generation

1. Euthanize animals following local animal welfare regulations.
2. Dissect testes from each animal and place them in GBSS in a dish.
3. Remove tunica albuginea from each testis.
4. Wash seminiferous tubules with GBSS briefly (*see* **Note 1**).
5. Transfer tubules from each testis to a tube containing 6 mL Collagenase solution.
6. Incubate tubules for 3 min at 32 °C with agitation.

7. Remove tubules from the incubator and allow them to settle to the bottom of the tube for 1–2 min.
8. Remove supernatant from the tube containing tubules (contains the interstitial somatic cells).
9. Add 6 mL Trypsin solution to each tube.
10. Incubate for 12 min at 32 °C with agitation.
11. Pipette the digests repeatedly using a disposable plastic Pasteur pipette.
12. Incubate tubes for an additional 12 min at 32 °C with agitation.
13. Pipette the digests repeatedly again; there should be no apparent clumps of cells visible following pipetting.
14. Add 1:10 of a volume (600 μL) of FBS to each tube to inactivate the trypsin.
15. Pool samples from multiple testes into a single tube and filter with a 40 μM filter.

3.2 Staining of Cell Suspension

1. Add an additional 10 μL (per testis) of DNase I solution to each tube to prevent clumping of cells.
2. Add 15 μL Hoechst 33342 (per testis) to each tube.
3. Add 8 μL SYTO16 (per testis) to the teach tube.
4. Incubate at room temperature for 40 min (up to 2 h), protected from light.
5. Centrifuge at 250 × G for 10 min at room temperature.
6. Remove supernatant.
7. Re-suspend cell pellets in 1 mL GBSS (per testis).
8. Add 2 μL DNAse I solution per testis to each tube.
9. Add 10 μL DRAQ7 per testis to each tube.
10. Filter cells through a 30 μM filter into a tube appropriate for cell sorting.

3.3 FACS of Stained Cells

1. Set up the FACS Aria III for sorting based on the manufacturer's recommended procedures (Standard sort settings are shown in Table 1).
2. Create a plot comparing FSC-A (linear) and DRAQ7-A (log) and draw a gate to remove very small debris (low FSC-A) and DRAQ7-A positive dead cells (Fig. 1) (*see* **Note 2**).
3. Taking cells gated as lacking DRAQ7 staining, plot Hoechst Blue-A (linear) vs. Hoechst Red-A (linear).
4. Draw gates separating cells with 1C and 4C DNA content using Hoechst Blue intensity. Cells with 4C DNA content are further separated based on their Hoechst red intensity into

Table 1
Settings of BD FACS Aria III used for sorting

Nozzle size	70 μM
Lasers	375 nM
	488 nM
	561 nM
	633 nM
Sheath pressure	70 psi
Sheath buffer	PBS (without Mg^{++} or Ca^{++})
Sort mode	4 Way Purity
Sort mode settings	Yield mask = 0
	Purity mask = 32
	Phase mask = 0
Drop frequency	86,900 Hz
Amplitude	11.8
Drop delay	44.09
Plate voltage	4500 V
Filter sets	Hoechst 33342 Blue: 450/20
	Hoechst 33342 Red: 610LP/670LP
	SYTO16: 502LP/530/30
	DRAQ7: 755LP/780/60

those in early vs. late prophase (early prophase = low Hoechst red intensity).

5. Select plot SYTO16-A (log) vs. FSC-A (log) for cells with haploid DNA content. Draw gates separating cells with low SYTO16 intensity (round spermatids) from those with high SYTO16 intensity (elongating spermatids) (*see* **Note 3**). Elongating spermatids can be further divided into early vs. late based on their size (FSC-A). Early elongating spermatids have higher FSC-A than late elongating spermatids.
6. For each population selected (2× for 4C meiotic spermatocytes and 3× for 1C haploid spermatids), create a plot of FSC-A vs. SSC-A. Draw gates to select the cells that form a high density in each population.
7. Sort cells into 1.5 mL tubes pre-coated with PBS + 2.5% BSA (overnight at 4 °C) containing a remaining 50 μL of PBS.

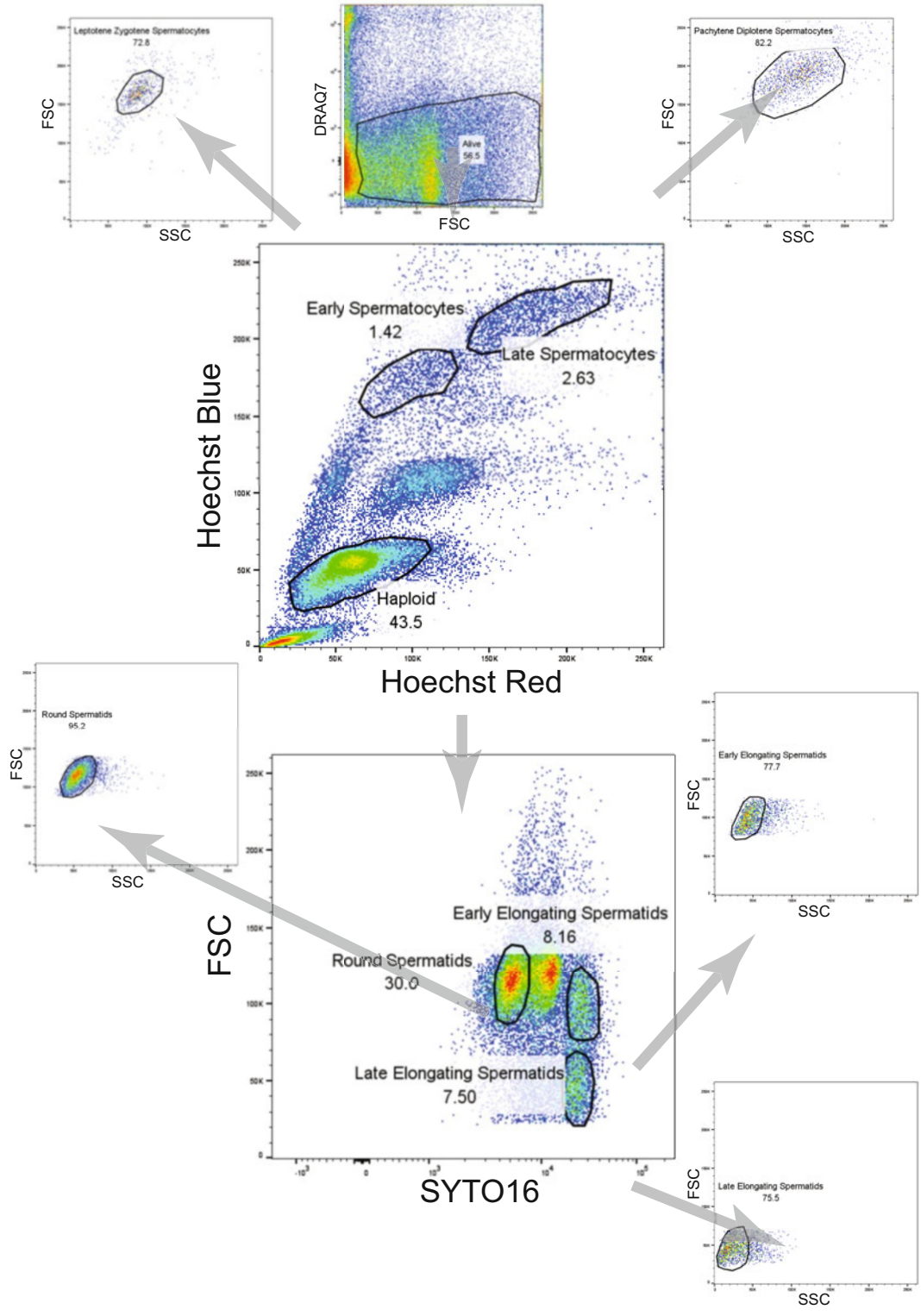

Fig. 1 Gating strategy for isolation of male germ cells. Scheme showing a flow chart of gating to isolate five distinct male germ cell populations. Plots used to separate multiple populations are shown in large plots, while those used to select only single populations are shown in smaller sizes

3.4 Quality Control of Sorted Cells by Immunofluorescence

3.4.1 Preparation of Meiotic Spermatocytes (Based on [11])

1. Place 5–10,000 cells to be analyzed (determined by FACS and/or manual counting with a hemocytometer) into a fresh 1.5 mL tube (usually around 5 μL of directly sorted cells).
2. Add 4× volume of Hypotonic buffer to each tube (typically around 20 μL).
3. Mix very gently by tapping the side of the tube.
4. Incubate for 10 min at room temperature.
5. Add 30 μL of PFA-T to each well of a 10-well slide.
6. Add 10 μL of hypotonically treated cells to each PFA-T-coated well.
7. Allow the cells to dry slowly in the fixative (1–2 h at room temperature or overnight).
8. If analysis is to be performed later, slides can be stored at −80 °C, or immunofluorescence can be performed immediately.

3.4.2 Preparation of Haploid Spermatids

1. Add 30 μL of PFA-T to each well of a 10-well slide.
2. Place 5–10,000 cells to be analyzed (determined by FACS and/or manual counting with a hemocytometer) onto each PFA-T-coated well.
3. Allow the cells to dry slowly in the fixative (1–2 h at room temperature or overnight).
4. If analysis is to be performed later, slides can be stored at −80 °C, or immunofluorescence can be performed immediately.

3.4.3 Immunofluorescence

1. Wash each dried well with 30 μL PBS three times rapidly, allowing the final wash to remain for 5 min at room temperature.
2. Block samples for 20 min at room temperature with 30 μL of Blocking solution.
3. Incubate samples for 1 h at room temperature or overnight at 4 °C with primary antibodies diluted in blocking solution.
   (a) For spermatocytes: Rabbit anti-SYCP3 (1:500) & mouse anti-γH2AX (1:5000).
   (b) For spermatids: Goat anti-TNP2 (1:200) & mouse anti-Prm1 (1:500).
4. Wash each well with 30 μL PBS three times rapidly, and allow the final wash to remain for 5 min at room temperature.
5. Incubate samples for 30 min at room temperature with secondary antibodies (and DAPI) diluted in Blocking solution (*see* **Note 4**).
   (a) For spermatocytes: Donkey anti-Rabbit AlexaFluor 568 (1:500). & Donkey anti-mouse AlexaFluor 647 (1:500) & DAPI (1:1000).

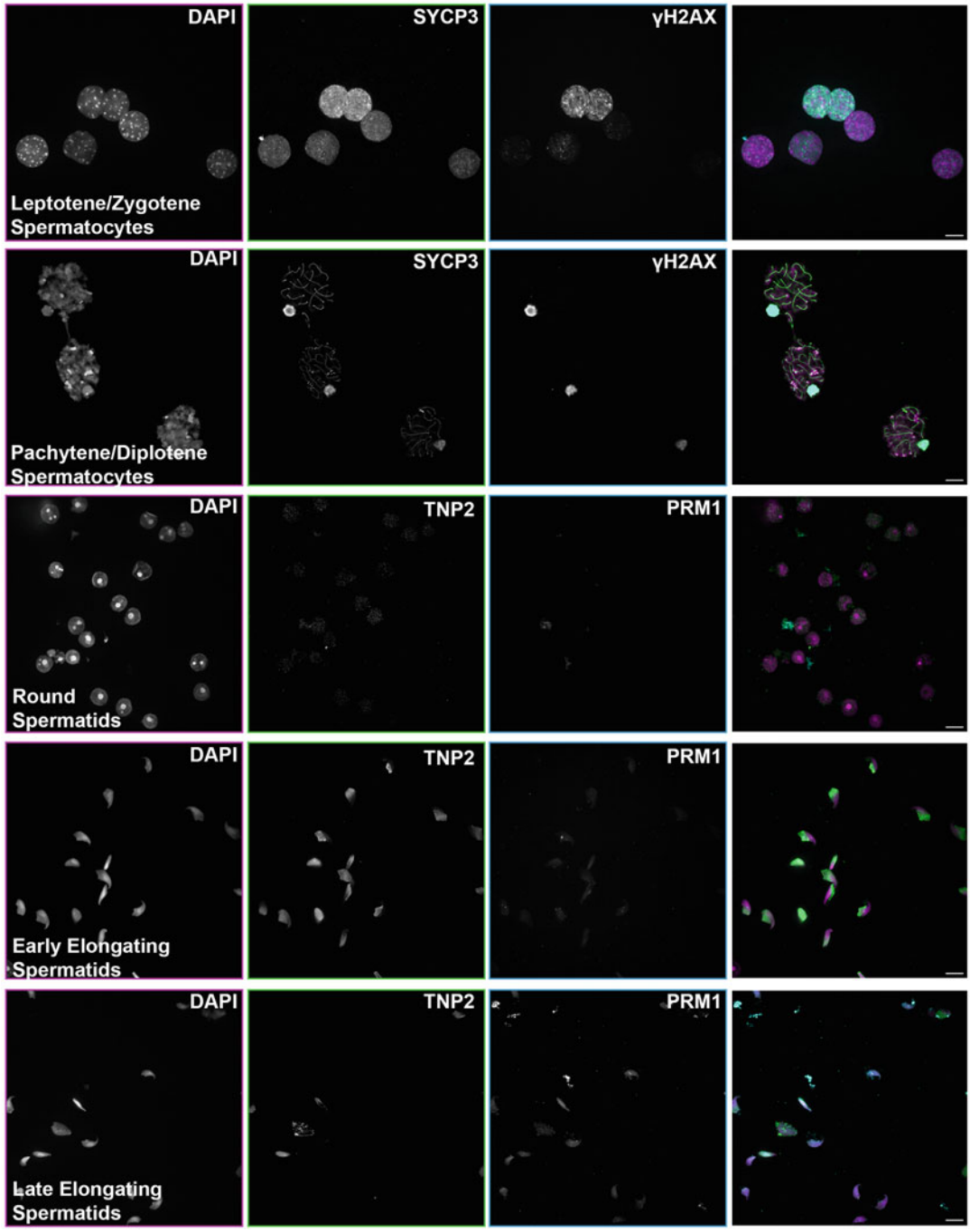

Fig. 2 Quality control of cell populations by immunofluorescence. Images of cells isolated by FACS and stained with antibodies indicate developmental stage. Spermatocytes (Leptotene/Zygotene & Pachytene/Diplotene) are stained with SYCP3 and γH2AX, while spermatids (round, early elongating and late elongating) are stained with TNP2 & PRM1. Scale bars indicate 10 μM

(b) For spermatids: Donkey anti-Goat AlexaFluor 568 (1:500) & Donkey anti-mouse AlexaFluor 647 (1:500) & DAPI (1:1000).

6. Wash each well with 30 μL PBS three times rapidly, and allow the final wash to remain for 5 min at room temperature.
7. Add 4 μL VectaShield mounting media to each well.
8. Cover the slide with a 50 mM coverslip.
9. Secure the coverslip to the slide with nail polish.
10. Image slides on a microscope with fluorescent filters appropriate for the fluorophores used in immunofluorescence.
11. For meiotic spermatocytes: The length and thickness of synaptonemal complexes (marked by SYCP3) can be used to identify which sub-stage of meiotic prophase a cell is in (10) (Fig. 2). The presence of γH2AX in a single nuclear focus (the XY body) is indicative of cells in late meiotic prophase (Pachytene and later), while the presence of γH2AX more broadly throughout the nucleus is indicative of early meiotic prophase.
12. For spermatids: Nuclear shape and size (seen with DAPI staining) can suggest the specific developmental stage (Fig. 2). Round spermatids possess a round nucleus while elongating spermatids show elongated, angular nuclei. Early elongating spermatids are larger than late elongating spermatids, but differences can be subtle. Staining with transition protein antibodies provides a clear distinction, as only early elongating spermatids are positive for this marker (Fig. 2). Protamine staining can be used as an additional marker as these proteins are more clearly abundant in late elongating spermatids than early elongating spermatids.

4 Notes

1. Be careful to minimize the breaking of seminiferous tubules during this step. Elongating spermatids are only weakly attached to the interior of the tubules, so each break of the tubules can potentially decrease the yield of these cells.
2. Elongating spermatids are quite small compared to most somatic cells. We thus keep the location of the gate on the FSC-A axis (to exclude debris) relatively close to the origin to avoid potentially excluding these cells from analysis.
3. Often cell populations with three distinct intensities of SYTO16 are visible in these plots. We generally isolate cells with the lowest and highest intensities. Cells in the middle population consist of a mixture of round and elongating spermatids. If pure separation of these populations is not required,

cells of intermediate SYTO16 levels can be included to increase the total yield of haploid cells.

4. The specific antibodies and fluorophores used in this protocol for quality control could be swapped depending on availability and suitable microscope filters. We generally exclude staining using fluorophores excited at 488 nM, as residual SYTO16 staining can remain after sorting and thus make interpretation of staining in this channel more challenging. If fluorophores in this range must be used for technical reasons, care should be taken to examine markers that are not localized diffusely in the nucleus, as this is the staining pattern observed for SYTO16.

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Chapter 6

Isolation and In Vitro Culture of Germ Cells and Sertoli Cells from Human Fetal Testis

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Abstract

In the human fetal testis, fetal germ cells (FGCs) are progressively surrounded by supporting Sertoli cells inside seminiferous cords. During the second trimester, the FGCs develop asynchronously and can be observed in several stages of development. However, the mechanism that regulates the transition between the different developmental stages as well as the formation of spermatogonia is currently not well understood. For this, it is necessary to develop suitable isolation protocols and a platform for in vitro culture of FGCs of different stages. Here, we report a method to isolate distinct populations of FGCs and Sertoli cells from second trimester human testis using a panel of conjugated antibodies for THY1, PDPN, ALPL, KIT, and SUSD2 for fluorescence-activated cell sorting (FACS) followed by in vitro culture up to 7 days. This platform provides the base for cellular and molecular characterization of the different testicular cell populations to investigate the transition between FGCs and spermatogonia and shed some light on crucial processes of early human gametogenesis unknown until now.

Key words Fetal germ cells, Sertoli cells, Human testis, Fluorescence-activated cell sorting, In vitro culture, Immunofluorescence

1 Introduction

In humans, the precursors of testicular fetal germ cells (FGCs), termed primordial germ cells (PGCs), have been recently identified in cultured 12 day human pregastrulation embryos as positive for NANOG, SOX17, POU5F1, and PDPN [1, 2] and in 16–18 day human gastrulating embryos using single-cell transcriptomics [3]. This is around week 2–3 of development (WD2–3), corresponding to week 4–5 of gestation (WG4–5). Thereafter, PGCs move into the dorsal part of the yolk sac wall and the hindgut

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endoderm and finally migrate toward the gonadal ridges, which they colonize around WD5–6 [4].

In the developing testis, PGCs become progressively enclosed within the seminiferous cords by pre-Sertoli cells; this starts around WD7 and continues up to the end of first trimester of gestation. Throughout the first trimester, PGCs are rather homogeneous and can be identified by the expression of markers such as POU5F1 (also known as OCT4), SOX17, TFAP2C, ALPL (also known as TNAP), and PDPN [5–7]. The total number of PGCs per testis shows an exponential increase between WD6–9 from an average of 10,647 to 74,626 [8].

During the second trimester, FGCs gain heterogeneity, and besides POU5F1+ FGCs (or multiplying M-prospermatogonia), there are also transitional and more mature FGCs (or arrested T1-prospermatogonia) that downregulate POU5F1 and become positive for DDX4 (also known as VASA) and MAGEA3 [6, 7, 9–12]. These changes are associated with the movement of prospermatogonia to the basal lamina of the seminiferous cords, where they give rise to mitotically quiescent T1-prospermatogonia (also termed prespermatogonia) [for a review, *see* [13]]. The migration toward the basal lamina starts around the end of the second trimester of gestation and continues up to 8 months after birth [13]. The number of FGCs at WD16–19 is about 1.5×10^6, and although proliferating rates decrease, low proliferation is still detected in the late gestational period [8]. In contrast to rodents, human prospermatogonia do not appear to undergo a period of complete proliferative quiescence, probably due to the presence of M-prospermatogonia throughout human testicular development.

The somatic gonadal cells, derived from mesoderm, include two testis-specific cell types essential for testicular function: Sertoli cells and Leydig cells. Leydig cells, present in the interstitial space of the testis, express CYP17A1 and STAR and are responsible for the production of testosterone, whereas Sertoli cells, together with the FGCs, are present inside the seminiferous cords and express SOX9 and AMH [9, 14].

Although several studies have characterized the molecular signature of human fetal testis during first and second trimesters using single-cell and spatial transcriptomics [6, 9, 15], very little is known regarding the mechanisms that control the transition from POU5F1+ FGCs to POU5F1−/DDX4+ FGCs and the role of the somatic niche in this transition [7]. This gap in knowledge is also reflected in the limited progress regarding human in vitro gametogenesis [16] and the current failure to maintain human spermatogonia in long-term culture [17] or to differentiate them into sperm cells in vitro. However, such knowledge is essential to develop methods for fertility preservation in boys and men as well as to investigate causes and potential treatments for male infertility [18].

In this study, we describe how to process second trimester intact human fetal testes (WD13–WD19) to isolate live populations of FGCs (KIT-high and KIT-low) as well as Sertoli cells by fluorescence-activated cell sorting (FACS) using a panel of FACS-antibodies for known FGCs markers, such as PDPN and ALPL [12], THY1 (also known as CD90) and SUSD2 [10], and KIT [6, 19]. Moreover, we propose a culture platform to maintain FACS-sorted FGCs with FACS-sorted Sertoli cells acting as a feeder layer, as described for mouse [20, 21]. For maintenance, the culture medium of choice was aRB27 in combination with a vitronectin-coated substrate [11], previously used to culture PGC-like cells (PGCLCs) differentiated from induced pluripotent stem cells (iPSCs) [22]. However, to promote further FGC maturation in culture, optimization with different culture medium, such as that used to induce meiosis in mouse male germ stem cells, is necessary [21]. Successful FACS sorting and in vitro culture were confirmed by immunofluorescence for POU5F1 and DDX4 to identify two different populations of FGCs and SOX9 to identify the Sertoli cells.

2 Materials

2.1 General Reagents, Disposables, and Equipment

2.1.1 Reagents

1. Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} and Mg^{2+} (DPBS−/−), store at room temperature (RT).
2. DPBS with Ca^{2+} and Mg^{2+} (DPBS+/+), store at RT.
3. Saline solution (0.9% NaCl in water), store at RT.
4. Phosphate-buffered saline (PBS), 1× pH 7.4, store at RT.
5. Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM-F12), store at 4 °C.
6. Advanced Roswell Park Memorial Institute (aRPMI) 1640 medium, store at 4 °C.
7. B27 Supplement, store at −20 °C (*see* **Note 1**).
8. GlutaMAX, store at RT.
9. Non-essential amino acids (NEAA), store at 4 °C.
10. MycoZap Plus-CL, store at −20 °C (*see* **Note 1**).
11. Penicillin-Streptomycin (PS) 100×, store at −20 °C (*see* **Note 1**).
12. RevitaCell (RC) 100×, store at −20 °C (*see* **Note 1**).
13. 80% EtOH in water (v/v), store at RT.

2.1.2 Disposables

1. Sterile syringe filter with luer lock (0.22 μm).
2. Syringe (1 mL and 5 mL).
3. Syringe with luer lock (60 mL).

4. Falcon polypropylene tubes (15 mL and 50 mL).
5. Falcon round-bottom polystyrene tubes with cell strainer snap cap (5 mL, for FACS).
6. Ultra-low attachment (ULA) plate (24 well).
7. Chambered μ-Slide 18 well slide with polymer coverslip bottom.
8. Petri dish (6 cm and 10 cm diameter).
9. Sterile pipette tips (10 μL, 200 μL, and 1000 μL).
10. Serological pipettes, plugged (5 mL and 10 mL).
11. Microcentrifuge tubes (1.5 mL).
12. Disposable nitrile gloves.
13. Aluminum foil.
14. Paper tissues.
15. Parafilm.

2.1.3 Equipment

1. Biosafety level 2 (BSL2) laminar flow cabinet.
2. Fume hood.
3. Bead or water bath set at 37 °C.
4. Swinging bucket rotor centrifuge with bucket adapters (for 5 mL, 15 mL, and 50 mL tubes).
5. Tabletop microcentrifuge.
6. Vortex.
7. Micropipettes (P10, P200, and P1000).
8. Pipet boy.
9. Incubator set at 5% CO_2 on air and 37 °C with humidified atmosphere.
10. Ice machine.
11. Benchtop lab water purification system (MilliQ).
12. Cold room or fridge (4 °C).
13. Freezer (−20 °C and −80 °C).
14. Liquid nitrogen storage container.
15. Inverted microscope for immunofluorescence, such as spinning disk confocal microscope (Andor Dragonfly).
16. BD FACS Aria III cell sorter or CytoFLEX SRT Benchtop sorter, to detect the fluorophores in Table 1 and equipped with a 100 nm nozzle.

2.1.4 Facility Rooms

1. BSL2 laboratory.
2. Laboratory space with fume hood.
3. Liquid nitrogen storage room.

Table 1
Fluorophore-conjugated FACS antibodies and reagents (store in dark)

	Conjugated antibody, storage temperature	Fluorophore	Brand, catalog no.	Dilution
1	Anti-human PDPN, 4 °C	PE	Invitrogen, 12-9381-42	1:3000
2	Anti-human KIT, 4 °C	APC	BD Biosciences, 55041	1:100
3	Anti-human SUSD2, 4 °C	VioBright-FITC	Miltenyi Biotec, 130-127-902	1:100
4	Anti-human THY1, 4 °C	Brilliant Violet 421	BioLegend, 328121	1:100
5	Anti-human ALPL, 4 °C	Brilliant Violet 786	BD Biosciences, 742714	1:100
6	Live/dead cell marker, 4 °C	7AAD	BioLegend, 420403	1:200
7	BD CompBeads Anti-Mouse Ig, κ/negative control compensation particles set, 4 °C	–	BD Biosciences, 552843	–

4. FACS facility room (BSL1 or BSL2).
5. Imaging facility room.

2.2 Preparation of Human Fetal Testis for Enzymatic Digestion

1. Human fetal testis (WD16-WD19) (*see* **Note 2**) in saline solution in a petri dish on ice.
2. Small sharp scissors, disinfected with 80% EtOH.
3. Small spoon, disinfected with 80% EtOH.
4. (Disposable) scalpel, disinfected with 80% EtOH.
5. BD Microlance3 needles 30G × ½ (0.3 mm × 13 mm).
6. Stereo microscope inside a BSL2 laminar flow cabinet.
7. aRB27 medium (aRB27): aRPMI 1640, 1× GlutaMAX, 1× NEAA, 1:100 B27, 1:1000 MycoZap Plus-CL; prepared inside a BSL2 laminar flow cabinet. Store at 4 °C up to 2 weeks.
8. aRB27 medium with RevitaCell (aRB27 + RC): aRB27, 1× RevitaCell; prepared inside a BSL2 laminar flow cabinet. Store at 4 °C up to 2 days.

2.2.1 Fresh Human Fetal Testis

2.2.2 Cryopreservation and Thawing Human Fetal Testis

1. Bambanker cell freezing medium.
2. Cryovials, round bottom (2 mL).
3. Freezing container.

4. DMEM-F12 with 1× PS (DMEM-F12 + PS), prepared inside a BSL2 laminar flow cabinet. Store at 4 °C up to 2 weeks.
5. aRB27 + RC.

2.3 Enzymatic Digestion of Human Fetal Testis Pieces into Single Cells

1. Fetal gonad dissociation stock solution (4×), prepared inside a BSL2 laminar flow cabinet. See components in Table 2 (*see* **Note 3**). Dissolve 100 mg Collagenase IV in 17,6 mL PBS+/+ and add 2 mL of 25 mg/mL Hyaluronidase and 0.4 mL of 5kU/mL DNase I, to make a total of 20 mL stock solution. Sterilize by passing through a 0.22 μm syringe filter. Store 1 mL aliquots at −20 °C (*see* **Note 1**).
2. 0.25% Trypsin-EDTA, store aliquots at −20 °C (*see* **Note 1**).
3. Nylon mesh cell strainer (70 μm).
4. FACS buffer: 0.5% bovine serum albumin (BSA) in DPBS−/−, store at 4 °C up to 6 months (*see* **Note 4**).

2.4 Isolation of FGCs and Sertoli Cells by FACS Sorting

1. Conjugated FACS antibodies (Table 1) (*see* **Note 5**).
2. Ice box to use in BSL2 laminar flow cabinet.
3. Negative and IgG-positive compensation beads (Table 1), store at 4 °C.
4. FACS tubes: Falcon round-bottom polystyrene tubes with blue cell strainer cap (5 mL).
5. BD FACS Aria III cell sorter or CytoFLEX SRT Benchtop sorter, with filters to detect the fluorophores in Table 1 and equipped with a 100 nm nozzle.
6. aRB27 + RC.

2.5 Culture of Sorted FGCs on Sorted Sertoli Cells

1. Chambered μ-Slide 18 Well slide with polymer coverslip bottom.
2. Human recombinant vitronectin XF (VTN), store at −20 °C (*see* **Note 1**).
3. FACS-sorted cells.
4. aRB27 + RC.

2.6 Immunofluorescence of Cultured Sorted FGCs and Sertoli Cells

1. 0.2 M phosphate buffer, pH 7.4, store at 4 °C (*see* **Note 6**).
2. 4% paraformaldehyde (PFA): 1:1 (v/v) of 8% PFA in MilliQ water (*see* **Note 7**) and 0.2 M phosphate buffer. Mix fresh and keep on ice until use.
3. Permeabilization buffer: 0.2% Triton X-100 in 1× PBS, store at RT up to 1 month.
4. Blocking buffer: 1% BSA and 0.1% Tween-20 in 1× PBS, store at 4 °C up to 2 weeks.

Table 2
Components of the fetal gonad dissociation stock solution (4×)

	Reagent, storage temperature	Brand, catalog no.	Concentration
1	DPBS+/+, 4 °C	Gibco, 14040117	1×
2	Collagenase IV, 4 °C	Gibco, 17104019	5 mg/mL
3	Hyaluronidase from sheep testes, −20 °C	Merck, H2126-100MG	2.5 mg/mL
4	DNase I from bovine pancreas, −20 °C	Merck, D4527-10KU	0.1 kU/mL

Table 3
Primary antibodies for immunofluorescence

	Cell Type	Primary antibody, storage temperature	Cellular compartment	Brand, catalog no.	Dilution
1	DDX4+ FGCs	Goat anti-human DDX4, 4 °C	Cytoplasm	R&D Systems, AF2030	1:1000
2	POU5F1+ FGCs	Mouse anti-human POU5F1, 4 °C	Nucleus	Santa Cruz, sc-5279	1:100
3	Sertoli cells	Rabbit anti-human SOX9, 4 °C	Nucleus	Chemicon, AB5535	1:150

Table 4
Secondary antibodies for immunofluorescence and reagents (store in dark)

	Fluorophore	Secondary antibody, storage temperature	Brand, catalog no.	Dilution
1	Alexa Fluor 647	Donkey anti-goat, 4 °C	Invitrogen, A-21447	1:500
2	Alexa Fluor 488	Donkey anti-mouse, 4 °C	Invitrogen, A-21202	1:500
3	Alexa Fluor 555	Donkey anti-rabbit, 4 °C	Invitrogen, A-31572	1:500
4	–	0.1 mg/mL (w/v) or 300 μM 4′,6-diamidino-2-phenylindole (DAPI) in water, 4 °C	Invitrogen, D3571	1:1000

5. Washing buffer: 0.1% Tween-20 in 1× PBS, store at RT up to 1 month.
6. Primary antibodies for immunofluorescence to identify FGCs and Sertoli cells (Table 3).
7. Secondary antibodies for immunofluorescence staining (Table 4).

3 Methods

Perform all procedures wearing disposable gloves and under a BSL2 laminar flow cabinet at RT unless stated otherwise.

3.1 Preparation of Human Fetal Testis for Enzymatic Digestion

If using freshly isolated testes, proceed to Subheading 3.1.1 and skip Subheadings 3.1.2 and 3.1.3. Alternatively, if it is desired to cryopreserve testis samples for later use, proceed to Subheading 3.1.2.

3.1.1 Using Freshly Isolated Human Fetal Testis

1. With the testis in a petri dish containing saline solution, remove the mesonephros/epididymis in case it is still attached to the testis, using small sharp scissors under a stereo microscope (Fig. 1a).
2. Pipette 300 μL of aRB27 + RC as a droplet in the inverted lid of a petri dish and place the testis in the drop using a small spoon.
3. Under a stereo microscope, pin the testis down using a needle attached to a 1 mL syringe (for easy holding), and using a scalpel make a sharp longitudinal incision through the tunica layer, but without cutting through the testis (Fig. 1a). Alternatively, attach a needle to another 1 mL syringe and bend the needle, using the tip to cut through the tunica layer (*see* **Note 8**).
4. To remove the tunica, use the bent needle (Fig. 1a) as a scraper. While keeping the testis pinned down with the straight needle, use the bent needle to carefully roll or scrape the testis in the opposite direction of where you hold it to "unwrap" it from the tunica (Fig. 1a). Continue along multiple edges as needed. Discard any pieces of tunica that may come loose during the process, but be careful to keep the seminiferous tubules. Discard the tunica, as this contains mostly fibroblasts.
5. Holding the testis with the straight needle, cut the testis with the scalpel into about 20 pieces (1 mm × 1 mm × 1 mm) (*see* **Note 8**) (Fig. 1a).
6. For one testis, pipette 1 mL/well aRB27 + RC into 3–4 wells of a 24 well ULA plate. Cut the tip of a P1000 tip to obtain a wide-bore pipette tip (using disinfected scissors). With the wide-bore tip, pipette 6–7 testis pieces per well (*see* **Note 9**).
7. Place the 24 well ULA plate with the testis pieces in the incubator overnight.

3.1.2 Cryopreservation and Thawing of Human Fetal Testis

1. Follow the steps described in Subheading 3.1.1, **steps 1–5**.
2. Add 500 μL Bambanker cell freezing medium to each cryovial.

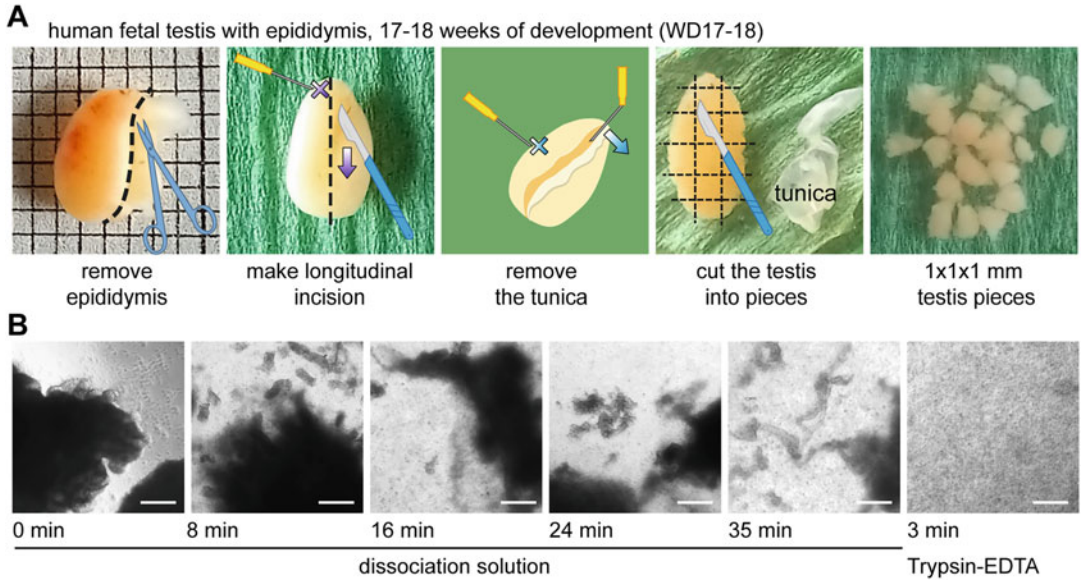

Fig. 1 Preparation of single cell suspension from human fetal testis. (**a**) From testis to small testis pieces. The mesonephros/epididymis is removed with surgical scissors and an incision is made through the tunica using a scalpel (purple arrow) while holding the testis with a needle (purple cross). The tunica is peeled off using a bent needle (blue arrow) while holding the testis with a needle (blue cross). The testis is then cut in 1 × 1 × 1 mm pieces with a scalpel. (**b**) From small testis pieces to single cells. Progression of the testis pieces during enzymatic digestion using dissociation solution followed by treatment with Trypsin-EDTA. Scale bars: 250 μm

3. From the testis pieces in the petri dish, remove as much aRB27 medium as possible and add 500 μL Bambanker to the testis pieces, not to dilute the freezing medium.
4. Using a wide-bore P1000 tip, pipette 6–7 testis pieces per cryovial. Ensure all pieces are submerged in freezing medium in the cryovial.
5. Place the cryovials in a freezing container and place it in a −80 °C freezer overnight. The following days, transfer the cryovials to liquid nitrogen for long-term storage.

3.1.3 Using Cryopreserved-Thawed Human Fetal Testis

Although it is possible to use cryopreserved-thawed human testis to isolate FGCs and Sertoli cells for culture, note that the total cell number per testis-equivalent and yield from the different cell populations is lower when compared to fresh testis (*see* Subheading 3.3.1).

1. Per testis-equivalent, warm 10 mL DMEM-F12 + PS in a 15 mL Falcon tube in a bead or water bath set to 37 °C.
2. Thaw the cryovial(s) in the bead or water bath until a small clump of "ice" remains and quickly place the cryovial(s) and the

Falcon tube with DMEM-F12 + PS in the BSL2 laminar flow cabinet.

3. Drop by drop, pipette 1 mL warm DMEM-F12 + PS into each cryovial. Pipette the liquid from the cryovial into the Falcon tube containing warm DMEM-F12 + PS, leaving the testis pieces in the cryovial. Repeat this process once more, then pipette the content of the cryovial, including the testis pieces, into the Falcon tube containing warm DMEM-F12 + PS, using a wide-bore P1000 tip if necessary.
4. Spin down the 15 mL Falcon tube for 3 min at 200 × *g* in a swinging bucket centrifuge. Discard the supernatant, add 5 mL of warm aRB27 + RC per testis-equivalent, and distribute 6–7 pieces/well (3 wells with about 1.5 mL aRB27 + RC/well) of a 24 well ULA plate, using a wide-bore P1000 tip if necessary.
5. Place the 24 well ULA plate with the testis pieces in the incubator overnight.

3.2 Digestion of Human Fetal Testis Pieces into Single Cells

1. With a wide-bore P1000 tip, pipette all the testis pieces from one testis-equivalent into a 15 mL Falcon tube, wash the wells with DPBS−/− and spin down the Falcon tube for 4 min at 200 × *g* in a swinging bucket centrifuge.
2. Prepare the fetal gonad 1× dissociation solution by thawing 1 mL aliquot of the dissociation stock solution (4×) and add 3 mL DPBS+/+.
3. Aspirate the medium from the Falcon tube and add 3 mL of 1× dissociation solution per testis-equivalent (20 pieces).
4. Transfer 1 mL 1× dissociation solution and 6–7 pieces per well of a 24 wells ULA plate with a wide-bore P1000 tip (total of 3 wells). Place the plate in the incubator.
5. Every 5 min, take the plate out of the incubator to mechanically disrupt the pieces with a P1000 pipette (*see* **Note 10**). Monitor progress under a stereomicroscope. Repeat until there are no big clumps left and most seminiferous cords have broken down into smaller pieces. This will take 25–35 min (Fig. 1b).
6. Transfer the digested tissue to a 15 mL Falcon tube, add 5 mL FACS buffer (*see* **Note 11**), to stop the enzymatic digestion (*see* **Note 12**).
7. Centrifuge for 6 min at 220 × *g*, carefully aspirate the supernatant, and resuspend the pellet in 500 μL of 0.25% Trypsin-EDTA per testis-equivalent. Transfer the cell suspension to one well of a 24 well ULA plate and place the plate in the incubator for 3 min.
8. Mechanically disrupt any pieces and confirm under the microscope that most of the tissue has been digested into single cells (Fig. 1b). If there are still clumps left, return the plate back into

the incubator for 2 additional minutes before again mechanically disrupting the tissue.

9. Transfer the cell suspension into a 15 mL Falcon tube with 5 mL FACS buffer (*see* **Note 11**) to quench the digestion. Wash the well with 1 mL additional FACS buffer.
10. Place a 70 μm nylon mesh cell strainer on a 50 mL Falcon tube and pre-wet with 1 mL FACS buffer (*see* **Note 13**). Use the P1000 micropipette to pass the cell suspension through the filter in one go, maintaining the tip in vertical orientation in contact with the cell strainer. Wash the filter with 1–2 mL FACS buffer. Remove the filter, place the cap of the 50 mL Falcon tube, and centrifuge the cells for 7 min at 220 × *g*.
11. Aspirate the medium and resuspend the cell pellet in 1 mL FACS buffer per sample.

3.3 Isolation of FGCs and Sertoli Cells by FACS Sorting

3.3.1 Testis Sample

1. For this procedure, keep all reagents and cells as much as possible on ice and covered with aluminum foil (dark).
2. Prepare the FACS antibody panel solution (*see* **Note 5**) (Fig. 2) by first preparing a 30× dilution of the PDPN antibody (1 μL antibody in 29 μL in FACS buffer). Thereafter, add 1 μL of the 30× dilution of PDPN antibody and 1 μL of each of the other conjugated antibodies per 100 μL final volume FACS buffer (Table 1).
3. From the 1 mL testis cell suspension in FACS buffer, keep cells to set the FACS gates and thresholds. For this, pipette 20–50 μL to microcentrifuge tubes for unstained control and for (optional) single conjugated antibody controls (*see* **Note 14**). If using compensation beads, *see* Subheading 3.3.2.
4. Transfer the rest of the cell suspension to be immunostained with the FACS antibody panel solution to a microcentrifuge tube, centrifuge for 5 min at 220 × *g*, and remove the supernatant.
5. Resuspend the cells to be FACS sorted in 100–200 μL FACS antibody panel solution and incubate on ice for 30 min with periodical mixing (Fig. 3). For the single staining control, use the same antibody concentration as for the panel solution.
6. After the incubation period, add 800 μL FACS buffer to each cell suspension sample.
7. Pre-wet the strainer cap FACS tubes with 300 μL FACS buffer (*see* **Note 13**). Transfer each sample from the microcentrifuge tube to a FACS tube passing it through the blue cell strainer cap.
8. Centrifuge the cell suspension with the FACS antibody panel for 5 min at 220 × *g*, remove supernatant (*see* **Note 15**), add 200 μL FACS buffer, and resuspend the cells.

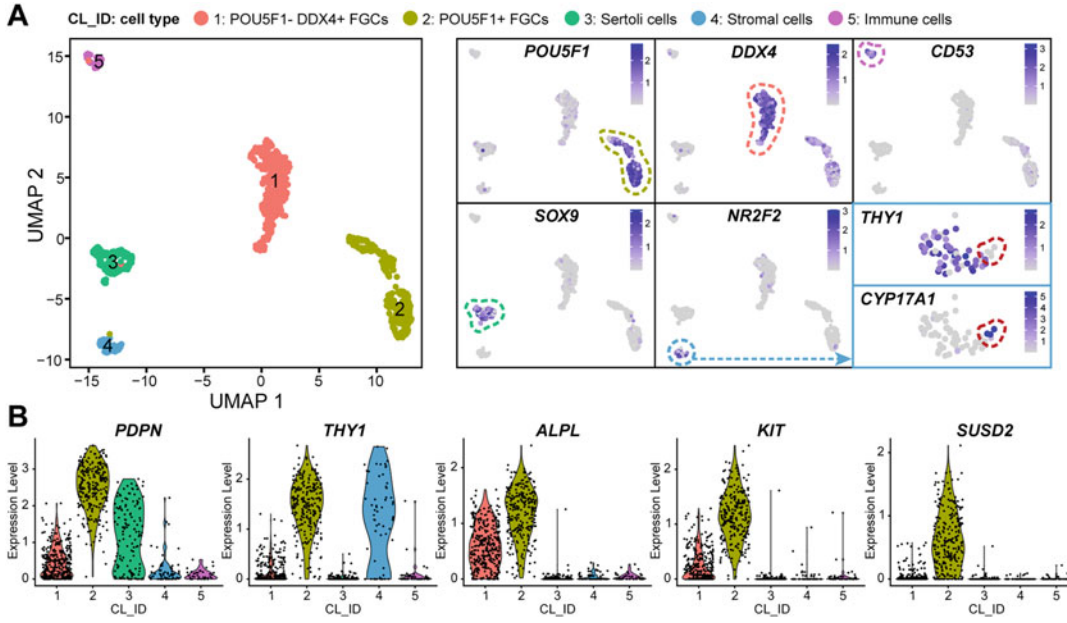

Fig. 2 Gene expression level associated to the selected FACS antibodies using single-cell transcriptomics data from human fetal testis. (**a**) Uniform manifold approximation and projection (UMAP) visualization of single-cell transcriptomics data from human fetal male gonads (WD17–WD23) extracted from an online available dataset [6]. As expected, five clusters (CL) corresponding to the main cell types in the testis were identified: CL1 corresponded to *POU5F1-DDX4+* FGCs, CL2 to *POU5F1+* FGCs, CL3 to *SOX9+* Sertoli cells, CL4 to *NR2F2+* stromal cells, which included a small subpopulation of *THY1−/CYP17A1+* Leydig cells (see blue box to the right), and CL5 to *CD53+* immune cells. (**b**) Violin plots depicting gene expression levels associated with the FACS antibodies used to identify the different main cell types present in the human fetal testis

9. Prepare microcentrifuge tubes with 300 μL aRB27 + RC to collect the different populations of FACS sorted cells.
10. Bring all the samples on FACS tubes, 7AAD reagent, collection microcentrifuge tubes, and a 15 mL Falcon tube with extra FACS buffer on ice to the FACS sorter (*see* **Note 16**).
11. One by one, analyze and record (*see* **Note 17**) the unstained sample and single stain controls and perform compensation. Add 1 μL 7AAD reagent just prior to FACS analysis.
12. From the bulk of cells, gate the population 1 (P1) from SSC-A and FSC-A, excluding cell debris. From the cells in P1, gate the population of singlets from SSC-H and SSC-W (P2) and FSC-H and FSC-W (P3). Intersect P1, P2, and P3 and subsequently gate the live cells (P4) from the 7AAD negative population (Fig. 4a).
13. Using the proposed combination of conjugated antibodies, there are several populations that can be FACS sorted, including THY1−/PDPN+ Sertoli cells (*see* **Note 18**) and THY1+/PDPN+ FGCs. THY1+/PDPN+ FGCs can be further

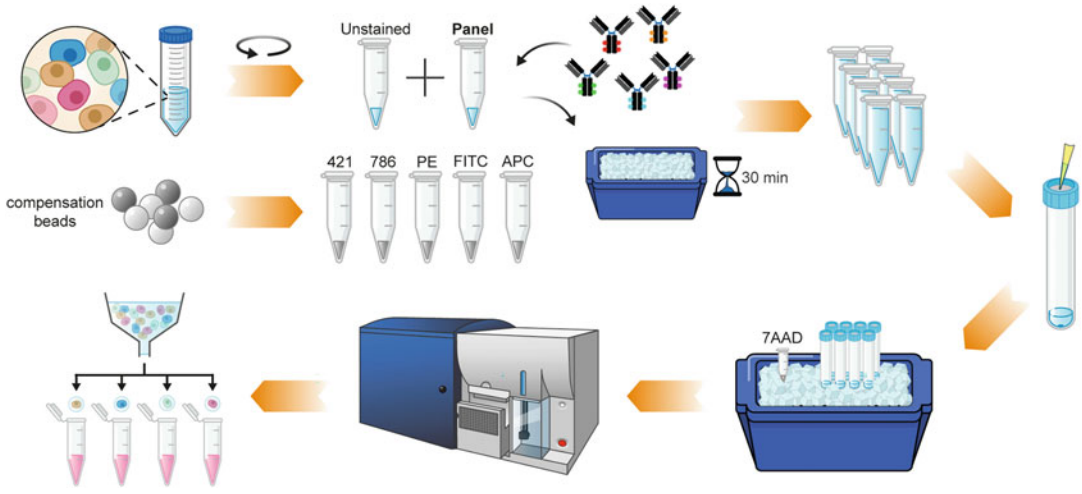

Fig. 3 Schematic representation of the FACS workflow. The cell suspension from one human fetal testis-equivalent (blue) and the compensation beads (grey) are divided over microcentrifuge tubes and incubated with the respective FACS antibodies. Immunostained cells are topped up with FACS buffer and the contents are pipetted through the filter cap of their respective FACS tubes. FACS tubes and 7AAD reagent are taken on ice to the FACS sorter where cells are sorted into collection microcentrifuge tubes

separated in SUSD2-high/KIT-high FGCs and SUSD2-low/KIT-low FGCs (Fig. 4b). Note that the combination PDPN/THY1 or PDPN/ALPL or KIT/THY1 is sufficient to isolate the three populations of interest (Sertoli cells, KIT-high FGCs and KIT-low FGCs) although with less purity (Fig. 4c).

14. If the flow rate is above 6000 events/second for FACS sorting using a 100 μm nozzle, add 100–300 μL FACS buffer to dilute the cell suspension and measure the flow rate (repeat until the flow rate is bellow 6000 events/second). The sorting efficiency decreases if the cell concentration is too high. If the cells are in the FACS tube for prolonged periods of time, they may clump together. To remove the clumps, pass the cells again through the blue cell strainer cap using a P200 pipette.
15. Due to the cryopreservation procedure, both the total number of cells per testis-equivalent (Fig. 4d) and the yield from the different cell populations are lower when compared to fresh testis (Fig. 4e).

3.3.2 Compensation Beads (If Using)

1. For this procedure, keep all reagents as much as possible covered with aluminum foil (dark).
2. Thoroughly vortex the negative and positive IgG compensation beads for single stain controls and add 1 drop of negative and 1 drop of positive compensation beads to a microcentrifuge tube containing 500 μL FACS buffer. Thoroughly vortex and pipette 100 μL diluted compensation beads into different microcentrifuge tubes.

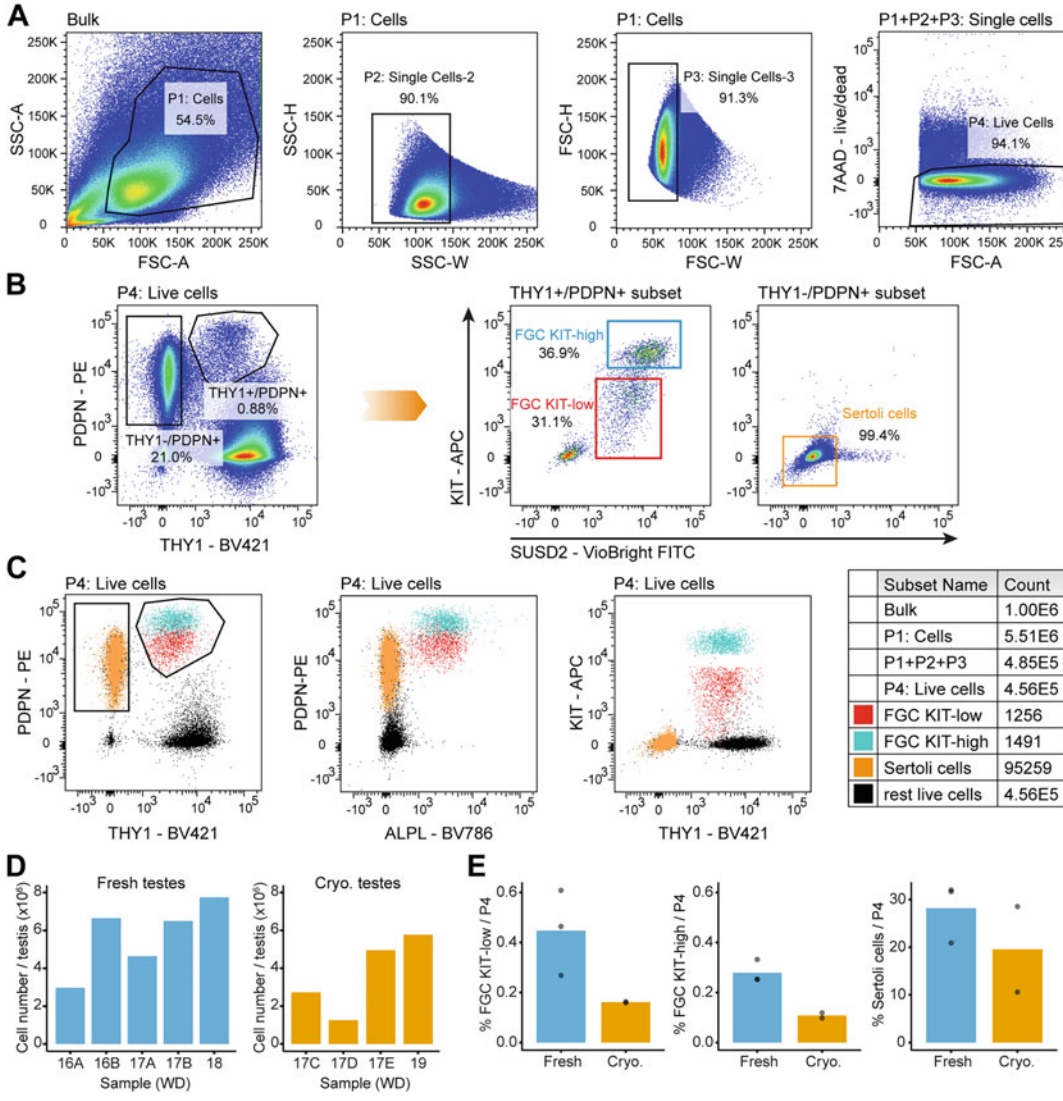

Fig. 4 FACS gating strategy used for human fetal testis. (**a**) FACS gating strategy to gate live cells from bulk, after compensation of all channels used. (**b**) FACS gating strategy used to separate FGC and Sertoli cell populations using the selected FACS antibodies (PDPN-PE and THY1-BV421 followed by KIT-APC and SUSD2-VBFITC). (**c**) FACS plots showing backgating of the three sorted populations (FGC KIT-high, FGC KIT-low, and Sertoli cells) in the different channel combinations depicted. The table indicates the cell number of each gated population per one million events analyzed (Bulk). (**d**) Bar plots show total cell counts obtained per fresh or cryopreserved (cryo.) testis equivalent from different weeks of development (WD) after digestion. Samples of equal WD were differentiated with a letter suffix. (**e**) Bar plots show percentage of the three sorted populations (FGC KIT-high, FGC KIT-low, and Sertoli cells) obtained per fresh or cryopreserved (cryo.) testis equivalent from P4 (live cells)

3. Per microcentrifuge tube, add each of the FACS antibodies using twice the concentration indicated in Table 1, and for PDPN-PE using 1:100 instead of 1:3000. Thoroughly vortex each tube and incubate at RT for 20 min.
4. Transfer contents of each microcentrifuge tube to FACS tubes (no filtering), add 2 mL FACS buffer, centrifuge at 200 × *g* for 10 min, and discard supernatant (*see* **Note 15**).
5. Per FACS tube, resuspend the pellet in 100 µL FACS buffer. Thoroughly vortex before using in the FACS.

3.4 Culture of FGCs on Sertoli Cells

1. Thaw an aliquot of VTN at RT and dilute 100× in DPBS−/− to a concentration of 10 µg/mL (*see* **Note 19**). Pipette 100 µL VTN dilution per well of the 18 Well µ-Slide and leave for 60 min at RT (*see* **Note 20**).
2. Spin down the microcentrifuge collection tubes with FACS sorted cells for 5 min at 220 × *g* and resuspend in adequate volume of aRB27 + RC (150 µL per 18 Well µ-Slide well) (*see* **step 4**). Do not pipette the cells up and down excessively.
3. Remove the VTN coating solution from the 18 Well µ-Slide.
4. Per well, plate 25,000–50,000 Sertoli cells (*see* **Note 21**) and 1000–5000 FGCs in a total volume of 150 µL aRB27 + RC. Shake the dish in quick, successive horizontal and vertical movements to distribute the cells evenly over the well.
5. Place the dish in the incubator.
6. Carefully refresh the medium with aRB27 the day after and every 2 days up to 7 days.

3.5 Immunofluorescence of Sorted FGCs on 18 Well µ-Slide

1. Perform all procedures at RT unless stated otherwise. Be careful with removing and adding solutions to and from the wells as the FGCs may come loose.
2. Remove the medium from each well. Add 50 µL 4% PFA per well and leave for 15 min (*see* **Note 7**).
3. Remove PFA (*see* **Note 7**) and add 150 µL PBS per well for 10 min.
4. Remove PBS and add 50 µL permeabilization buffer per well for 10 min.
5. Remove permeabilization buffer and add 50 µL blocking buffer per well for 30 min.
6. Prepare 50 µL primary antibody master mix (Table 3) in blocking buffer per well.
7. Remove blocking buffer, add the primary antibody master mix, and incubate overnight at 4 °C in a humidified chamber (*see* **Note 22**).

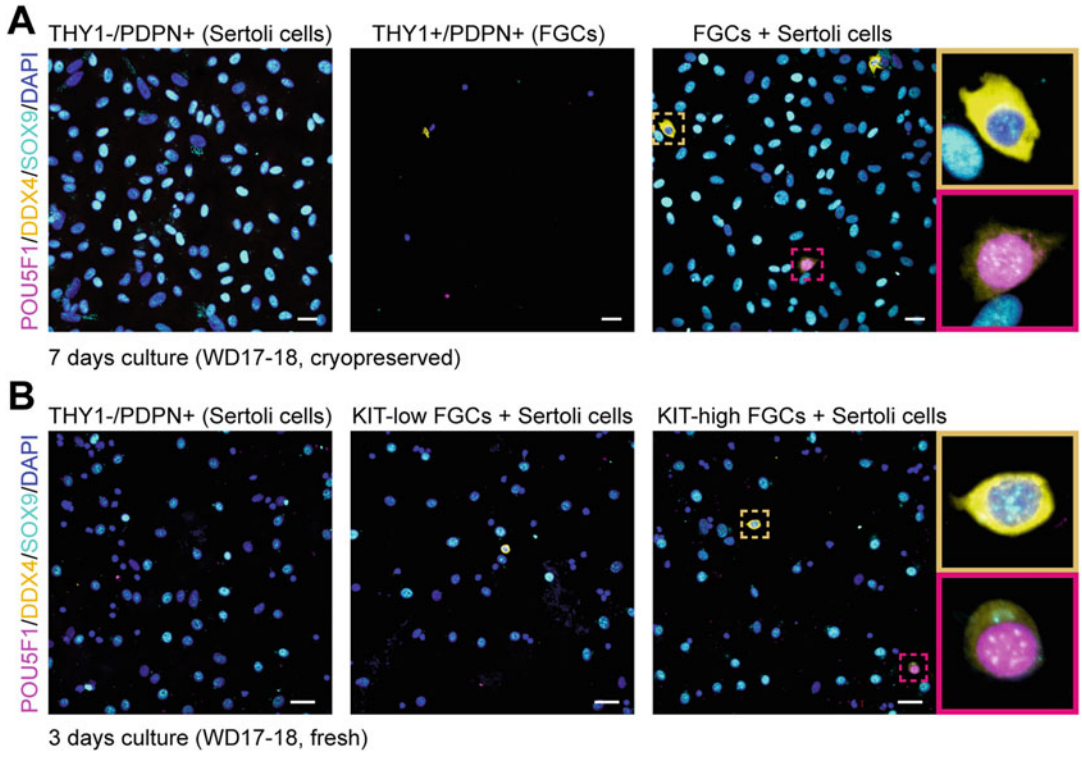

Fig. 5 Identification of FACS sorted populations of the human fetal testis by immunofluorescence. (**a**) Immunofluorescence for SOX9, POU5F1, and DDX4 on FACS sorted THY1−/PDPN+ cells (Sertoli cells), THY1+/PDPN+ cells (FGCs), and a combination of the two after 7 days in culture. Cells were FACS sorted from a WD17–18 testis that has been cryopreserved. Yellow and magenta dashed boxes magnified (right) show a POU5F1-/DDX4+ FGC and a POU5F1+ FGC. Scalebars: 25 μm. (**b**) Immunofluorescence for SOX9, POU5F1, and DDX4 on FACS sorted THY1−/PDPN+ cells (Sertoli cells) alone or cultured with THY1+/PDPN+/KIT-high FGCs or THY1+/PDPN+/KIT-low FGCs for 3 days. Cells were FACS sorted from a freshly isolated WD17–18 testis. Yellow and magenta dashed boxes magnified (right) show a POU5F1-/DDX4+ FGC and a POU5F1+ FGC. Scalebars: 25 μm

8. Remove the primary antibodies and wash with 150 μL washing buffer per well, 2× for 10 min.
9. Prepare 50 μL secondary antibody master mix with DAPI (Table 4) in blocking buffer per well.
10. Remove the washing buffer and add the secondary antibody master mix. Cover with aluminum foil (or in the dark) and incubate for 60 min.
11. Remove the secondary antibodies, wash twice with 150 μL washing buffer per well, and store in 150 μL PBS. The plate should be sealed with parafilm and stored in the dark at 4 °C until ready to be imaged using an inverted microscope for immunofluorescence, such as spinning disk confocal microscope.

12. Immunofluorescence revealed that after 7 days in culture THY1+/PDPN+ FGCs (both POU5F1+ FGCs and POU5F1−/DDX4+ FGCs) did not survive without a co-coculture with THY1−/PDPN+ Sertoli cells (Fig. 5a). In addition, after 3 days in the used (co)culture conditions the KIT-high FGC subset contained both POU5F1+ FGCs and POU5F1−/DDX4+ FGCs, whereas the KIT-low FGC subset contained POU5F1−/DDX4+ FGCs (Fig. 5b).

4 Notes

1. Thaw upon arrival and distribute into smaller aliquots inside an BSL2 laminar flow cabinet. Store aliquots at −20 °C and after thawing the aliquot store at 4 °C. Avoid freeze/thaw cycles.
2. Ethical permit from the local medical ethical committee is required to use human material from elective abortions (without medical indication), as well as signed informed consent from all the donors and a signed material transfer agreement with the institution where the procedure took place. For this work, a letter of no objection was obtained from the Medical Ethical Committee of the Leiden University Medical Center (B21.052). For guidance, the ISSCR has developed standards for informed consent regarding the donation of human fetal tissue for research [23].
3. Prepare aliquots of hyaluronidase and DNase I upon arrival on a BSL2 laminar flow cabinet: dissolve 100 mg hyaluronidase in 4 mL PBS+/+ (25 mg/mL) and freeze aliquots of 2 mL; dissolve 10kU DNase I in 2 mL PBS+/+ (5kU/mL) and freeze aliquots of 0.4 mL. Store single-use aliquots of both components at −20 °C. Avoid freeze/thaw cycles.
4. For 500 mL FACS buffer, weigh 2.5 g BSA fraction V in a 50 mL Falcon tube and dissolve first in 40 mL DPBS−/− from a new, previously unopened bottle of DPBS−/− inside an BSL2 laminar flow cabinet. Filter the BSA solution through a 0.22 μm sterile syringe filter using a 60 mL syringe directly into the freshly opened DPBS−/− bottle.
5. The choice of specific conjugated antibodies for FACS sorting is essential for the isolation of pure population of cells. For this, it is useful to explore the expression of genes of interest using available single-cell transcriptomics datasets from human fetal male gonads (WD17–WD23), such as that provided by Li and colleagues [6]. First, select the appropriate developmental period, in this case second trimester. Then define cell clusters and validate those by plotting the expression of known markers of the cell types of interest (Fig. 2a). Next, plot the expression

of the genes of interest (Fig. 2b) to have an indication of what to expect across the different cell populations in the testis. Note that gene expression can differ from protein expression.

6. To make 0.2 M phosphate buffer, prepare 0.2 M NaH_2PO_4.H_2O [16,56 g in 600 mL water (MilliQ)] and 0.2 M Na_2HPO_4.H_2O [64,68 g in 1800 mL water (MilliQ)]. Add the 0.2 M Na_2HPO_4.H_2O (acid) to the 0.2 M NaH_2PO_4.H_2O (base), until pH 7.4. Store at 4 °C for 6 months.
7. PFA is hazardous: it should be always handled in a fume hood and disposal of solid and liquid waste should follow laboratory safety guidelines. Avoid inhalation and contact. To prepare 8% PFA (w/v) in water, warm water (MilliQ) to about 70–80 °C and add three quarters of the water to the PFA powder, while stirring. After a couple of minutes add 5 drops of 5 N NaOH and observe that the solution changes from milky to transparent. Cool down, add the rest of the water and store at 4 °C for 3 months.
8. Be careful not to drag the testis across the petri dish with the needles and not to cut into the plastic as small plastic shards are difficult to remove afterwards. Once the tunica layer is removed, the testis is very soft and does not need much pressure to be cut. Use single, gentle cuts to cut the testis while holding it still with the needle.
9. Collect all the testis pieces using extra aRB27 + RC if necessary, but avoid collecting plastic shards.
10. To disrupt the pieces, start by repeatedly pipetting the pieces up and down in the solution. When the pieces start breaking up, take up all the solution and pipette down while holding the tip firmly against the bottom of the well.
11. Keep FACS buffer on ice as much as possible.
12. When using cryopreserved testis samples, the treatment with dissociation solution may be sufficient to digest the testis into single cells. In that case, skip the Trypsin digestion step (in Subheading 3.2 skip **steps 7–9**, proceed directly to **step 10**).
13. Pre-wetting the cell strainer with FACS buffer prevents cells from sticking to the filter.
14. As the FGCs have low abundance in the testis (roughly 1–3%) and human fetal testis are difficult to obtain, it is advised to use compensation beads instead to set up compensation thresholds (*see* Subheading 3.3.2).
15. To remove the supernatant, prepare a clean paper tissue and place inside the laminar hood. Carefully remove the cap of the FACS tube without disrupting the pellet. In a single and smooth motion, flip and empty the FACS tube over a waste

tube, and dry the FACS tube opening, still inverted, with the tissue. Do not flip the tube back upright during this process, as this will dislodge the pellet. Approximately 50 μL FACS buffer will remain in the FACS tube. Then invert and place on ice.

16. The CytoFLEX SRT Benchtop sorter uses 15 PSI pressure to take up the sorted sample, whereas the BD FACS Aria III sorter uses 20 PSI. The CytoFLEX sorter is therefore gentler on the FGCs.
17. Record at least 10,000 events of unstained and single immunostained controls (or compensation beads) to set gates and adjust compensation and at least 10,000 events of the immunostaining panel.
18. The Sertoli cells can be used as a feeder layer to culture FGCs.
19. Mix gently and do not vortex. Use immediately to coat the wells.
20. In case the coated wells are not for immediate use, seal the culture dish containing the VTN coating solution with parafilm and store at 4 °C up to 1 week. Place an 18 Well μ-Slide inside a 10 cm petri dish for easy handling.
21. If culturing for less than a week, plate 50,000 Sertoli cells per well.
22. To make a humidified chamber, wet paper tissue with tap water and place it inside a petri dish or small closed container to surround the 18 Well μ-Slide placed inside.

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Part II

Establishing In Vitro Systems for Studying Primordial (and) Germ Cells

Chapter 7

Human Primordial Germ Cell-Like Cell Induction from Pluripotent Stem Cells by SOX17 and PRDM1 Expression

Naoko Irie, Toshihiro Kobayashi, and M. Azim Surani

Abstract

Human primordial germ cell (PGC) development initiates about 2 weeks after fertilization during embryogenesis. Unique molecular events follow, including epigenetic resetting, to establish functional gametes (egg and sperm). Due to the inaccessibility of human embryos, it is essential to have an amenable experimental platform to investigate the mechanisms and potential dysfunctions of the events. We previously established a PGC-like cell (PGCLC) differentiation method using human pluripotent stem cells (PSCs) via induction of precursor cells followed by stimulation with a cytokine cocktail including BMP. We also revealed that the expression of PGC specifiers, SOX17 and PRDM1, can robustly induce PGCLCs from PSCs without the cytokines. The balance of SOX17 and PRDM1 is critical for germ cell fate since the two factors also regulate endoderm differentiation. Here we describe a detailed procedure for PGCLC differentiation with the balanced induction of SOX17 and PRDM1. The protocol can be used for PGC induction in other mammalian species exhibiting PGCs with SOX17 expression. Together, these studies will advance the understanding of germ cell biology and its applications in reproductive technology and medicine.

Key words Human primordial germ cells, Human pluripotent stem cells, Primordial germ cell-like cells, SOX17, PRDM1

1 Introduction

Germ cells, eggs, and sperm pass genetic and epigenetic information to the following generations. In humans, the specification of primordial germ cells (PGCs) occurs around 2 weeks after fertilization at the onset of gastrulation [1–3]. Nascent PGCs migrate through the developing body to reach the gonads, ovary, or testis, where germ cells become functionally mature at puberty. During early development, PGCs undergo unique molecular programming, including epigenetic programming, regulation of non-coding genomic regions, and gene-based regulatory networks [4–9].

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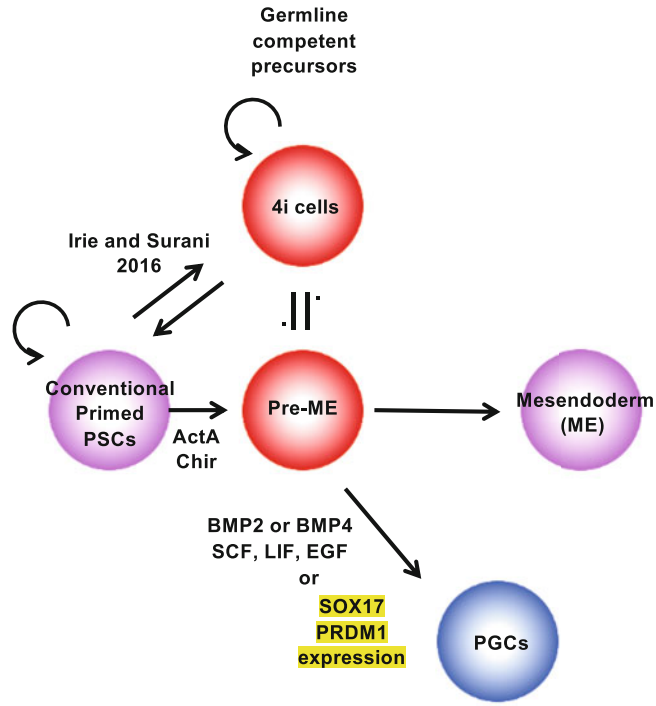

Fig. 1 Overview of PGCLC induction SOX17/PRDM1 from PSCs. PGCLC induction from human PSCs can be performed by inducing precursors (Pre-ME and 4i cells) [25] that are competent for PGC fate in the presence of cytokines BMP2/4, SCF, LIF, and EGF. PGCLCs can also be induced by the transgene expression of SOX17 and PRDM1 from PGC precursor cells

However, the lack of amenable experimental models and the inaccessibility of early human embryos and fetuses have hampered research on human germline development. To address this problem, we developed a robust cell culture model to recapitulate early germline development using human pluripotent stem cells (PSCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) [10, 11]. There are currently two ways for generating PGC precursors: 4i culture [10], and Pre-ME (precursors of mesendoderm, also known as incipient mesoderm-like cells; iMeLCs) [11, 12]. These cells can respond to cytokine cocktails for PGC specification, BMP2 or BMP4, SCF, LIF, and EGF to differentiate into PGCLCs (Fig. 1).

By combining the characterization of in vivo human fetal germ cells and human germ cell cancer, seminoma, we identified transcriptional factors SOX17 and PRDM1 to be essential for PGC specification [10]. Notably, the involvement of SOX17 in the early germline was also observed in a non-human primate, pig, rhinoceros, and rabbit [11, 13–16] but not in rodents, mice and rats [17–20], which express SOX2 instead of SOX17 in PGCs. Rodents and

non-rodent mammals also show significant differences in early embryogenesis, reflecting evolutionary divergence [21].

Strikingly, human PGC fate can be induced by a balanced induction of SOX17 and PRDM1 transgenes in the precursor cells both for 4i cells and Pre-ME cells in the absence of cytokines (Fig. 1) [11]. Alternatively, forced expression of SOX17 with TFAP2C/GATA2/GATA3 or CRISPR-mediated activation of endogenous SOX17 with PRDM1/TFAP2C also allows differentiation of PGCs without external stimuli [11, 22, 23].

Here, we describe a detailed protocol for the induction of PGC fate via forced expression of SOX17/PRDM1. A similar system can induce germ cell lineage for animals exhibiting SOX17 expression in PGCs, including endangered species and livestock animals. Indeed, a recent report showed that these transgenes helped to establish optimal conditions for PGC fate induction from common marmoset PSCs [24]. The alternative protocol for human PGC induction provides a broad opportunity for further exploring human germ cell biology, elucidating the molecular mechanisms of normal development and those leading to disorders, and uncovering the impact on human health and reproduction and the transmission of genetic and epigenetic information to the following generation.

2 Materials

2.1 Reagents

1. Vitronectin (VTN-N) Recombinant Human Protein, Truncated.
2. Lipofectamine™ 2000 Transfection Reagent.
3. Lipofectamine™ Stem Transfection Reagent.
4. Opti-MEM I Reduced Serum Medium.
5. PBS, pH 7.2.
6. DMEM.
7. FCS.
8. L-Glutamine.
9. Penicillin-Streptomycin solution.
10. Essential 8 Medium.
11. Advanced RPMI 1640 medium.
12. B-27 Supplement (50×), serum-free (B27).
13. MEM Non-Essential Amino Acids Solution (100×) (NEAA).
14. TrypLE Express Enzyme (1×), no phenol red.
15. Trypsin-EDTA (0.25%), phenol red.
16. Poly(vinyl alcohol) (PVA).

17. Activin A.
18. CHIR99021.
19. Y-27632 dihydrochloride.
20. Dexamethasone.
21. Doxycycline hyclate.
22. DMEM-F/12.
23. Collagenase Type IV.
24. DNaseI.

2.2 Preparation of Culture Medium

1. MEF medium: 500 mL of DMEM, 50 mL of FCS, 5 mL of L-Glutamine and 5 mL of Penicillin-Streptomycin solution. Store the solution at 4 °C for up to 1 month.
2. 10% (wt/vol) stock solution of PVA: Add 10 g of PVA to 100 mL water (tissue culture grade) with vigorous shaking and then autoclave it at 121 °C for 20 min. Then, store the solution at 4 °C for up to 6 months.
3. aRB27: 38 mL of Advanced RPMI 1640 medium, 400 μL of B27, 400 μL of NEAA, 400 μL of L-Glutamine, 400 μL of Penicillin-Streptomycin solution, and 400 μL of PVA. Store the solution at 4 °C for up to 1 week.
4. Pre-ME medium: 10 mL of aRB27, 100 ng/mL Activin A, 3 μM CHIR99021, and 10 μM Y-27632. Freshly prepare the medium before use.
5. 2× Collagenase/DNaseI solution: dissolve Collagenase Type IV (5.2 mg/mL) in DMEM-F/12, filter the solution through 0.22 μm membrane, and add DNase I (20 U/mL). Make aliquots of an appropriate volume for 1–2 uses (~1 mL) and store at −20 °C.

3 Methods

Please see Irie et al. 2016 [25] for "Maintenance of conventional and 4i PSCs", "Conversion of conventional and 4i PSCs", and "Induction of PGCLCs by cytokines".

3.1 Transfection of Transgenes in 4i PSCs (see Fig. 2)

1. PiggyBac transposon-based plasmid DNA are constructed for dexamethasone (DEX) inducible SOX17, fusion protein with glucocorticoid receptor (*see* **Note 1**), and doxycycline (dox) inducible PRDM1 [11] together with drug-resistant cassettes such as hygromycin B, puromycin or neomycin. Freshly isolated DNA plasmids stored at −20 °C are used for transfection.

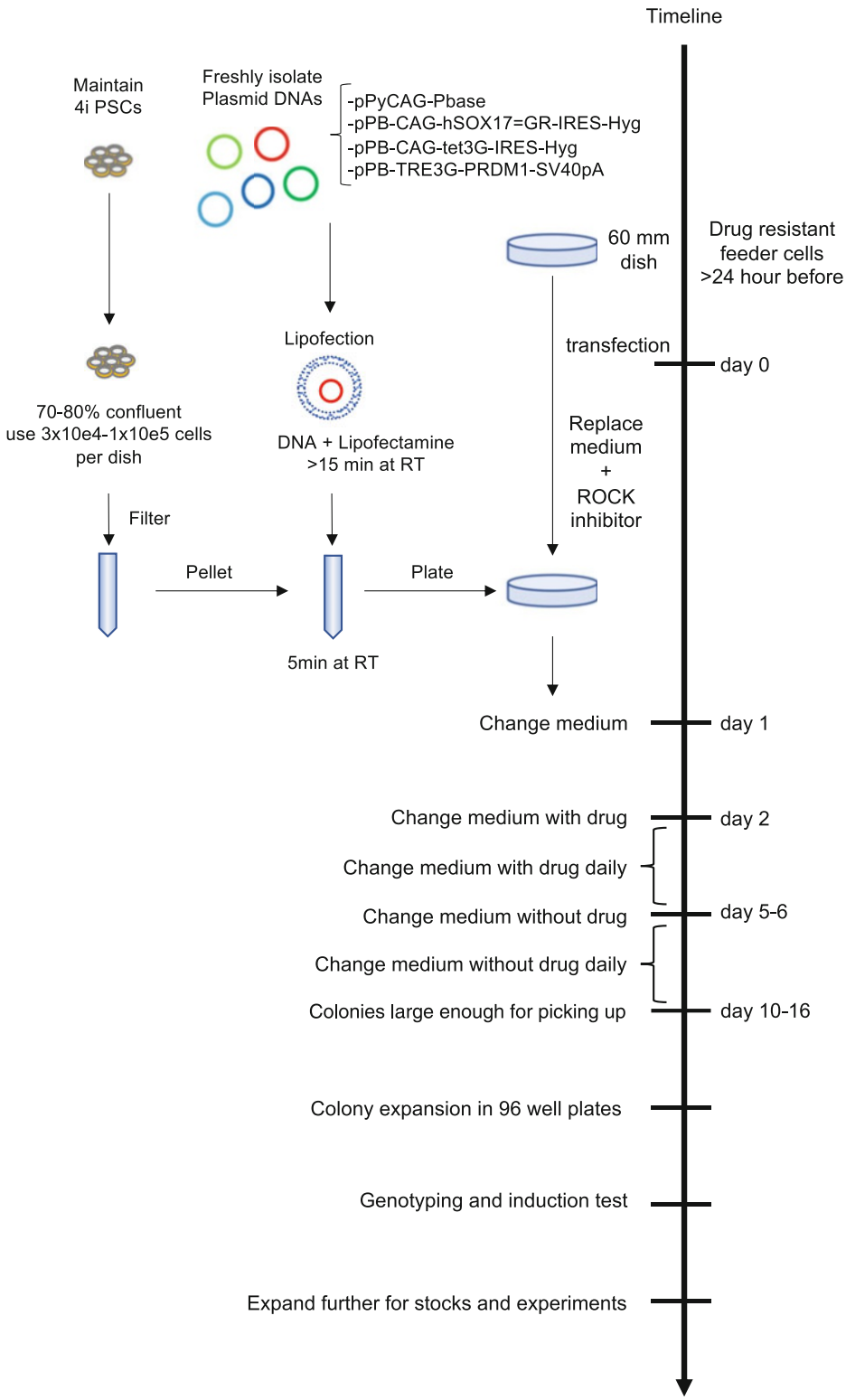

Fig. 2 Procedure for generating inducible SOX17/PRDM1 transgenic PSCs. Experimental timeline for establishing SOX17=GR and PRDM1 dox inducible human PSC clones for PGCLC induction

2. Prepare drug-resistant feeder MEFs in a 60 mm dish >24 h before transfection for using 4i cells.
3. Day 0 of transfection: Prepare DNA and Lipofectamine™ 2000/Lipofectamine™ Stem Transfection Reagent complex. Total 1.2 μg of DNA plasmids: 0.3 μg pPyCAG-PBase, 0.3 μg pPB-CAG-tet3G-IRES-Hyg, 0.3 μg pPB-CAG-hSOX17 = GR-IRES-hyg, 0.3 μg pPB-TRE3G-PRDM1 in 50 μL Opti-MEM are combined with 1.2 μL Lipofectamine™ 2000/Stem Transfection Reagent in 50 μL Opti-MEM and the mixture is incubated for 15 min at room temperature.
4. 70–80% confluent 4i PSCs are washed with PBS once and dissociated with TrypLe (200 μL per 1 well in 6-well plates) by incubating for 3–5 min at 37 °C, 5% CO_2. Add MEF media (1000 μL per 1 well in 6-well plates) to quench the enzymatic activity. The cells are dissociated into single cells and passed through a 50 μm filter and collected in 15 mL conical tubes.
5. Count cell numbers and use $3 \times 10e^4$–$1 \times 10e^5$ cells for transfection. The cell density is essential for picking up the clonal colonies. It is recommended to prepare several dishes with different cell numbers in parallel.
6. Centrifuge and pellet the cells at 500 rcf for 5 min.
7. Remove supernatant as much as possible.
   (Optional: Centrifuge the cells again at 500 rcf, 3 min to collect the remaining liquid to be removed further).
8. Add DNA + Lipofectamine complex from **step 3**, resuspend the cell pellet, and incubate for 5 min at room temperature. It is not recommended to incubate longer than 5 min to avoid damaging the cells.
9. Replace the MEF media in 60 mm dish from **step 2** with 4i media + ROCK inhibitor (10 μM) for 4i cells.
10. Add cell suspension with DNA + Lipofectamine complex to the dish with the appropriate medium, equally distribute the cells across the dish by rocking the dish, and place the dish in the humidified incubator, 37 °C, 5% CO_2.

3.2 Clonal Isolation of Transgene Transfected PSCs (see Fig. 2)

1. Day 1 after the transfection, change media with fresh 4i medium.
2. Day 2 after the transfection, change media with fresh 4i media with appropriate antibiotic drugs with an optimal concentration (Perform drug concentration test in advance to identify the lowest concentration that efficiently kills the cell line in about 3–4 days).
3. Day 3–5 or 6 after the transfection, change media with fresh media with antibiotic drug once a day. Some cell death should be observed (untransfected cells).

4. Day 5 or 6 after the transfection and onwards, replace media with fresh media without the drug once a day until surviving colonies become visible and large enough for manually picking up but not too big as this could induce some undesired differentiation in the undifferentiated colonies.
5. Choose a dish with a colony density sparse enough to be able to isolate individual colonies.
6. Prepare 96-well plate with feeder MEFs 1 day before picking colonies.
7. Prepare 96 U-bottomed plate with 15 μL TrypLe or Trypsin/EDTA 0.25%.
8. Pick colonies with P20 tips or 27G needles with a syringe under the dissection microscope and transfer the detached colony to 96 U-bottomed well with 15 μL TrypLe or 0.25% Trypsin/EDTA.
9. After picking 8–16 colonies, incubate at 37 °C, 5% CO_2 humidified incubator for 10–15 min. Pipette and dissociate the colonies into single cells with 100–150 μL of 4i media with ROCK inhibitor (10 μM) using P200 tips and transfer all the solution to 96-well plates with MEF feeders for 4i cells.
10. Change media daily until colony sizes are ready for passaging.
11. Prepare 24-well plates with feeder cells in advance.
12. Dissociate the cells with TrypLe. Half of one well in 96-well plates can be used for expanding in 24-well plates and the other half can be used for either genotyping for the transgene integration or for functional induction test. The differentiation test is recommended for at least 10–20 clones (*see* **Note 2**), and see the following section for the procedure.

3.3 Induction of PGCLCs with SOX17 and PRDM1 Transgenes from 4i and Pre-ME Cells

The induction of PGCLC by SOX17/PRDM1 can be performed from 4i cells and Pre-ME PGC precursors (Fig. 1). Here we describe induction of Pre-ME and PGCLC induction methods using 4i and Pre-ME cells.

3.3.1 Induction of Pre-ME Cells

1. Aspirate medium and wash PSCs cultured in E8 with PBS.
2. Add 0.2–0.3 mL 0.25% Trypsin/EDTA per well in a 6-well plate.
3. Incubate at 37 °C for 3 min.
4. Add 1 mL of MEF medium and pipet up and down until cells are dissociated into single cells.

   (Optional: use 50–70 μm cell strainer to remove cell clumps.)
5. Centrifuge at 500 rcf for 5 min.

6. Aspirate supernatant and suspend cells in Pre-ME medium (0.2–1 mL).
7. Subject cells to pipetting up and down a few times.
8. Count the number of cells.
9. Seed 2 × 10e^5 cells/well (12-well plate) on a vitronectin-coated plate.
10. Proceed after 10–12 h (Pre-ME) with PGCLC induction.
    (Caution: The precise timings may vary with different cell lines or culture conditions. We recommend determining optimal timing of PGC induction with cytokines with either BMP2 or BMP4 supplemented with LIF, SCF, and EGF.)

3.3.2 Induction of PGCLC by SOX17/PRDM1 from Pre-ME or 4i Cells

1. Aspirate medium and wash cells (Pre-ME cells: normally 12 h after Pre-ME induction) or 4i cells (80–90% confluent) with PBS.
2. Add 0.2–0.3 mL 0.25% Trypsin/EDTA or TrypLE Express per well in a 6-well plate.
3. Incubate at 37 °C, 5% CO_2 for 3 min.
4. Add 1 mL of MEF medium and dissociate into single cells by repeated pipetting.
5. Filter cells through 50 μm sterile single-pack CellTrics filters (Sysmex) into 15 mL conical centrifuge tubes.
6. Centrifuge at 500 rcf for 5 min.
7. Aspirate supernatant and suspend cells in aRB medium.
8. Pipette cells a few times.
9. Count the number of cells.
10. Dilute the cell suspension in aRB medium with final concentration of dexamethasone at 100 μM and doxycycline at 1 μg/mL and adjust the concentration of cells to 4 × 10e^3–1.2 × 10e^4 cells/100 μL/well.
11. Add 100 μL of the cell suspension into a single well of Corning® Costar® Ultra-Low attachment 96-well plates (Corning, 7007).
12. Centrifuge the plate at 1200 rpm for 1–2 min to collect the cells at the bottom of the wells to ensure the aggregates are well formed.
13. Culture for 2 days. The small aggregates are formed within 24 h and develop further over 2 days.

3.4 Isolation and Detection of PGCLCs

1. Collect day 2 aggregates into 15 mL conical centrifuge tubes using wide bore pipette tips and centrifuge at 400 rcf for 3 min to collect the aggregates.

2. Aspirate the supernatant, add PBS to wash the aggregates and centrifuge at 400 rcf for 3 min.
3. Aspirate the supernatant and dissociate the aggregates with 0.5–1 mL 0.25% Trypsin/EDTA for 5–20 min at 37 °C by pipetting every 5–10 min. When the aggregates are difficult to dissociate, add the same amount of 2× collagenase/DNaseI solution to the aggregates in Trypsin/EDTA and incubate for 5–20 min at 37 °C.
4. Pipette the aggregates to break them into single cells and dilute the cell suspension 2–10 times with MEF medium.
5. Centrifuge the cells at 500 rcf, 5–10 min.
6. Aspirate the supernatant and suspend the dissociated cells in 50 μL of FACS solution containing 3% (v/v) FBS in PBS.
7. Incubate the cells for 30–60 min with 5 μL of AlexaFluor647 Mouse anti-Human Alkaline Phosphatase antibody (BD Pharmingen, 561500) or the antibody with fluorochrome of choice depending on the fluorescence indicator in the cell lines if present, and in accordance with the detection setup of the flow cytometer.
8. Dilute to wash the cells + antibodies with PBS and centrifuge at 500 rcf for 5–10 min.
9. Aspirate the supernatant and resuspend the cells in 350–500 μL FACS solution with 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole). It is recommended to filter cells before the analysis to avoid blocking the flow cytometry system.
10. Analyze by flow cytometers such as BD LSRFortessa (B.D. Biosciences) or sort PGCLC population by cell sorter, such as BD FACSAria III (B.D. Biosciences). Gating strategies to detect and analyze the PGCLC population are shown in Irie et al. [25].

4 Notes

1. Inducible system for SOX17 and PRDM1 works best for using GR system for SOX17 and dox system for PRDM1. Examination for using dox inducible systems for both SOX17 and PRDM1 showed low efficiency for inducing PGC fate.
2. As the balance for expression of SOX17 and PRDM1 is essential for PGCLC induction, selecting optimal clones is important. This can be evaluated by the induction test of at least 10–20 clones for PGCLCs in the presence of DEX 100 μM and dox 1 μg/mL. The concentration of DEX and dox can be optimized for experimental purposes.

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Chapter 8

Induction of Primordial Germ Cell-Like Cells from Rat Pluripotent Stem Cells

Mami Oikawa, Masumi Hirabayashi, and Toshihiro Kobayashi

Abstract

In vitro induction of primordial germ cell like-cells (PGCLCs) from pluripotent stem cells (PSCs) is a robust method that will contribute to understanding the fundamentals of cell fate decisions, animal breeding, and future reproductive medicine. Here, we introduce this system established in the rat model. We describe a stepwise protocol to induce epiblast-like cells and subsequent PGCLCs by forming spherical aggregates from rat PSCs. We also describe a protocol to mature these PGCLCs from specified/migratory to the gonadal stage by aggregation with female gonadal somatic cells.

Key words Rat pluripotent stem cells, Epiblast-like cells, Primordial germ cell-like cells, Gonadal somatic cells, Cell aggregations

1 Introduction

Primordial germ cells (PGCs), the precursors of sperm and eggs, emerge from the pre-gastrulating epiblast in mammals. Understanding the molecular basis of PGC specification offers insights into cell fate decisions, the development of novel techniques for animal breeding, and future reproductive medicine. However, limited access to early-stage embryos and the small number of specified PGCs (~40 in mice) have hindered our ability to study the early steps of germ cell specification and development. To overcome this limitation, techniques promoting the induction of pluripotent stem cells (PSCs) to PGC-like cells (PGCLCs), counterparts of in vivo PGCs, have been developed [1]. These techniques have facilitated the analysis of conserved and divergent mechanisms of PGC specification in several mammalian species [2–6]. In this chapter, we describe the PGCLC induction system we established in the rat (*Rattus norvegicus*), a widely used model animal in biomedical

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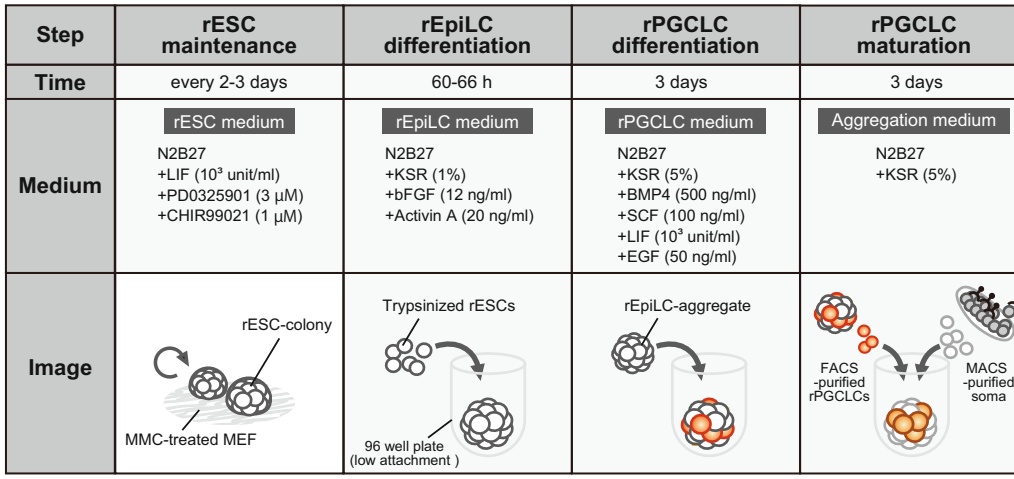

Fig. 1 Summary of experimental procedures (Oikawa et al. [5])

research (Fig. 1 shows schematics of our protocol). Rat PSCs differentiate into epiblast-like cells (EpiLCs) via the formation of spherical aggregates, unlike adherent monolayers in mice PSCs. Subsequently, we demonstrate that transferring the rat EpiLC (rEpiLC)-aggregates into wells filled with medium containing BMP4, a critical cytokine for mammalian PGC specification, leads to efficient induction of rat PGCLCs (rPGCLCs). Detection of PGC-specific marker expression by various methods such as flow cytometry, immunofluorescence staining, and reverse transcription polymerase chain reaction confirmed the successful induction of rPGCLCs. These rPGCLCs are closely equivalent to specified/migratory in vivo rat PGCs at embryonic day (E) 9.5–11.5. Co-culture with gonadal somatic cells supports the maturation of PGCLCs [7] to late-stage gonadal PGCLCs. In rats, depletion of c-KIT- but not SSEA1-positive endogenous PGCs by Magnetic Activated Cell Sorting (MACS) enriches gonadal somatic cells from E15.5 gonads. rPGCLCs mature into late-stage PGCs upon aggregation culture of purified rPGCLCs (both male and female) with female but not male gonadal somatic cells for 3 days and are equivalent to in vivo rat PGCs at E12.5–15.5. The expression of late-stage PGC markers such as *Dazl* and *Ddx4*, and the detection of epigenetic reprogramming-related changes that occur during PGC development indicate successful maturation of PGCLCs. We believe our in vitro PGCLC induction and maturation method will broaden our understanding of pluripotency and germline development in rats and provide insights into conserved and divergent mechanisms across mammals.

2 Materials

Prepare all solutions before starting the experiments. Store the stock solution and medium according to each storage condition. Follow the ethical rules on animal experiments determined by each institution. Diligently follow the regulations on laboratory waste disposal.

2.1 Maintenance of Rat Pluripotent Embryonic Stem Cells (rESCs)

1. Mouse embryonic fibroblast (MEF) medium: Mix 100 mL of DMEM with 10 mL of FBS, 1 mL of GlutaMAX, and 1 mL of penicillin/streptomycin. Store at 4 °C for up to 4 weeks.
2. N2B27 medium: Mix 50 mL of D-MEM/F12 (1:1) with 50 mL of Neurobasal Medium, 1 mL of B-27 serum-free supplement, 0.5 mL N-2 serum-free supplement, 0.5 mL of GlutaMAX, and 0.5 mL of penicillin/streptomycin. Store at 4 °C for up to 2 weeks.
3. CHIR99021 stock solution: Prepare 30 mM solution in DMSO. Make 5 μL aliquots and store them at −20 °C for up to 6 months.
4. PD0325901 stock solution: Prepare 10 mM solution in DMSO. Make 5 μL aliquots and store them at −20 °C for up to 6 months.
5. ESGRO® Recombinant Rat LIF Protein (rLIF; Merck) stock: Prepare 10^7 units/mL per vial. Make 5 μL aliquots and store them at −20 °C for up to 6 months.
6. rESC medium: Mix 40 mL of N2B27 with 4 μL of CHIR99021 stock solution, 4 μL of PD0325901 stock solution, and 4 μL of rat LIF protein stock. Store at 4 °C for up to 2 weeks.
7. Trypsin-EDTA (0.25%), phenol red.
8. 12-well cell culture plate.
9. Phosphate buffered saline, free of Ca^{++} and Mg^{++} (PBS).
10. 0.1% gelatin/PBS. Sterilization by filtration. Store at room temperature.
11. Mitomycin C (MMC)-treated MEF prepared in house. Briefly, expanded primary MEF established from E13.5 ICR mouse fetus are treated with 10 μg/mL MMC for 2.5 h. Then, MMC-treated MEF are stocked in a liquid nitrogen tank until use.
12. rESCs (*see* **Note 1**).
13. 15 mL centrifuge tubes.

2.2 rEpiLCs Differentiation Via Spheroid Culture

1. Recombinant Human FGF-basic (bFGF) stock solution: Prepare a 50 μg/mL solution following the manufacturer's protocol (*see* **Note 2**). Make 25 μL aliquots and store them at −20 °C for up to 6 months.
2. Recombinant Human/Murine/Rat Activin A (Activin A) stock solution: Prepare a 500 μg/mL solution following the manufacturer's protocol (*see* **Note 2**). Make 10 μL aliquots and store them at −20 °C for up to 6 months.
3. KnockOut™ Serum Replacement (KSR).
4. rEpiLC differentiation medium: Mix 10 mL of N2B27 medium with 100 μL of KSR, 2.4 μL of bFGF stock solution, and 0.4 μL of Activin A stock solution. Prepare the medium just before use. We normally store the medium at 4 °C for up to 3 days.
5. PBS.
6. rESCs.
7. 96-well low attachment cell culture plate (U-bottom).
8. 15 mL centrifuge tubes.
9. Cell counter plates.

2.3 rPGCLCs Differentiation

1. Recombinant Mouse SCF Protein (SCF) stock solution: Prepare 100 μg/mL solution following the manufacturer's protocol (*see* **Note 2**). Make 20 μL aliquots and store them at −20 °C for up to 6 months.
2. Recombinant mouse EGF (EGF) stock solution: Prepare 200 μg/mL solution following the manufacturer's protocol (*see* **Note 2**). Make 10 μL aliquots and store them at −20 °C for up to 6 months.
3. Recombinant Human BMP-4 (BMP4) stock solution: Prepare 100 μg/mL solution following the manufacturer's protocol (*see* **Note 2**). Make 10 μL aliquots and store them at −20 °C for up to 6 months.
4. rPGCLC differentiation medium: Mix 9.5 mL of N2B27 medium with 500 μL of KSR, 1 μL of rLIF stock, 10 μL of SCF stock solution, 2.5 μL of EGF stock solution, and 50 μL of BMP4 stock solution. Prepare the medium just before use.
5. PBS.
6. rEpiLC-aggregates.
7. 96-well low attachment cell culture plate (U-bottom).
8. 4-well or 24-well cell culture plate.
9. Stereoscopic microscope.
10. 15 mL centrifuge tubes.

11. 5 mL tube for FACS.
12. Anti-c-KIT antibody (R&D Systems, cat# AF1356).
13. Anti-goat secondary antibodies conjugated with fluorophore.
14. DAPI solution (1 mg/mL).

2.4 rPGCLCs Maturation Using Gonadal Somatic Cells

1. Aggregation medium: Mix 9.5 mL of N2B27 medium with 500 μL of KSR.
2. rPGCLCs, 3 days after induction (d3 rPGCLCs).
3. E15.5 pregnant rats (*see* **Note 3**).
4. Fine scissors and forceps.
5. Sharpened tungsten needles (*see* **Note 4**).
6. Filter paper.
7. Anti-c-KIT antibody (R&D Systems).
8. Biotin Labeling Kit (Dojindo). The biotinylation procedure is conducted according to the manufacturer's protocol.
9. Streptavidin magnetic beads.
10. Mini MACS Starting Kit (Miltenyi Biotec).
11. MS columns.
12. Cell strainer, 70 μM filter.
13. 1.5 mL tubes.
14. 15 mL centrifuge tubes.
15. 5 mL tube for FACS.
16. Box filled with ice.
17. 96-well low attachment cell culture plate (U-bottom).
18. 3% fetal bovine serum (FBS) in PBS.
19. 7.5% BSA solution.
20. MACS buffer: Mix 13.94 mL of PBS with 1 mL of 7.5% BSA solution and 60 μL of EDTA stock solution. Prepare the buffer just before use.
21. FACS buffer: Mix 14.8 mL of PBS with 200 μL of 7.5% BSA solution. Prepare the buffer just before use. 3%FBS/PBS can substitute for this buffer.
22. MEF medium. The composition is the same as Subheading 2.1.
23. 10 cm petri dish.

3 Methods

3.1 rESCs Maintenance

1. Coat the wells of a 12-well cell culture plate with 500 μL of 0.1% gelatin/PBS at room temperature. After 5–10 min, aspirate and discard the 0.1% gelatin/PBS.
2. Seed the MEFs (4–5 × 10^5 cells per well) into the gelatin-coated plates. Incubate the plate at 37 °C in a 5%CO_2 incubator.
3. On the next day, aspirate the MEF medium. Rinse with 1 mL of PBS and aspirate. Pipette 1 mL of rESC medium into the well and incubate the plate until seeding the rESCs.
4. Seed the frozen-thawed rESCs at 2–8 × 10^4 cells per well.
5. After 2 days, top up each well with 1 mL of rESC medium. Culture 2–3 days before induction.
6. Routinely passage rESCs every 2–3 days. Dissociation of rESC-colonies into single cell follows Subheading 3.2, **steps 3–11**. Seed rESCs on MMC-treated MEF at 2–4 × 10^4 cells per well of 12-well plates.

3.2 rEpiLC Differentiation

1. Pipette 100 μL of rEpiLC medium per well, avoiding the corner and edge wells. To avoid evaporation of the medium, pipette 100 μL of PBS into corner, edge and side wells. Incubate the plate at 37 °C in a 5%CO_2 incubator until before use (*see* **Note 5**).
2. Prepare the rESCs for rEpiLC induction. Carefully remove excess rESC medium from the rESC culture plate wells by using a P1000 pipette. We normally leave 1 mL of medium per well of a 12-well plate. Make sure not to aspirate rESC colonies.
3. Shake the plate gently from side to side for rESC colonies to detach, then collect rESCs and their medium into a 15 mL tube. Rinse the well with 1–2 mL of PBS and collect it into the same 15 mL tube.
4. Centrifuge the tube at 250 × g, 20 °C, for 5 min.
5. Discard the medium gently while avoiding aspiration of the cell pellets.
6. Pipette 200 μL of 0.25% Trypsin/EDTA and incubate at 37 °C for 3 min in the water bath.
7. Add 1–2 mL of MEF medium to quench the reaction and pipette up and down gently to dissociate rESC-colonies into single cells.
8. Centrifuge the tube at 250 × g, 20 °C, for 5 min.
9. Aspirate the medium.

10. Pipette 200 μL of N2B27 medium and suspend the cell pellets.
11. Count the cell number.
12. Seed the 4×10^3 cells per well into the 96-well plate containing rEpiLC medium. Incubate the plate at 37 °C in a 5%CO_2 (*see* **Note 6**).
13. On day 1 after the induction of rEpiLCs, top up every well with 100 μL of rEpiLC medium.
14. On day 2, carefully discard 100 μL of rEpiLC medium from the well. Then, add 100 μL of fresh rEpiLC medium. The induction of rEpiLCs takes about 60–66 h in total.

3.3 rPGCLC Differentiation and Validation by Flow Cytometry

1. Pipette 100 μL of rPGCLC medium per well (*see* **Note 7**). As in Subheading 3.2, **step 1**, pipette 100 μL of PBS into corner, edge and side wells. Incubate the plate at 37 °C in 5% CO_2 until use.
2. To transfer the rEpiLC aggregates, use 20–200 μL pipette tip or glass capillaries. Rinse the inside of the tips or capillaries with N2B27 medium to prevent cell attachment.
3. Pipette 500 μL of N2B27 medium into the two wells of a 4-well plate or 24-well plate.
4. Collect the rEpiLC-aggregates into one of the wells under the stereomicroscope.
5. Rinse the rEpiLC-aggregates twice in wells containing N2B27 medium.
6. Transfer the rEpiLC-aggregates into wells of the 96-well plate containing rPGCLC medium (one rEpiLC-aggregate per well). Incubate the plate at 37 °C in 5% CO_2.
7. On day 2 after the induction of rPGCLCs, top up each well with 100 μL of rPGCLC medium.
8. On day 3, confirm successful rPGCLC induction (d3 rPGCLC; left images in Fig. 2b) by flow cytometry analysis or immunofluorescence staining (*see* **Note 8**).
9. For flow cytometry analysis or cell sorting, collect aggregates into 1.5 mL tube containing 500 μL of PBS.
10. Centrifuge the tube at 250 × g, 20 °C, for 1 min.
11. After removal of the supernatant, pipette 100 μL of 0.25% Trypsin-EDTA and incubate the tube at 37 °C for 5 min in a water bath.
12. Add 900 μL of MEF medium and pipette up and down gently to quench the reaction. Filter the cells using a cell strainer to remove cell clumps.
13. Centrifuge the tube at 250 × g, 20 °C, for 5 min.

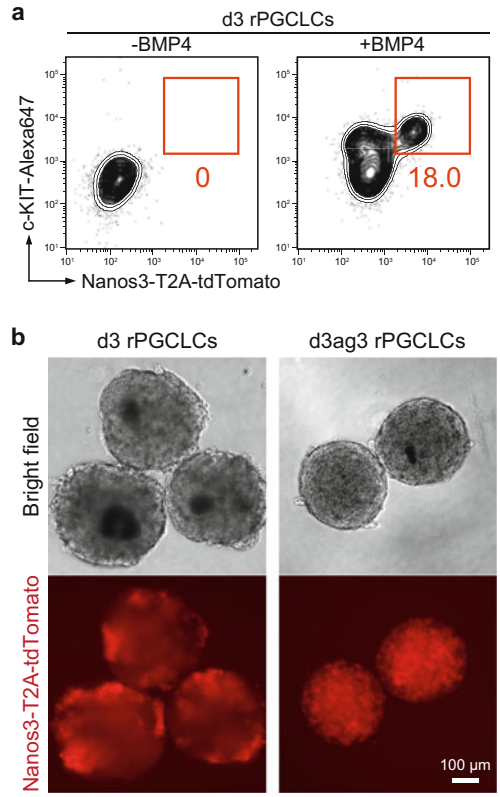

Fig. 2 rPGCLC induced from rESCs harboring Nanos3-T2A-tdTomato reporter. (**a**) FACS patterns of 3 days after rPGCLC induction with or without BMP4. Cells were stained with anti-c-KIT antibody. (**b**) Morphology and fluorescence of d3 and d3ag3 rPGCLCs (Oikawa et al. [5])

14. After removal of the supernatant, Resuspend the cells into 300–500 μL of FACS buffer.
15. Count the cell number.
16. Add 0.25 μL of anti-c-KIT antibody (non-biotinylated) per 1×10^5 cells. Incubate the tube on ice for 30 min.
17. Add 1 mL of FACS buffer and pipette up and down gently.
18. Centrifuge the tube at 250 × g, 20 °C, for 5 min.
19. After removal of the supernatant, add 2.5 μL of 1:100 diluted fluorescent-dye conjugated anti-goat IgG per 1×10^5 cells. Incubate the tube on ice for 30 min.
20. Add 1 mL of FACS buffer and pipette up and down gently.
21. Centrifuge the tube at 250 × g, 20 °C, for 5 min.
22. Aspirate the supernatant and resuspend the cells into 300–500 μL of FACS buffer containing DAPI (10 μg/mL).

23. Set up the flow cytometer according to the manufacturer's instructions.
24. Run the BMP4(−) rPGCLC samples and mark a gate for the negative cell population.
25. Run the BMP4(+) rPGCLC samples. Analyze the induction efficiency based on the reporter- or c-KIT-positive cell population (Representative FACS patterns of Nanos3-T2A-tdTomato reporter and c-KIT are shown in Fig.2a.).

3.4 Preparation of Gonadal Somatic Cells

1. Anesthetize pregnant rats at 15 days post-coitum with isoflurane and then euthanize them. Remove the uterus, including the blood and fat, on a filter paper.
2. Transfer the uterus to a 10 cm petri dish and pour 3% FBS/PBS to prevent drying.
3. Remove the uterine muscular wall gently. Remove the placenta and amniotic membrane from the fetus. Collect gonads-mesonephros complexes (representative images of Nanos3-T2A-tdTomato reporters are shown in Fig.3). In this experiment, only female gonads are used (*see* **Note 9**).
4. Separate a gonad (dashed line in Fig.3) from the mesonephros using sharpened tungsten needles.
5. Transfer all gonads into a 15 mL tube containing 10 mL of PBS.
6. Centrifuge the tube at 250 × g, 20 °C, for 5 min.
7. Discard the medium gently, avoiding aspiration of the gonad pellet.
8. Pipette 500 μL of 0.25% Trypsin-EDTA into the tube containing the gonads. Incubate the tubes at 37 °C for 8 min in a water bath. After 5 min, tap the tubes gently, and then incubate the tubes for 3 more min.
9. Add 4.5 mL of MEF medium into the tube to inactivate the trypsin. Pipette up and down gently to dissociate the gonadal tissue into single cells.
10. Filter the cell suspension through a cell strainer and collect the cells into a new 15 mL tube.
11. Centrifuge the tube at 250 × g, 20 °C, for 5 min.
12. Discard the medium.
13. Pipette 100 μL of MACS buffer and suspend the cells gently. Transfer the suspension into a 1.5 mL tube. Count the cell number.
14. Add 0.25 μL of biotinylated c-KIT antibody stock per 1×10^5 cells. Incubate the tube on ice for 30 min.
15. Add 1 mL of MACS buffer to dilute the antibody.

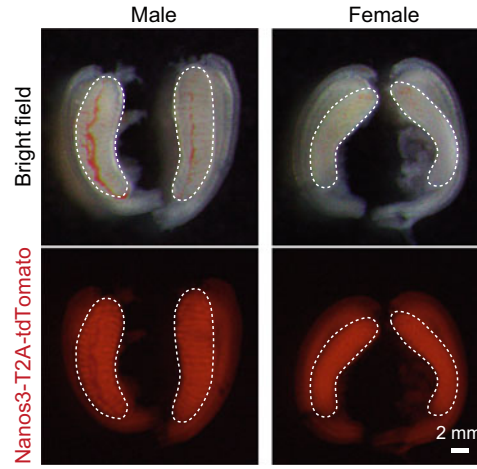

Fig. 3 Morphology of E15.5 rat gonads with mesonephros obtained from Nanos3-T2A-tdTomato reporter rat. Dashed line shows gonads (Oikawa et al. [5, 9])

16. Centrifuge the tube at 250 × g, 20 °C, for 5 min.
17. Discard the medium.
18. Add 2.5 μL of Streptavidin magnetic beads per 1×10^5 cells. Incubate the tube on ice for 30 min.
19. Add 1 mL of MACS buffer.
20. Centrifuge the tube at 250 × g, 20 °C, for 5 min.
21. Discard the medium.
22. Pipette 1 mL of MACS buffer and suspend the cells gently. Keep the cell suspension on ice.
23. Place the MACS MS Column on a MiniMACS separator.
24. Pipette 500 μL of MACS buffer into the column. Discard the flow-through.
25. Apply all gonadal cell suspensions into the column. Collect the flow-through into a 15 mL centrifuge tube.
26. Add 500 μL of MACS buffer and collect the flow-through into the 15 mL centrifuge tube. Repeat this step once.
27. Add 8 mL of aggregation medium to the collected flow-through.
28. Centrifuge the tube at 250 × g, 20 °C, for 5 min.
29. Discard the medium.
30. Pipette 0.5–1 mL of aggregation buffer and count the cells. Keep the cell suspension on ice.

3.5 rPGCLC Maturation by Aggregation with Gonadal Somatic Cells

1. Transfer the d3 rPGCLC-aggregates into a 15 mL tube containing 5 mL PBS.
2. Centrifuge the tube at 250 × g, 20 °C, for 1 min.
3. Discard the medium gently, then add 500 μL of Trypsin-EDTA. Incubate at 37 °C, for 5 min using a water bath.
4. Add 4.5 mL of MEF medium and pipette up and down gently to quench the reaction.
5. Filter the cell suspensions using cell strainers to remove undissociated cell clumps.
6. Centrifuge the tube at 250 × g, 20 °C, for 5 min.
7. Discard the supernatant carefully. Pipette 500 μL of FACS buffer and suspend the cells gently.
8. Collect the rPGCLCs by cell sorting based on fluorescent reporters or antibody staining. The sorting method follows Subheading 3.3, **steps 15–25**. Collect rPGCLCs into a 1.5 mL tube filled with 500 μL of FACS buffer.
9. Transfer the collected cells into a 15 mL tube. Pipette 4.5 mL of aggregation medium.
10. Centrifuge the tube at 250 × g, 20 °C, for 5 min. Remove the supernatant carefully.
11. Suspend the purified rPGCLCs into 500–1000 μL of aggregation medium.
12. Adjust the cell concentration of rPGCLC suspension to 5×10^4 cells per mL.
13. Adjust the cell concentration of female gonadal somatic cell suspension (Subheading 3.4, **steps 1–30**) to 5×10^4 cells per mL.
14. Mix an equal volume of rPGCLC suspension and gonadal somatic cell suspension. Pipette up and down gently.
15. Pipette 200 μL of mixed cell suspension into the 96-well plate. Incubate the plate at 37 °C in a 5% CO_2 incubator for 3 days.

3.6 Validation of Matured rPGCLCs

1. Collect rPGCLCs-gonadal somatic cell aggregates (d3ag3 rPGCLC; right images in Fig. 2b) into 3% FBS/PBS.
2. Transfer the aggregates into 4% PFA/PBS. Fix the aggregates at 4 °C for 4–6 h or at room temperature for 10 min.
3. Stain the sample with antibodies against in vivo late-stage rat PGCs, following the general immunofluorescence procedure (*see* **Note 10**).

4 Notes

1. We recommend using well-characterized rESC lines (i.e. tested for pluripotency, karyotype, and germline competency etc.). Using rESCs derived from PGC-reporter rats [5, 8, 9] makes validation of PGCLC specification easier.
2. The reconstitution procedure is slightly different among providers. Follow the protocol described in each provider's datasheet.
3. We normally use Wistar or SD rats. Follow the ethical guidelines and regulations of the affiliated institution for the use of experimental animals.
4. Morohaku et al. [10] describe details of the sharpening procedure of tungsten needles.
5. We normally do not use corner, edge and side wells of 96-well plates since the medium in these wells usually evaporates.
6. For the validation of rPGCLC specification, we normally seed rESCs to 6–12 of the wells for EpiLC induction. We seed rESCs to 30- to 60 of the wells for the aggregation experiment. Adjust the plate size depending on the purpose.
7. At least 4–6 of the wells should contain rPGCLC medium without BMP4 for negative controls for rPGCLC induction.
8. PGCLC specification can be also validated by several ways, such as immunofluorescence staining and gene expression analysis.
9. The sex of the gonads is morphologically distinguishable at this stage. Male gonads show elliptical and stripe patterns. At the same time, female gonads look thinner and pebble-like.
10. We normally use DDX4 or DAZL antibodies to detect PGCLC maturation. These marker genes are not detected in in vivo early PGC and d3PGCLCs.

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Chapter 9

Induction of Meiotic Initiation in Long-Term Mouse Spermatogonial Stem Cells Under Retinoid Acid and Nutrient Restriction Conditions

Xiaoyu Zhang and Ning Wang

Abstract

Spermatogonial stem cells (SSCs) produce haploid sperm via mitosis and meiosis in vivo. Although the technique to culture mouse SSCs has been well established, induction of meiosis in vitro has remained a challenge. Retinoic acid (RA) is required for meiosis in vivo; however, RA alone is not sufficient to induce meiosis in vitro. Here, we describe a method in which nutrient restriction and RA synergistically induce meiotic initiation into meiotic prophase I in cultured mouse SSCs.

Key words Spermatogonial stem cells, Germline stem cells, Meiotic initiation, Meiosis, Retinoic acid

1 Introduction

The long-term culture of stem cells with germline potential has broad applications for advancing basic science research, fertility preservation, and regenerative medicine. In addition to spermatogonial stem cells (SSCs) [1], recent progress in the derivation of primordial germ cell-like cells (PGCLCs) from pluripotent stem cells (PSCs) has also provided an alternative source of germ stem cells (GSCs) [2]. Similar to embryonic stem (ES) cell culture, GSCs are usually seeded onto a monolayer of supporting cells (e.g., STO cells, mouse embryonic fibroblasts or MEFs) that provide essential nutrients and growth factors [3]. Alternatively, male GSCs are cultured in defined culture media, optimized to support their maintenance and proliferation. These media typically contain specific growth factors, such as glial cell line-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF or FGF2), which are known to promote the survival and proliferation of male GSCs [4, 5].

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SSCs are capable of self-renewal to maintain the stability of GSCs and differentiation to produce mature spermatozoa through meiosis. Meiosis is a defining feature of germ cells, during which replicated parental chromosomes undergo homologous recombination during prophase I, followed by two rounds of divisions to halve chromosome contents [6].

Despite the progress in culturing GSCs in vitro, induction of meiosis in cultured SSCs has remained a challenge [7]. It is widely known that retinoic acid (RA) is required for meiosis in vivo, in that meiosis does not initiate and spermatogenesis stalls at the stage of undifferentiated spermatogonia in mice fed with vitamin A-deficient (VAD) diet [8] or administrated with compounds, e.g., WIN 18 446, that inhibit retinaldehyde dehydrogenases to block RA production from retinal [9]. However, RA alone is not sufficient to induce meiosis in vitro under normal culture condition [10] or in vivo [11]. Thus, meiotic induction usually requires transplantation of culture SSCs back to seminiferous tubules.

In our laboratory, we found that STRA8, an essential gatekeeper of meiotic initiation downstream of RA, acts as a suppressor of autophagy and that *Stra8*-deficient germ cells fail to initiate meiosis showing aberrant autophagy activation [12]. These results revealed for the first time a link between autophagy, a protein and cellular organelle recycling process through lysosomes [13], and the beginning of meiosis in mammalian male germ cells. Interestingly, nutrient restriction, probably the most potent inducer of autophagy [14], initiates meiosis in yeasts [15, 16]. On this basis, we devised and describe here in detail a simple culture system in which nutrient restriction and RA synergistically induce meiotic initiation in cultured mouse SSCs, faithfully recapitulating the transcriptomic and cytological features of the first stages of meiotic prophase I occurring in vivo [10].

2 Materials

2.1 Establishment of Long-Term Mouse SSC Culture

1. 3–4 male mice in C57BL/6 X DBA/2 F1 hybrid background at postnatal days 6–8 (*see* **Note 1**).
2. Sterile 70% ethanol.
3. Sterilized surgical tools (sharp scissors, forceps, spring scissors, etc.).
4. Sterile 1.5-mL microcentrifuge tube.
5. Sterile 15 and 50 mL polypropylene conical tubes.
6. Phosphate buffered saline (PBS) (pH 7.4) (Gibco Catalog No. 10-010-072).
7. Hanks' Balanced Salt Solution (HBSS) (no calcium, no magnesium, no phenol red) (Gibco Catalog No. 14175-095).

Table 1
Components of SSC media

	Company	Catalog number	SSC media
IMDM	Thermo Fisher	12440-053	
N2	R&D Systems	AR-009	1X
Pyruvic acid	Sigma	P2256	200 μg/mL
D-(+)-glucose	Sigma	G7021	6 mg/mL
DL-Lactic acid	Sigma	L4263	1 μL/mL
Bovine serum albumin (BSA)	MP Biomedicals	810661	5 mg/mL
L-Glutamine	Sigma	G7513	2 mM
2-Mercaptoethanol	Sigma	M3148	5×10^{-5} M
MEM vitamin	Thermo Fisher	11120-052	1X
NEAA	Thermo Fisher	11140-050	1X
Ascorbic acid	Sigma	A4544	10^{-4} M
d-Biotin	Sigma	B4501	10 μg/mL
β-Estradiol	Sigma	E2758	30 ng/mL
Fetal bovine serum	Hyclone	SH30396.03	10 μL/mL
KSR	Thermo Fisher	10828-028	50 μL/mL
Human FGF2	Peprotech	100-18B	10 ng/mL
Rat GDNF	Peprotech	450-51	15 ng/mL

8. Microcentrifuge, preferably with temperature control, to process samples at 4 °C.
9. CO_2 incubator.
10. Collagenase, Type IV (Gibco Catalog No. 17104-019). Freshly prepared as 2 mg/mL solution in HBSS on the day of use.
11. Deoxyribonuclease I from bovine pancreas (DNase I) (Sigma DN25). Prepared as 2 mg/mL solution in HBSS. Kept frozen until use.
12. Trypsin-EDTA (0.25%) (Gibco Catalog No. 25200-056).
13. Cell strainer (40 μm pore size) (Corning Catalog No. 352340).
14. Cell counter (Invitrogen Countess II).
15. Tissue culture dishes (vacuum-gas plasma tissue-culture treated polystyrene, 60 mm).
16. 0.1% gelatin solution (ATCC Catalog No. PCS-999-027).

17. Mitotically arrested mouse embryonic fibroblasts (MEFs) (*see* **Note 2**).
18. Media for MEF cells: DMEM (Gibco 11995-065), Fetal bovine serum (FBS) Gibco A3160501).
19. SSC media (see Table 1).

2.2 Meiotic Induction Media

1. Earle's Balanced Salt Solution (EBSS), calcium, magnesium, phenol red (Gibco Catalog No. 24010-043).
2. 0.1 mM all-trans retinoic acid (Sigma, R2625) dissolved in DMSO.

2.3 Meiotic Progression Media

1. Stock solutions: 10^{-4} M melatonin (Sigma M5250) in DMSO; 10^{-1} M 5α-Dihydrotestosterone (DHT) (APExBIO B8214) in DMSO; Bovine pituitary extract (BPE) (Gibco 13028-014); 200 μg/mL follicle-stimulating hormone (FSH) (Sigma F4021) in IMDM.

3 Methods

3.1 Establishment of Mouse SSC Culture

1. Euthanize 3–4 mouse male pups under CO_2.
2. Testes are collected in a 60 mm dish containing 2 mL sterile HBSS using the aseptic technique on ice.
3. After the removal of tunica albuginea, testes are cut into small pieces using spring scissors.
4. Tissues are transferred to a 15 mL tube by using a transfer pipette containing 2 mL of 2 mg/mL Collagenase IV solution (final collagenase IV concentration: 1 mg/mL). The tubules are gently dispersed by pipetting.
5. Tissues are incubated in 37 °C water bath for 20 min until tissues are separated (gently pipetting if necessary).
6. Tubule fragments are washed with the addition of 4 mL of HBSS, followed by centrifugation at 300× *g* for 5 min at 4 °C. The supernatant is removed.
7. Tubule fragments are incubated in 1 mL of 0.25% trypsin-EDTA solution, 1 mL of HBSS, and 2 mL of 2 mg/mL DNase I in 37 °C water bath for 5 min.
8. After incubation, 1 mL of FBS is added to neutralize trypsin.
9. Gently pipette the mixture to generate a single-cell suspension.
10. Pass the cell suspension through a 40-μm cell strainer.
11. Collect cells by centrifugation at 300× *g* for 5 min at 4 °C. Discard the supernatant.
12. Add 4 mL of HBSS to the cell pellet. Wash cells by gentle pipetting.

13. Collect cells by centrifugation at 300× *g* for 5 min at 4 °C. Discard the supernatant.
14. Resuspend testicular cells in 4 mL of SSC media. Measure cell concentration by cell counter.
15. Plate 1×10^6 testicular cells on 0.1% gelatin-coated 60 mm dish with 4 mL SSC media. Incubate overnight in CO_2 incubator at 37 °C.
16. Plate MEFs in 60 mm dishes in 4 mL of DMEM containing 10% FBS at 70–80% confluency. The number of MEFs used to achieve this confluency should be empirically determined.
17. The next day, non-adherent cells (germ cells) are collected into 15 mL conical tubes. Cells are collected by centrifugation at 300× *g* for 5 min at 4 °C. The supernatant is discarded. Adherent cells (somatic cells, e.g., Sertoli cells) are discarded. Based on our experience, this procedure—differential plating [17]—is an effective step to enrich SSCs.
18. Remove media from MEF feeder cells.
19. Resuspend the collected non-adherent cells (germ cells) in 4 mL of SSC media. Plate the cells on MEFs.
20. Change media every other day by removing all media from plates using gentle pipetting, followed by adding 4 mL of fresh SSC media.
21. When SSCs reach 70–80% confluency, split the cells by standard cell culture procedure. Plate cells onto new MEFs.

3.2 Induction of Meiotic Initiation

1. To induce meiotic initiation, 1.0×10^6 cells from SSC culture are plated on 60-mm dishes coated with MEF feeder cells (*see* **Note 3**).
2. Next day, remove SSC media and add 4 mL of meiotic induction media. Meiotic induction media is composed of 10% of SSC media, 90% EBSS (e.g., 1 mL of SSC media and 9 mL of EBSS), and 100 nM retinoic acid (1:1000 dilution from the stock solution) (*see* **Note 4**).
3. Incubate SSCs in meiotic induction media in CO_2 incubator at 34 °C for 2 days (*see* **Notes 5** and **6**).
4. At this time, early meiotic events, such as activation of key meiotic genes and DNA double-strand breaks (DSBs), can be detected as reported (Fig. 1) [10].

3.3 Meiotic Progression

1. To further promote meiotic progression through leptonema and up to the zygotene stage, replace meiotic induction media with meiotic progression media. Meiotic progression media is composed of SSC media (Table 1) without GDNF and FGF2

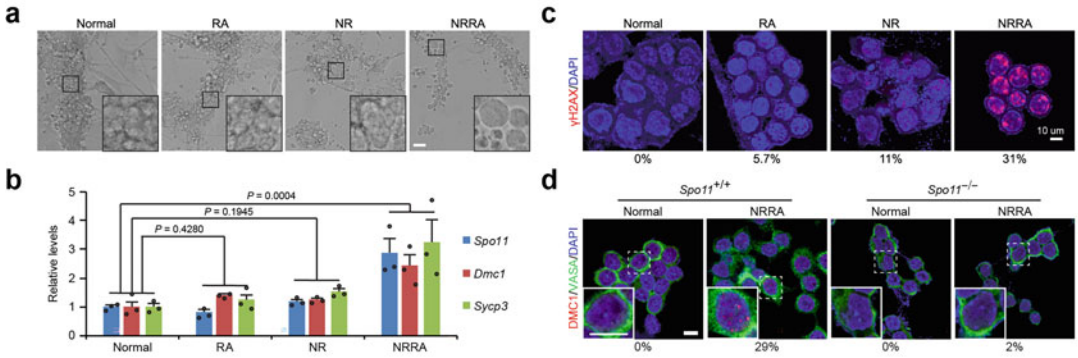

Fig. 1 Nutrient restriction synergizes with RA to induce meiotic initiation in SSC culture. (**a**) Bright-field images of SSC culture with indicated treatments for 2 days. Scale bars, 50 μm. (**b**) Relative expression of *Spo11*, *Dmc1*, and *Sycp3* against *Gapdh* analyzed by qRT-PCR in SSC culture with indicated treatments for 2 days. (**c**) Immunostaining for γH2AX (red) and DAPI (blue) with indicated treatment for 2 days. Scale bars, 10 μm. (**d**) Immunostaining for DMC1 (red), DDX4 (green), and DAPI (blue) in $Spo11^{+/+}$ and $Spo11^{-/-}$ SSC culture. Scale bars, 10 μm. In panels **c** and **d**, the numbers below the immunofluorescent images indicate the percentage of cells seen with the relevant staining under different conditions

supplemented with 10^{-7} M melatonin, 10^{-6} M DHT, 50 ng/mL BPE, and 200 ng/mL FSH (*see* **Note 7**).

2. Incubate cells with meiotic progression media at 34 °C for 4 days (*see* **Notes 5** and **6**).
3. At this time, prophase I leptotene and zygotene events, such as the formation of the lateral and central elements of the synaptonemal complex, can be examined as reported [10] (Fig. 2) (*see* **Note 8**).

4 Notes

1. The genetic background of donor mice has a significant influence on the establishment of mouse SSC culture. In our experience, testicular cells from either C57BL/6 X DBA/2 F1 hybrid background or CD1 background produce SSC cultures with rapid growth, while testicular cells from C57BL/6 inbred background do not sustain in vitro growth after 1 month. The age of the donor mouse pups is preferentially to be between 6- and 8 days of age. In a typical preparation, testes from 3 to 4 pups are used. Testes from fewer animals are also able to establish SSC culture, but smaller dishes, e.g., 35 mm dishes, should be used for the initial culture.
2. Commercially available MEF cells, e.g., R&D system Catalog No. AR005, are used. According to the manufacturer's website, MEF cells are collected from outbred CF1 mice, mitotically arrested by irradiation.

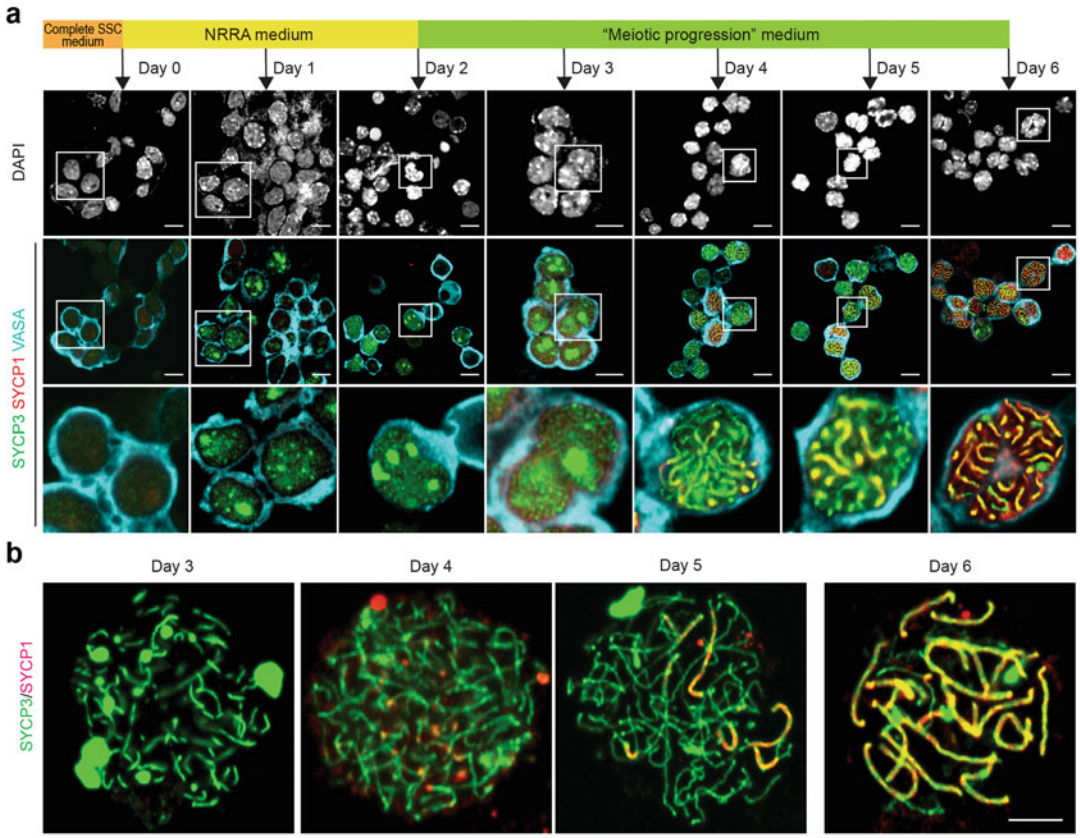

Fig. 2 Nutrient restriction and RA-induced meiotic initiation supports meiotic progression. (**a**) DAPI staining and immunostaining for SYCP1 (red), SYCP3 (green), and VASA (cyan) in SSC cultures with indicated treatment. Scale bars, 10 μm. (**b**) Representative chromosome spreads stained by SYCP1 (red) and SYCP3 (green) from SSC cultures following the indicated treatment are shown

3. MEF cells may produce cytokines that inhibit cell differentiation. Use MEF cells at lower density.
4. Reducing the concentration of FGF2 and GDNF with RA treatment is not sufficient to induce meiosis. FGF2/GDNF are not inhibitors of meiosis and cultured SSCs do not spontaneously initiate meiosis without FGF2 and GDNF. This is consistent with past literature, which has shown that the targets of GDNF and FGF2 are mostly related to cell cycle regulators and spermatogonial stem cell self-renewal, but not directly related to meiosis [18–21]. Moreover, in F9 embryonic carcinoma cells that do not require GDNF and FGF2 in the culture system, RA treatment under nutrient restriction conditions was sufficient to induce meiotic initiation, suggesting that this process does not involve GDNF and FGF2.
5. If the density of cultured SSCs is high, refresh media every day.

6. It is known that temperature plays a role in male germ cell differentiation in vivo. Culturing cells at 34 °C, rather than 37 °C, can greatly improve cell survival under meiotic induction media and meiotic progression media.
7. RA is not used in the whole experiment: RA is used only in meiotic induction media but not in the meiotic progression media.
8. Instead of using trypsin to harvest cultured cells, TIM buffer [22] mixed with 1 mg/mL collagenase IV can be used to directly harvest cells by gentle pipetting and subsequent chromosome spread analysis.

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Chapter 10

Lipofection-Based Delivery of CRISPR/Cas9 Ribonucleoprotein for Gene Editing in Male Germline Stem Cells

Mariella Obermeier, Vera Rogiers, Tamara Vanhaecke, and Yoni Baert

Abstract

Gene editing in the murine germline is a valuable approach to investigate germ cell maturation and generate mouse models. Several studies demonstrated that CRISPR/Cas9 alters the genome of cultured male mouse germline stem cells delivered by electroporation of plasmids. Recently, we showed proof-of-principle that gene knockout can be effectively targeted in mouse germline stem cells by lipofecting Cas9:gRNA ribonucleoproteins. In this protocol, we describe a simple, fast, and cheap workflow for gene editing via the lipofection of non-integrative ribonucleoproteins in murine male germline stem cells.

Key words CRISPR/Cas9, Germline stem cell, Gene editing, Ribonucleoprotein, Germline genome editing, Gene knockout, Spermatogonial stem cell, Oligodeoxynucleotide

1 Introduction

Gene editing in the murine germline is an efficient tool for establishing mouse models and investigating the physiological and pathogenic processes involved in germ cell maturation [1–3]. Male mouse germline stem (GS) cells are the most primitive postnatal germ cells cultured in vitro [4]. Cells can be kept in culture for more than two years, while preserving their genetic stability and spermatogenic potential for self-renewal and differentiation [5, 6]. Cultures of male GS cells open up opportunities for comprehensive experiments such as invitro differentiation of GS cells into fertilization-capable sperm cells and systematic CRISPR screenings [7, 8].

Several studies have demonstrated that CRISPR/Cas9 is able to modify the genome of mouse GS cells. After the gene editing, cells could differentiate in vivo and produce healthy, genetically altered non-mosaic offspring [9–11]. CRISPR/Cas is an engineered nuclease-inducing site-specific DNA double-strand breaks,

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which triggers DNA repair, including non-homologous end joining (NHEJ) and homology-directed repair (HDR) [12]. Using these repair mechanisms, CRISPR/Cas produces gene knockouts and mutations (NHEJs), as well as specific insertions incorporating a DNA template (HDR) [13].

The editing efficiency of the CRIPSR/Cas system strongly depends on its delivery route, enabling the engineered nuclease to enter the cell nucleus without inducing massive damage to the host cell. CRISPR/Cas can be transported to the cell nucleus in the form of DNA, RNA, or proteins and bridge the cell membrane through viruses, lipids or electrical carriers [14]. Studies in rodent GS cells delivered CRISPR/Cas9 mostly through the electroporation of plasmids [9–11, 15]. However, plasmids are limited by broad off-target activities and possible genome integration [16, 17].

Non-integrative ribonucleoproteins (RNP) present a quick and efficient alternative to gently deliver CRISPR/Cas9 in the form of protein:RNA (Cas9:gRNA) complexes [18]. On top of that, the lipid-mediated transfer of RNP is simple and cheap and does not involve special equipment compared to viral or non-viral alternatives.

Recently, we showed proof-of-principle of RNAiMAX-mediated Cas9:gRNA RNP transfection in male mouse GS cells, targeting *Egfp* (enhanced green fluorescent protein) and inducing loss of EGFP expression, which corresponded to EGFP knockout. Lipofection of ribonucleoproteins reached knockout rates similar to those of systems using plasmids, with the advantage of being non-integrative, less prone to off-target effects, and simple to use [19]. Here, we describe the lipofection of non-integrative Cas9:gRNA RNP transfection in male mouse GS cells, including the initiation of a stable GS cell culture derived from testicular tissue.

2 Materials

2.1 Testes Collection and Cryopreservation

2.1.1 Compounds

1. Testicular tissue from mice, 5–7 days post-partum (*see* **Note 1**).
2. Dulbecco's Modified Eagle Medium (DMEM)/F-12.
3. Cryomedium (Table 1).

2.1.2 Equipment

1. Balance.
2. Sterilization filter (0.2 μm).
3. Pipettes (10 and 50 mL).
4. Conical tube (15 mL).
5. Petri dish (e.g., 90 mm diameter).

Table 1
Ingredients to prepare 100 mL *cryomedium*

Ingredient	Amount
DMEM F12	89.3 mL
DMSO	10.7 mL
Sucrose	2.4 g

Dissolve 2.4 g sucrose in 89.3 mL DMEM/F12 and sterilize by filtering. The solution can be stored at −20 °C. Add 10.7 mL dimethyl sulfoxide (DMSO) shortly before usage

6. Cryovials (1.8 mL).
7. Dissection set.
8. Two sterile forceps.

2.2 Cell Culture

2.2.1 Compounds

1. GS cell medium (Table 2).
2. Mitomycin inactivated C57BL/6 mouse embryonal fibroblasts (MEF; A34962; Thermo Fisher Scientific; Merelbeke, Belgium).
3. MEF medium: DMEM, 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine.
4. Collagenase from *Clostridium histolyticum*.
5. TrypLE™.
6. 1× PBS.
7. 0.4% (w/v) gelatine in PBS.
8. DMEM.
9. FBS.

2.2.2 Equipment

1. Balance.
2. 12-well culture plate (for GS cell culture).
3. 48-well culture plate (for GS cell transfection).
4. Sterilization filter (0.2 μm).
5. Conical tubes (15 and 50 mL).
6. Pipettes (100 μL, 5, 10, and 50 mL).
7. Sterile forceps.
8. 70 μm cell strainer.
9. Syringe.

Table 2
***GS cell medium* to prepare 250 mL medium is based on Kanatsu-Shinohara et al. [20]**

Ingredient	Final conc.	REF number and company	
StemPro™-34 SFM		10640019	Thermo Fisher Scientific
Bovine serum albumin	5 mg/mL	10735094001	Roche
Glucose	6 mg/mL	G7021	Sigma
L-Glutamine	2 mM	G7513	Sigma
Minimal Essential Medium Vitamin solution	1% (v/v)	M6895	Sigma
Minimal Essential Medium Nonessential amino acid solution	1% (v/v)	M7145	Sigma
β-Mercaptoethanol	5×10^{-5} M	M7522	Sigma
Putrescine	60 μM	P7505	Sigma
Ascorbic acid	10^{-4} M	A4544	Sigma
Pyruvate	340 μM	P2256	Sigma
Lactic acid	1 μL/mL	L4263	Sigma
Biotin	10 μg/mL	B4501	Sigma
Insulin	25 μg/mL	I1882	Sigma
Transferrin	100 μg/mL	T1147	Sigma
Sodium selenite	30 nM	S5261	Sigma
B-estradiol	30 ng/mL	E2758	Sigma
Progesterone	60 ng/mL	P8783	Sigma
StemPro-34 supplement	2.6% (v/v)	10640019	Thermo Fisher Scientific
Pen/Strep	1%	15140122	Thermo Fisher Scientific
FBS	1%	10500056	Thermo Fisher Scientific
Human EGF	20 ng/mL	PHG0311L	Thermo Fisher Scientific
Murine bFGF	10 ng/mL	13256029	Thermo Fisher Scientific
Human GDNF	10 ng/mL	PHC7045	Thermo Fisher Scientific

Dissolve bovine serum albumin and D-glucose in StemPro-34 medium and sterilize by filtering. Subsequently, add L-Glutamine, vitamin solution, amino acids, β-mercaptoethanol, putrescine, ascorbic acid, pyruvate, lactic acid, biotin, insulin, transferrin, sodium selenite, β-estradiol, and progesterone. This mixture can be stored up to four weeks at 4 °C. Add StemPro supplement, FBS, penicillin/streptomycin (Pen/Strep), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and glial-derived neurotrophic factor (GDNF) to complete the GS cell medium. Consider that the complete medium is only stable for up to two weeks at 4 °C

2.3 Lipofection of Cas9:gRNA Ribonucleoproteins

2.3.1 Compounds

1. Alt-R® S.p. Cas9 Nuclease V3 (224675276; IDT; Leuven, Belgium).
2. Alt-R® CRIPSR/Cas9 sgRNA (IDT) (*see* **Note 2**).
3. Ultramer® DNA Oligo single-stranded oligodeoxynucleotides (IDT), only if gene conversion is targeted (*see* **Note 2**).
4. Nuclease-free IDTE buffer (pH 7).
5. OptiMEM (2329619; Thermo Fisher Scientific).
6. RNAiMAX.

2.3.2 Equipment

1. Conical tubes (5 and 15 mL).
2. Eppendorf tubes (5 mL).
3. Pipettes (10, 100, 1000 μL).

3 Methods

3.1 Testes Collection and Cryopreservation

1. Harvest testes from sacrificed mice (5–7 days post-partum). Place all testes in ~6 mL DMEM/F-12 in a 15 mL conical tube directly after removal.
2. While working under a sterile light microscope, gently remove the tunica albuginea of each testis using two forceps. Testicular tissue can be placed in a sterile petri dish with DMEM/F-12 medium for easy handling during this step.
3. Digest the testicular tissue directly after removal or cryopreserve until usage. For cryopreservation, transfer the testicular tissue into cryovials containing 1.5 mL cryoprotectant (Table 1) and incubate for 10 minutes (min) at room temperature (RT). Place the cryovials into an appropriate freezing container and incubate minimum 2 h at −80 °C. Cryovials can be stored afterward in liquid nitrogen and thawed when needed (*see* **Note 3**).

3.2 Initiation and Maintenance of a GS Cell Culture

(Based on Wang et al. [3] and Shinohara et al. [20]; with modifications)

1. Testicular tissue can be digested directly after extraction or thawed after storage in liquid nitrogen. For thawing, place cryovials in a 37 °C water bath (2 min). Transfer testicular tissue immediately after thawing in DMEM/10% FBS and wash twice. Avoid toxicity against the testicular tissue from thawed cryomedium by working fast.
2. To digest testicular tissue, place it into a 15 mL conical tube containing 9.5 mL DMEM. Add 0.5 mL collagenase type I (20 mg/mL sterile stock) to the 15 mL tube and incubate in a 37 °C water bath for 15 min. Gently pipette up and down after 7.5 and 15 min.

3. Centrifuge (5 min, 300*g*) and discard supernatant.
4. Wash precipitate with 5 mL sterile PBS. Then, centrifuge (5 min, 300*g*) and discard the supernatant.
5. Resuspend the precipitate in 3 mL TrypLE to dissociate cell-cell contacts. Incubate in a 37 °C water bath (10 min).
6. After incubation, add 3 mL DMEM/10% (v/v) FBS to stop trypsinization. Pipette up and down to segregate remaining cell attachments. Before proceeding with the next step, verify that cells are present in a single-cell format (*see* **Note 4**).
7. Filtrate the obtained cell suspension through a 70 μm cell strainer. Centrifuge (5 min, 300*g*) and discard supernatant.
8. Resuspend the precipitate in 1.5–3 mL GS cell medium (Table 2) and keep it on ice. The exact amount of medium depends on cell yield and should not exceed 2.5×10^5 cells/mL.
9. Coat a 12-well culture plate by adding 500 μL 0.4 % (w/v) gelatine in PBS per well. Incubate for 30 min (37 °C) and aspirate solution afterwards.
10. Seed testicular cells in the gelatine-coated 12-well culture plate. Calculate 5×10^4 cells/cm^2 (~2×10^5 cells/well in 0.8 mL GS cell medium). Incubate the plate overnight (37 °C, 5% CO_2). Many somatic cells will adhere to gelatine, while germ cells will float in the medium or weakly stick to the somatic cell layer (*see* **Note 5**).
11. On the next day, transfer the medium containing floating GS cells, well per well, into a non-coated 12-well plate. Detach GS cells which stick to the somatic cell layer by intense pipetting using a 1000 μL pipette.
12. Replace culture medium every 2–3 days. GS cells will attach to an endogenous feeder build by remaining somatic testicular cells, and will form GS cell colonies (Figure 1A).
13. Passage plates 10–14 days after culture initiation at a ratio of 1:1 (P1 → P2). For cell passaging, wash cells two times in PBS, trypsinize (400 μL TrypLE/well in a 12-well culture plate) and incubate for 10 min (37 °C). Flush wells carefully with trypsin cell mixture before collecting medium containing GS cells in a 15 mL conical tube. Mix to dissociate cells, inactivate trypsinization, and filter through a 70 μm cell strainer. Centrifuge (5 min, 300*g*) and discard supernatant. Dilute GS cells in GS cell medium and seed 5×10^4 cells/cm^2 into a 12-well culture plate.
14. Change the medium every 2–3 days. When cells reach confluency, passage at a ratio of 1:2 (P2 → P3).

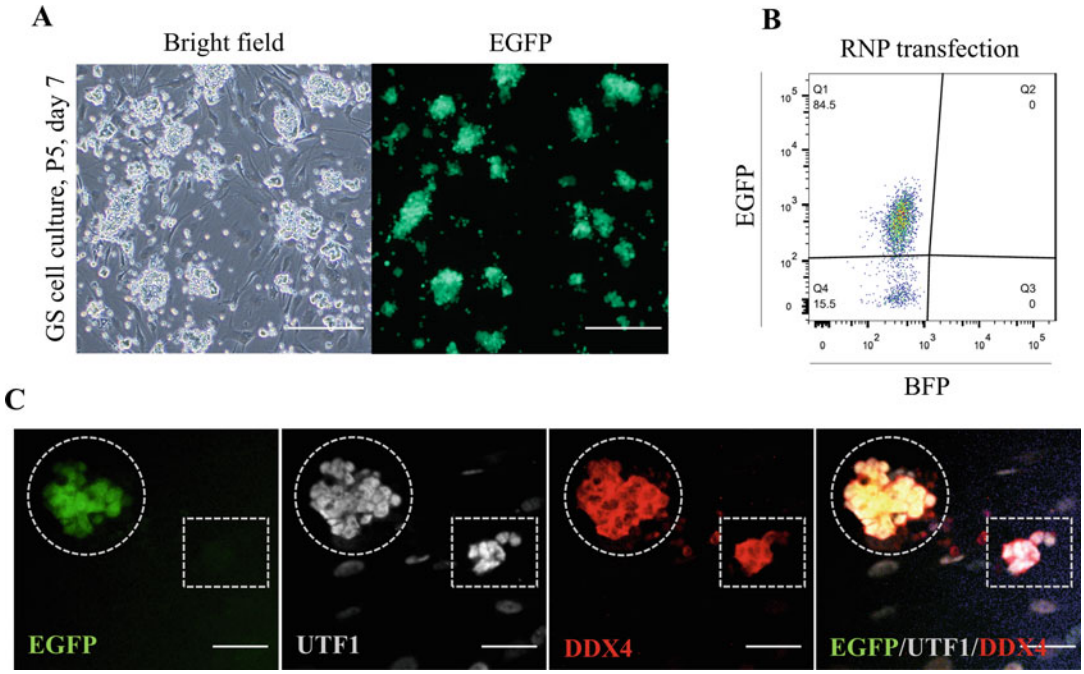

Fig. 1 GS cell cultures (**a**) and GS cells after gene editing (**b**, **c**). (**a**) GS cells grow and form colonies on mouse embryonic fibroblasts. (**b**) Flow-cytometry confirmed the loss of EGFP in GS cells (Q4) ten days after transfection. (**c**) Unmodified ($EGFP^+$, circle) and gene-edited ($EGFP^-$, square) GS cell colonies maintained their characteristics of undifferentiated germ cells ($UTF1^+/DDX4^+$) ten days after transfection. Scale bars represent 200 μm in pictures of GS cell culture and 50 μm in pictures of GS cell colonies after gene transfection

15. After 2–3 passages, the endogenous feeder will gradually disappear, slowing GS cell growth in the culture. GS cells should then be seeded onto a MEF feeder. Prepare the MEF feeder (~7.6×10^4 cells/cm^2) 24 h before usage following the manufacturer's instructions.
16. Passage GS cell culture when plates reach confluency or shortly before feeder detachment (*see* **Note 6**).
17. GS cells can be kept in culture for up to two years [5]. Cells can be used for transfection after establishing a stable and well-growing cell culture.

3.3 Preparation of CRISPR/Cas9 Transfection Complexes

(Based on the IDT protocol "Alt-R CRISPR-Cas9 System: Cationic lipid delivery of CRISPR ribonucleoprotein complexes into mammalian cells.")

1. Seed MEF-feeder (~7.6×10^4 cells/cm^2) in a 48-well culture plate 24 h before usage (~18 h before transfection start).
2. The next day, dissolve CRISPR gRNA in nuclease-free IDTE buffer (pH 7) and Cas9 in OptiMEM to a working solution of 1 μM (*see* **Note 7**).

Table 3
Preparation of transfection complexes

Ingredients	Amount (in μL)	
	Gene knockout	*Gene conversion*
RNP (180 nM)	2000	2000
RNAiMAX	96	96
OptiMEM	1904	1899
ssODN (100 μM)	–	5
Total volume	4000	4000

We established a protocol targeting gene knockout and conversion in *Egfp*. Most optimal concentrations identified in the study [19] are shown here. Concentrations of RNP (90 nM) and ssODN (125 nM) in transfection complexes were three times higher than the end concentrations. When using different gRNA, ssODN and/or gene targets, optimization of given concentrations might be required

3. Dilute Cas9 and gRNA in a ratio of 1:1 in OptiMEM. Calculate the concentration six times higher than the intended end concentration (e.g., 30 nM RNP), as it will get further diluted in the lipofection (1/2) and culture (1/3) media. For instance, to prepare 180 nM RNP solution in a total volume of 2500 μL, add 450 μL RNP (1 μM) + 450 μL Cas9 (1 μM) + 1600 μL OptiMEM into a tube. Incubate the mix for 5 min at RT to enable Cas9:gRNA complexing (*see* **Note 7**).
4. Mix RNP and the lipid-based carrier RNAiMAX (2.5 μL/cm^2) in OptiMEM. When specific gene editing via HDR is targeted, also include single-stranded oligodeoxynucleotides (ssODN). Incubate the mix for 20 min at RT to form transfection complexes.

Importantly, calculate RNP and ssODN three times higher than the intended end concentration. Recently, we targeted *Egfp* in EGFP-GS cells and identified an end concentration of 30 nM RNP and 42 nM ssODN as the most optimal condition for our system [19] (Table 3).

3.4 Reverse Transfection of GS Cells (Fig. 2)

1. During incubation of transfection complexes, harvest GS cells. Wash, trypsinize, and filter cells. Dilute GS cells in GS cell medium without penicillin/streptomycin to improve cell viability after transfection (*see* **Note 8**). Calculate ~8 × 10^4 cells/well and 200 μL medium/well when working in a 48-well culture plate.
2. Add 100 μL transfection complexes per well in an uncoated 48-well culture plate. Ensure to include all necessary controls (e.g., untreated, vehicle only, no donor DNA) and replicates.

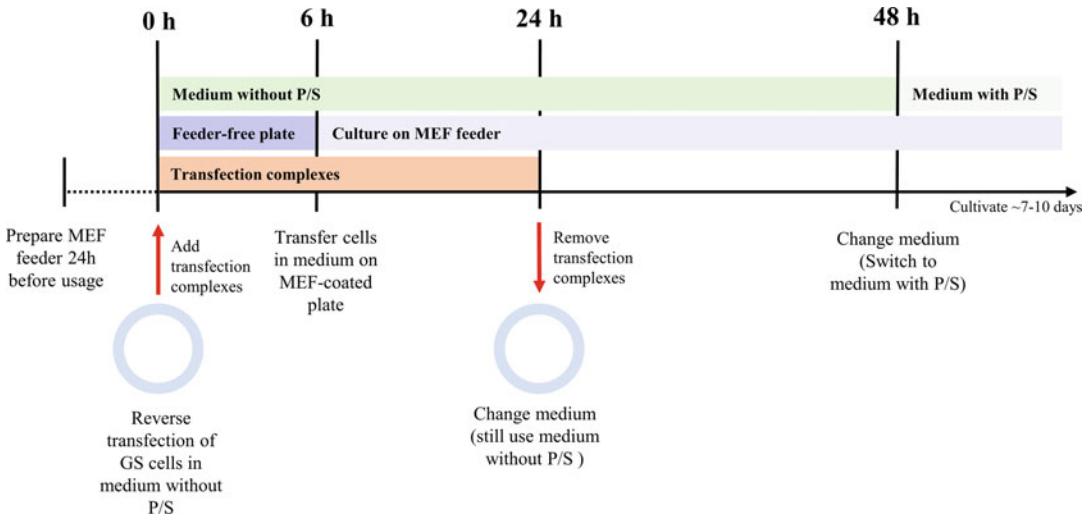

Fig. 2 Workflow of transfection protocol. Incubate GS cells with transfection complexes in an uncoated 48-well plate in a medium without penicillin/streptomycin (P/S) (6 h, 37 °C, 5% CO_2). Subsequently, transfer the medium containing GS cells and transfection complexes, well per well, onto a MEF-coated 48-well plate. The next day, remove transfection complexes by changing the medium. Forty-eight hours after transfection starts, switch to GS cell medium with P/S

3. Subsequently, seed ~8 × 10^4 GS cells/well in 200 µL GS cell medium without penicillin/streptomycin onto transfection complexes. Incubate for 6 h (37 °C, 5% CO_2). During this time, transfection complexes will enter GS cells (*see* **Note 9**).
4. After incubation, the medium containing GS cells and transfection complexes can be transferred well per well to the earlier prepared 48-well culture plate containing a MEF feeder. Carefully flush the wells before transferring the medium into the new plate. Incubate overnight (37 °C, 5% CO_2).
5. The next day, as GS cells stick to the MEF feeder, the transfection complexes can be removed by medium change. Still, use GS cell medium without penicillin/streptomycin. Incubate 24 h (37 °C, 5% CO_2).
6. Change medium. Switch to GS cell medium with penicillin/streptomycin.
7. Gene-edited GS cells can be expanded for up to 10 days or until the plate reaches confluency. Afterward, cells can be analyzed, sorted, expanded and/or used for further experiments (*see* **Note 10**).

4 Notes

1. We isolated testes from mice expressing EGFP in every cell containing a cell nucleus [19]. The heterozygous mouse F1 generation (B6D2F1/2) was used, which was bred from female C57BL/6-Tg(CAG-EGFP)13Osb/LeySopJ and male DBA/2J mice; an efficient strain to establish an EGFP-GS cell line from [20].
2. In our study, we used *Egfp*-gRNA (5′-CTCGTGACCACCCT GACCTA-3′) and *Bfp*-ssODN (5′-ACCCTGAAGTTCATCT GCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACC CTCGTGACCACCCTGAGCCACGGGGTGCAGTGCTTC AGCCGCTACCCCGACCACATGAAGCAGCACGACTTC TTCAAGTCCGCCATGCC-3′) [19].
3. Place not more than four testes per cryovial and ensure that all testes sank to the bottom of the vial before transferring them into the −80 °C freezer.
4. When testicular cells are dissociated insufficiently, big cell clumps will stick in the 70 μm cell strainer, strongly decreasing cell yield. A longer trypsinization time and harsher cell pipetting in trypsin will improve cell dissociation and, consequently, increase cell yield.
5. Poor colony formation during GS cell initiation can be improved by seeding higher cell starting concentrations per well (up to 8×10^5 cells/well in a 12-well culture plate).
6. Poor cell growth and survival in GS cell cultures is often caused by a deficient feeder cell layer. We cultured GS cells on mitomycin-inactivated mouse embryonal fibroblasts and in feeder-free laminin-coated plates, which was also described by Kanatsu-Shinohara et al. [20]. Alternatively, the cultivation on radiation-inactivated MEF is described in literature [3].
7. The working solution of gRNA can be stored up to six months at −20 °C. RNP complexes are stable for up to 4 weeks at 4 °C and up to 6 months at −80 °C.
8. Transfection in the presence of the antibiotic mixture penicillin/streptomycin caused massive cell toxicity when tested in HEK293T cells. Transfection complexes presumably formed small cellular entrance sides for the antibiotics, which caused the cell death. This mechanism was avoided when transfecting in an antibiotic-free medium.
9. GS cells are transfected in feeder-free conditions to avoid absorbance of transfection complexes by the feeder, which would be accompanied by a dramatic reduction of transfection efficiency.

10. Recently, we showed proof-of-principle of RNP-based CRIPSR/Cas9 lipofection by knocking out *Egfp*, which resulted in the loss of green fluorescence and was quantified using flow cytometry (Figure 1B). Expression of the stem cell marker UTF1 and the germ cell marker DDX4 showed that unmodified EGFP positive, but also gene-edited EGFP negative GS cell colonies maintained their undifferentiated germ cell phenotype after gene editing (Fig. 1c).

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Chapter 11

Mouse In Vitro Spermatogenesis on 3D Bioprinted Scaffolds

Guillaume Richer, Tamara Vanhaecke, Vera Rogiers, Ellen Goossens, and Yoni Baert

Abstract

Testes have a complex architecture that is compartmentalized into seminiferous tubules with a diameter of approximatively 200 μm in which the germ cells differentiate, surrounded by a basement membrane and interstitium. 3D bioprinting might be used to recreate the compartmentalized testicular architecture in vitro. Directed by a software program, pneumatic microextrusion printers can deposit 3D layers of hydrogel-encapsulated interstitial cells in a controlled manner by applying pressure. Once macroporous-shaped scaffolds resembling seminiferous tubules have been bioprinted with interstitial cells, the epithelial cell fraction can be seeded in the macropores to resemble the in vivo testicular architecture. Moreover, macropores can serve as a delimitation for all testicular cells to reorganize and improve the supply of nutrients to cells through the 3D constructs.

Key words Spermatogonial stem cells, Testicular organoid, In vitro spermatogenesis, Tissue engineering, Alginate, Scaffold, 3D bioprinting

1 Introduction

In vitro spermatogenesis (IVS) has already been successfully achieved in rodent testicular organ cultures [1]. In particular, organ cultures preserve the testicular architecture, but do not allow cell manipulation to carry out mechanistic studies, thus limiting their applicability [2, 3]. Because IVS greatly depends on the provision of a 3D testicular microenvironment, a new culture system was developed in the mouse using 3D (bio)printing of testicular cells and an alginate-based hydrogel, which allows single-cell input to restore testicular compartmentalization [4]. The macropores of printed cell-free scaffolds (CFS) were seeded with all testicular cells (TCs) from prepubertal C57BL/6J$^{Acr\text{-}EGFP}$ mice (TC/CFS, Fig. 1). In addition, testicular constructs were produced by seeding magnetic-activated cell sorting (MACS)-enriched C57BL/6J$^{Acr\text{-}EGFP}$ mouse CD49f^{+} epithelial testicular cells in macropores of interstitial cell-laden bioprinted scaffolds

Marco Barchi and Massimo De Felici (eds.), *Germ Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 2770, https://doi.org/10.1007/978-1-0716-3698-5_11,

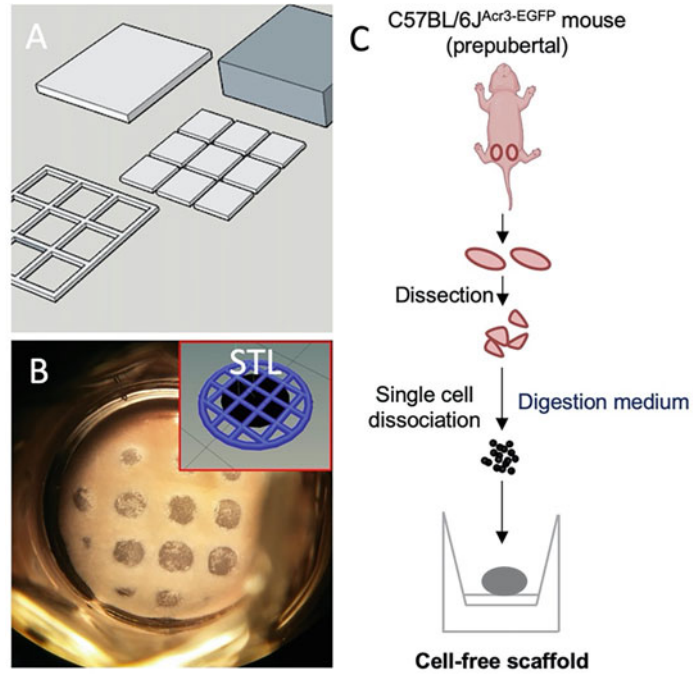

Fig. 1 Study design TC/CFS. (**a**) Sketchup software was used to draw the design of scaffolds. (**b**) CFS in hanging culture inserts in a 24-well plate. Insert shows STL file of CFS. (**c**) Seeding of testicular cells from prepubertal mice in the macropores of CFS

(CD49f$^+$/CLS, Fig. 2). Aggregation of cell suspensions into spheroidal-shaped aggregates was observed in the pores of TC/CFS and CD49f$^+$/CLS constructs in the weeks following cell seeding. Using immunofluorescence staining for EGFP and the postmeiotic markers cAMP responsive element modulator (CREM) or peanut agglutinin (PNA) (Fig. 3), elongated spermatids could be observed in some testicular cell aggregates in 66% of TC/CFS constructs. Differentiation up to the level of round and elongated spermatids was confirmed in 100% and 33% of CD49f$^+$/CLS constructs, respectively.

This chapter describes the first protocol adopted for achieving IVS in mice through the use of 3D bioprinting [4]. In the future, the efficiency of germ cell differentiation could be increased by improving the self-reorganization of testicular cells and by optimizing the layout of the scaffold layout and cell seeding parameters [5].

2 Materials

2.1 Testis Collection and Cryopreservation

1. Prepubertal (<7 dpp) and adult (6 months) C57BL/6J$^{Acr3\text{-}EGFP}$ mice.

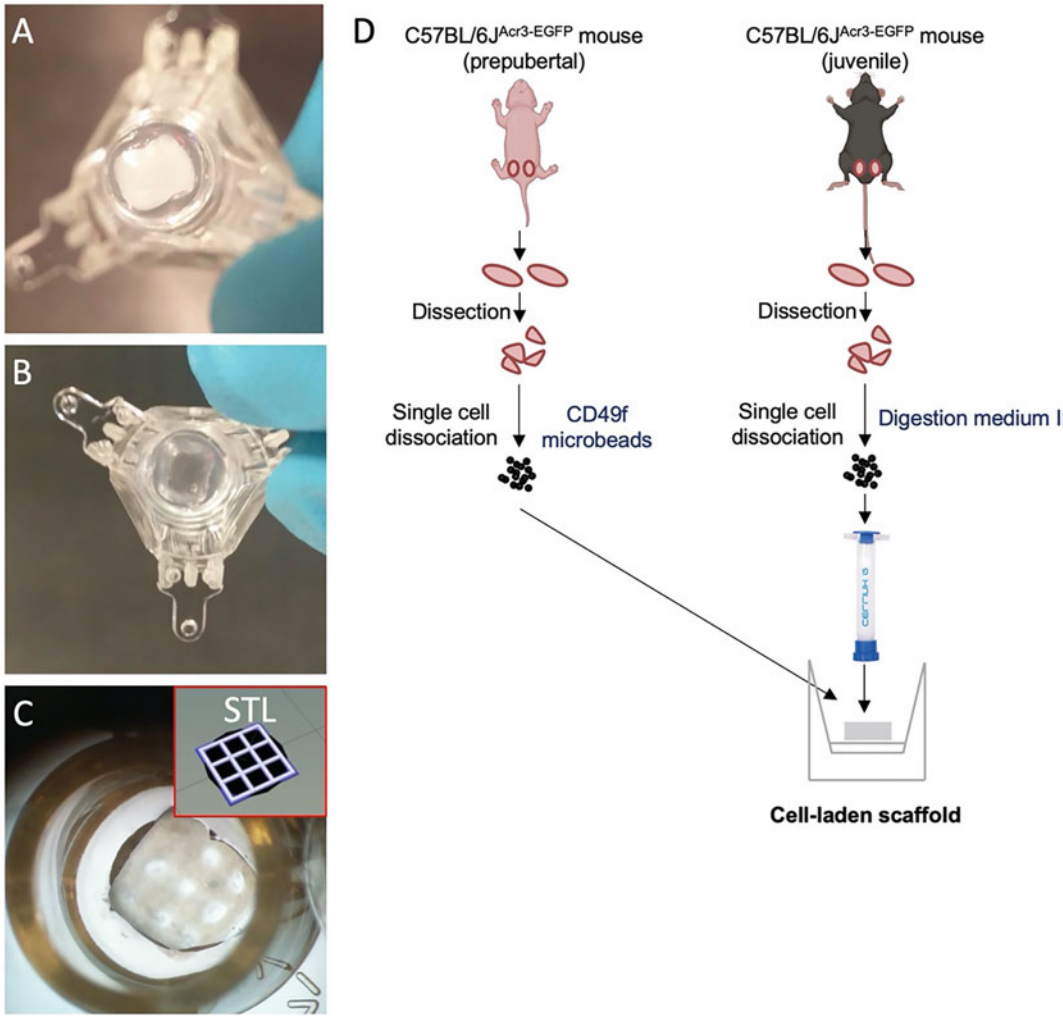

Fig. 2 Study design CD49f/CLS. (**a**, **b**) A square-shaped mold (**a**) was used to create an "agarose socket" (**b**) in which CLS can be placed. (**c**) Insert shows STL file of CLS. (**d**) Seeding of MACS-enriched CD49f^{+} testicular cells from prepubertal mice in the macropores of CLS containing interstitial cells from adult mice

2. Cryopreservation medium: DMEM/F12, 0.07 M sucrose, 1.5 M dimethyl sulfoxide (DMSO). Dissolve 1.20 g sucrose in 44.65 mL DMEM/F12 in a 50 mL polypropylene tube (*see* **Notes 1–3**). Filter-sterilize the solution in another 50 mL polypropylene tube. Complete the cryopreservation medium by adding 5.35 mL sterile DMSO (*see* **Note 4**).
3. "Mr. Frosty" freezing container (*see* **Note 5**).
4. 10 cm cell culture dishes.
5. 1.5 mL cryovials.
6. 15 mL, 50 mL polypropylene tubes.
7. 10 mL, 20 mL, 50 mL eccentric syringes.

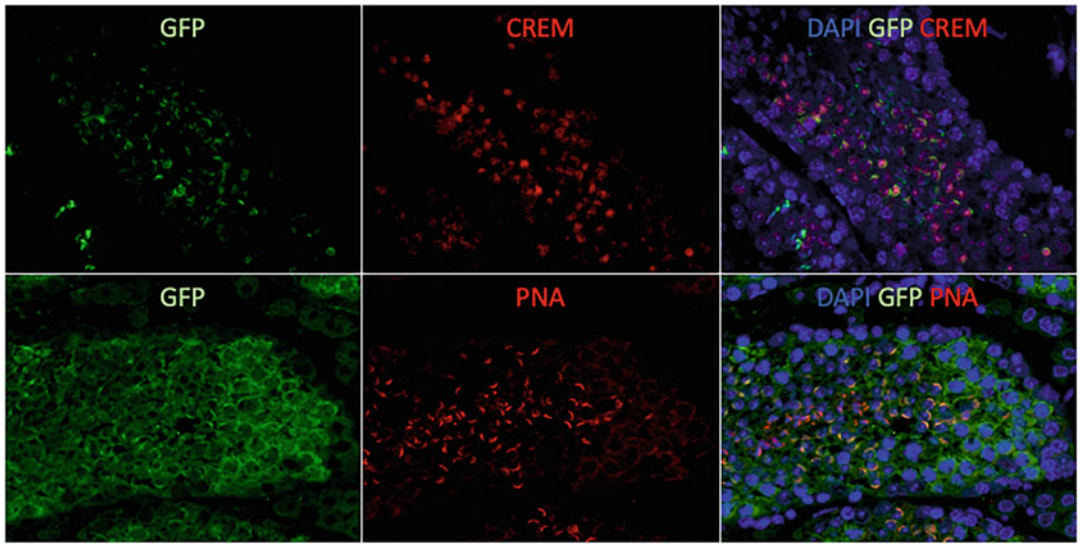

Fig. 3 Examination of germ cell differentiation. Control fluorescent staining on adult C57BL/6J$^{Acr\text{-}EGFP}$ for postmeiotic markers GFP (Acrosin) in combination with CREM or PNA

8. −80 °C freezer.
9. Liquid nitrogen cryotank.
10. Sterile surgical set and scissors.

2.2 Culture of 3D Constructs

2.2.1 3D (bio)Printing Process and Parameters for Scaffolds and Mold Preparation

1. Sketchup software (Fig. 1a) was used to draw the designs of the cell-free scaffold (CFS), cell-laden scaffold (CLS), and mold–CFS: diameter 7 mm, 0.15 mm height, strand distance 1.3 mm, strand thickness 0.6 mm; CLS: 3.5 mm^2, 0.15 mm height, strand distance 1.1 mm, strand thickness 0.6 mm; mold: 4.2 mm^2, 0.15 mm height, no strand distance (*see* **Note 6**)—and generate STereoLithopraphy files (STL, inserts of Figs. 1b and 2c). STL files were translated into a G-code using Heartware software.
2. 3D (bio)printing parameters of CFS, mold, and CLS: Cellink®-RGD bioink was dispensed using the Inkredible+ microextrusion bioprinter with a 25 G conical nozzle at room temperature using Heartware software and a pressure of 7–10 kPa and a plotting speed of 175–200%.

2.2.2 Enzymatic Digestion of Testicular Tissue

1. Dilution buffer: DMEM/F12, 10% (v/v) fetal bovine serum (FBS).
2. Digestion medium I to collect all testicular cells: αMEM, 1 mg/mL collagenase type 1A, 0.5 mg/mL DNase, 0.5 mg/mL hyaluronidase (*see* **Note 7**).
3. Digestion medium II to collect interstitial cells only: αMEM, 1 mg/mL collagenase type 1A.

4. Storage buffer: αMEM, 0.5 mg/mL DNase (*see* **Note 8**).
5. MACS buffer: 1× PBS, 0.5% (w/v) bovine serum albumin (BSA), 2 mM trypsin-ethylenediaminetetraacetic acid (EDTA).
6. Cell culture medium αMEM, 10% (v/v) knockout serum replacement (KSR), 0.1 μM melatonin, 1% (v/v) penicillin/streptomycin.
7. 0.4% (v/v) trypan blue.
8. Neubauer counting chamber.
9. Coverslips.
10. Centrifuge.
11. MACS separator.
12. MS columns.
13. 15 mL, 50 mL polypropylene tubes.
14. FITC rat anti-CD49f antibody.
15. Anti-FITC microbeads.
16. Phase-contrast microscope.
17. Shaking water bath.
18. Pipetboy.
19. 5 mL, 10 mL, 25 mL pipettes.
20. Parafilm.
21. 40 μm cell strainer.
22. Hanging culture inserts, polyethylene terephthalate 1 μm, 24-well.
23. CO_2 incubator.

2.2.3 3D (bio)Printing

1. Cell culture medium: αMEM, 10% KSR, 0.1 μM melatonin, 1% penicillin/streptomycin.
2. Agarose gel stands: prepare 0.35% (v/v) agarose by diluting 0.7% (w/v) agarose in sterile culture medium (*see* **Notes 9** and **10**). Cover the membrane of hanging culture inserts with 50 μL liquid 0.35% (v/v) agarose (*see* **Note 11**). Place the hanging culture inserts in the wells of a 24-well plate.
3. 1× PBS: 10× PBS in ddH_2O.
4. Hot plate.
5. 24-well plate.
6. 50 mL, 100 mL screw top bottle.
7. 10 cm dishes.
8. 15 mL, 50 mL polypropylene tubes.
9. Cellink® RGD bioink.
10. Cellink® crosslinking agent.

11. Inkredible+ 3D printer.
12. CO_2 incubator.
13. Vortex.
14. Centrifuge.

2.3 Gross Histological Analysis and In-Depth Examination

1. 1× tris-buffered saline (TBS): 50 mM Tris–HCl, 150 mM NaCl. Dissolve 6.06 g Tris and 8.76 g NaCl in 800 mL ddH_2O. Adjust solution to pH 7.5 using 1 M HCl and make up volume up to 1 L with ddH_2O.
2. Antigen retrieval buffer: 0.1 M dihydrate trisodium citrate, 0.0175 M citric acid, 0.05% (v/v) Tween-20. Dissolve 25.8 g of dihydrate trisodium citrate and 19.2 g citric acid in 800 mL ddH_2O to make 0.1 M citrate buffer. Adjust solution to pH 6.0 using 1 M HCl and make up volume up to 1 L with ddH_2O. Add 0.5 mL Tween-20 and mix well.
3. Blocking solution: 1× TBS, 20% (v/v) normal chicken serum (NChS), 5% (w/v) BSA.
4. Primary antibodies: goat polyclonal anti-GFP (Abcam), rabbit polyclonal anti-CREM.
5. Secondary antibodies: horseradish peroxidase (HRP)-conjugated chicken anti-rabbit, donkey anti-goat 488 (*see* **Note 12**).
6. Alexa Fluor 594-conjugated PNA.
7. TSA™ Cy3 plus system.
8. 2% (w/v) agarose.
9. Diamond pen.
10. ImmEdge hydrophobic barrier pen.
11. Alcohol, formalin, acetic acid (AFA) fixative.
12. 10% (v/v) neutral buffered formalin.
13. Sterile surgical set.
14. Tissue cassettes.
15. Microtome.
16. Microscope slides.
17. 2-Propanol.
18. Xylene.
19. Oven.
20. Hot plate.
21. Embedding station.
22. Mold for embedding cassettes.
23. Diamond pen.

24. ToupView software (ToupTek Photonics).
25. PAS kit.
26. Schiff's reagent.
27. Hematoxylin.
28. Acid–alcohol (1% [v/v] HCl).
29. Saturated lithium carbonate.
30. Entellan mounting medium.
31. SlowFade™ gold antifade mountant with 4′,6-diamidino-2-phenylindole (DAPI).
32. Nail polish.
33. Water bath.
34. Fluorescent microscope with emission filters: DAPI, GFP (FITC, Alexa Fluor 488), Texas Red (Alexa Fluor 594).
35. TissueTek VIP automated processor.
36. Paraffin.

3 Methods

3.1 Testes Collection and Cryopreservation

1. Prepare fresh *ready-to-use* cryopreservation medium or complete the frozen working solution (*see* **Notes 2** and **4**).
2. Sacrifice prepubertal mice by decapitation using scissors and adult mice by neck dislocation.
3. Immediately remove testes and collect in a 15 mL polypropylene tube filled with DMEM/F12.
4. Wash testes in a 10 cm dish filled with new DMEM/F12.
5. Immobilize the testes using a set of tweezers and carefully cut and tear apart the tunica albuginea to squeeze out the seminiferous tubules (*see* **Note 13**).
6. Collect the testicular tissue in a new dish filled with DMEM/F12 (*see* **Note 14**).
7. Transfer four testicular tissues to each cryovial filled with 1.5 mL cryomedium.
8. Keep the cryovials at 4 °C for 10 min.
9. Place the cryovials in a "Mr. Frosty" freezing container and freeze at −80 °C freezer for a minimum of 2 h.
10. Once frozen, store the samples in liquid nitrogen.

3.2 Culture of TC/CFS Constructs

3.2.1 3D Printing CFS

1. Print circle-shaped CFS in 10 cm dishes (*see* **Note 15**).
2. Crosslink the Cellink®-RGD with 100 mM $CaCl_2$ for 5 min and rinse CFS with culture medium.

3. Cover the membrane of hanging culture inserts with 50 µL liquid 0.35% (v/v) agarose.
4. Transfer CFS on 0.35% (v/v) agarose gel stands in hanging culture inserts in the wells of a 24-well plate using a sterile spatula (Fig. 1b).
5. Add 600 µL culture medium to the basolateral compartment of the hanging culture insert (*see* **Note 16**).

3.2.2 Fabrication TC/CFS

1. After retrieving the cryovials from the liquid nitrogen storage, immediately thaw the prepubertal mouse tissues for 1–2 min in a water bath at 37 °C.
2. Remove cryoprotective medium and wash the tissue in dilution buffer for 5 min.
3. Transfer the seminiferous tubules to new DMEM/F12 in a 10 cm dish.
4. Transfer the samples into a polypropylene tube filled with 2 mL digestion medium I (1.0 mg/mL collagenase 1A, 0.5 mg/mL hyaluronidase, and 0.5 mg/mL DNase in DMEM/F12) per cryovial of four testes (Fig. 1c).
5. Disrupt the seminiferous tubules by pipetting up and down using a pipetboy (*see* **Note 17**).
6. Isolate the lid of the polypropylene tube with parafilm and place the tubes in a shaking (132 rpm) water bath at 37 °C for 25–30 min (*see* **Notes 18** and **19**).
7. Resuspend the cell suspension up and down until cell clumps are broken.
8. Run the cell suspensions through a 40 µm cell strainer to remove remaining cell clumps.
9. Centrifuge the cell suspension for 5 min at 300× *g* and dilute the pellet in storage buffer (*see* **Note 20**).
10. Determine the viable cell seeding concentrations using the 0.4% trypan blue exclusion test and a Neubauer counting chamber using the following formula:

$$\text{Viable cell count} = \frac{\text{Number of live cells counted}}{\text{Number of large corner squares counted}} \times \text{dilution factor} \times 10,000$$

11. Centrifuge the desired number of cells in storage buffer for 5 min at 300× *g*.
12. Resuspend the testicular cell pellet into fresh culture medium at a concentration of 10^8 cells/mL.
13. Seed 5×10^5 testicular cells/5 µL drop of culture medium per CFS (Fig. 1c).

14. Refresh culture medium from the basolateral side of the hanging culture inserts.
15. Incubate at 35 °C in a humidified atmosphere containing 5% CO_2 and refresh the culture medium weekly.

3.3 Culture of CD49f/CLS Constructs

3.3.1 Agarose Sockets

1. Print square-shaped molds (dimensions: 4.2 mm × 4.2 mm, 1.5 mm height) in 10 cm dishes.
2. Crosslink the hydrogels with 100 mM $CaCl_2$ for 5 min and rinse the molds with culture medium.
3. Place the mold on the 0.35% (v/v) agarose gel stands in the culture inserts and add a second layer of 35 µL 0.35% (v/v) agarose around it (Fig. 2a).
4. When agarose has gelled, remove the mold using sterile tweezers to create an "agarose socket" (Fig. 2b) (*see* **Note 21**).

3.3.2 Digestion Adult Mouse Testes

1. Immediately thaw the adult mouse tissues for 1–2 min in a water bath at 37 °C.
2. Remove cryoprotective medium and wash the tissue in dilution buffer for 5 min.
3. Transfer the testicular tissues to new DMEM/F12 in a 10 cm dish.
4. Transfer the samples into a polypropylene tube filled with 2 mL of digestion medium II (1.0 mg/mL collagenase 1A in DMEM/F12) per cryovial of four testes (Fig. 2d) (*see* **Note 7**).
5. Disrupt the seminiferous tubules by pipetting up and down using a pipetboy (*see* **Note 17**).
6. Isolate the lid of the polypropylene tube with parafilm and place the tubes in a shaking (132 rpm) water bath at 37 °C for 10 min (*see* **Notes 18** and **19**).
7. Resuspend the cell suspension up and down.
8. Allow the seminiferous tubules fragments to sediment for 10 min.
9. Collect supernatant enriched in testicular interstitial cells.
10. Run the cell suspensions though a 40 µm cell strainer to remove remaining cell clumps.
11. Centrifuge the interstitial cell suspension for 5 min at 300× *g* and dilute the pellet in storage buffer (*see* **Note 20**).
12. Determine the viable interstitial cell concentrations using the 0.4% trypan blue exclusion test and a Neubauer counting chamber.
13. Dilute Cellink®-RGD bioink with adult interstitial cells at a concentration of 2×10^7 cells/mL (Fig. 2d) (*see* **Note 22**).

3.3.3 3D Bioprinting CLS

1. Print CLSs made of Cellink®-RGD diluted with adult interstitial cells (dimensions: 3.5 mm × 3.5 mm, 0.15 mm height, strand distance 1.1 mm, strand thickness 0.6 mm) in 10 cm dishes.
2. Crosslink the hydrogels with 100 mM $CaCl_2$ for 5 min and rinse the CLSs with culture medium.
3. Transfer the CLS on 0.35% (v/v) agarose gel stands in hanging culture inserts using a sterile spatula (Fig. 2c).
4. Add 600 µL culture medium to the basolateral compartment of the hanging culture insert and incubate at 35 °C in a humidified atmosphere containing 5% CO_2 for 15 min or longer to equilibrate.

3.3.4 MACS-Mediated Enrichment CD49f+ Epithelial Testicular Cells

1. After retrieving the cryovials from the liquid nitrogen storage, immediately thaw the prepubertal mouse tissues for 1–2 min in a water bath at 37 °C.
2. Remove cryoprotective medium and wash the tissue in dilution buffer for 5 min.
3. Transfer the seminiferous tubules to new DMEM/F12 in a 10 cm dish.
4. Transfer the samples into a polypropylene tube filled with 2 mL digestion medium I (1.0 mg/mL collagenase 1A, 0.5 mg/mL hyaluronidase, and 0.5 mg/mL DNase in DMEM/F12) per cryovial of four testes (Fig. 1c).
5. Disrupt the seminiferous tubules by pipetting up and down using a pipetboy (*see* **Note 17**).
6. Isolate the lid of the polypropylene tube with parafilm and place the tubes in a shaking (132 rpm) water bath at 37 °C for 25–30 min (*see* **Notes 18** and **19**).
7. Resuspend the cell suspension up and down until cell clumps are broken.
8. Run the cell suspensions through a 40 µm cell strainer to remove remaining cell clumps.
9. Centrifuge the cell suspension for 5 min at 300 *g* and dilute the pellet in storage buffer (*see* **Note 20**).
10. Determine the viable cell seeding concentrations using the 0.4% trypan blue exclusion test and a Neubauer counting chamber using the following formula:

$$\text{Viable cell count} = \frac{\text{Number of live cells counted}}{\text{Number of large corner squares counted}} \times \text{dilution factor} \times 10,000$$

11. Centrifuge the desired amount of single cells for 5 min at 300× *g*.
12. Stain by resuspending 10^6 cells in 100 μL MACS buffer and 40 μL FITC rat anti-CD49f antibody.
13. Incubate for 20 min in the dark on ice.
14. Wash unbound primary antibody by adding 1 mL MACS buffer per 10^7 cells and centrifuge at for 5 min at 300× *g*.
15. Resuspend 10^6 cells in 90 μL MACS buffer and 10 μL anti-FITC microbeads.
16. Incubate for 15 min on ice.
17. Wash the cells by adding 1–2 mL MACS buffer per 10^7 cells and centrifuge for 5 min at 300× *g*.
18. Remove supernatant and resuspend up to 10^8 cells in 500 μL MACS buffer.
19. Place the MS column in the magnetic field of a MACS separator.
20. Rinse the column with 500 μL MACS buffer.
21. Apply cell suspension from **step 9** onto the MS column.
22. Collect the unlabeled CD49f$^-$ effluent in a 15 mL polypropylene tube.
23. Perform three washing steps with 500 μL MACS buffer.
24. Transfer the MS column from the MACS separator on a 15 mL polypropylene tube.
25. Flush out the magnetically labeled CD49f$^+$ cell fraction from the MS column by adding 1 mL MACS buffer and immediately applying the plunger.
26. Repeat **step 25**.
27. Determine the viable CD49f$^+$ cell concentrations using the trypan blue exclusion test and a Neubauer counting chamber using the following formula:

$$\text{Viable cell count} = \frac{\text{Number of live cells counted}}{\text{Number of large corner squares counted}} \times \text{dilution factor} \times 10,000$$

3.3.5 Fabrication CD49f/CLS

1. Centrifuge the CD49f$^+$ cell fraction enriched in epithelial cells in storage buffer for 5 min at 300× *g*.
2. Resuspend the CD49f$^+$ cell pellet into fresh culture medium at a concentration of 1×10^8 cells/mL.
3. Seed 5×10^5 MACS-enriched CD49f$^+$ cells/5 μL drop of culture medium per CLS (Fig. 2d).

4. Refresh culture medium from the basolateral side of the hanging culture inserts.
5. Incubate at 35 °C in a humidified atmosphere containing 5% CO_2 and refresh the culture medium weekly.

3.4 Histochemistry and Immunofluorescence

3.4.1 Fixation of Constructs

1. Remove hanging culture inserts from the 24-well plate at the end of the culture periods using tweezers.
2. Add 2% (w/v) liquid agarose to the adluminal part of the culture inserts and allow agarose gelation to ensure the cellular aggregates do not escape the constructs.
3. Add 150 μL of AFA on solidified agarose to the adluminal part of the culture inserts for 30 min.
4. Cut the membrane of the culture insert with a scalpel and harvest the constructs, place into a cassette.
5. Submerge the cassette in 10% (v/v) neutral buffered formalin until dehydration.
6. Dehydrate the constructs automatically in eight baths of graded ethanol: 70%, 70%, 85%; 85%, 95%, 95%, 100%, 100% (30 min each), clear in two baths of xylene (1 h each), and impregnate with paraffin wax using a TissueTek VIP automated processor.

3.4.2 Paraffin Embedding and Tissue Sectioning

1. Coat the bottom layer of a mold for embedding cassette with melted paraffin.
2. Orient sample as desired in the mold on "cold side" of the embedding station.
3. Place the cassette on top of the mold and completely fill the mold with fresh melted paraffin.
4. Allow paraffin to harden at 4 °C.
5. Remove the mold from the cassette.
6. Slice the samples in 5 μm-thick sections at different depths using a microtome and place them on microscope slides.
7. Dry the slides at 37 °C overnight.

3.4.3 PAS Staining and Morphological Evaluation

1. Before staining, deparaffinize the sections twice in xylene, 5 min each.
2. Rehydrate the sections through 100%, 100%, 90%, 70% (v/v) ethanol and wash the sections twice in 1× PBS, 5 min each.
3. Incubate the samples in periodic acid for 5 min.
4. Wash the sections in running tap water for 3 min and twice in 1× PBS, 5 min each.
5. Incubate the samples in Schiff's reagent for 15 min.
6. Wash the sections in running tap water for 3 min and twice in 1× PBS, 5 min each.

7. Counterstain with hematoxylin for 5 min and rinse in running tap water.
8. Immerse the sections 1 min in 1% (v/v) HCl and rinse in running tap water.
9. Immerse the sections 1 min in lithium carbonate and rinse in running tap water.
10. Dehydrate in 70%, 90%, 100%, 100% (v/v) ethanol and twice in xylene, 5 min each.
11. Mount the sections in mounting medium.

3.4.4 Fluorescence Staining and In-Depth Examination of Germ Cell Differentiation

1. Mark the area around the tissue section with a diamond pen.
2. Deparaffinize the sections twice in xylene, 5 min each.
3. Rehydrate the sections through 100%, 100%, 90%, 70% (v/v) ethanol and wash the sections twice in 1× PBS, 5 min each.
4. Perform antigen retrieval using 0.01 M citrate buffer (pH 6.0) containing 0.05% (v/v) Tween-20 in a water bath at 95 °C for 30 min.
5. Cool down the sections at room temperature for 30 min.
6. Wash the sections twice in 1× TBS, 5 min each.
7. Dry the area around the sections using paper and draw a hydrophobic barrier using a ImmEdge pen.
8. Block endogenous peroxidase by methanol/3% H_2O_2 for 30 min at room temperature.
9. Block the sections with blocking solution containing 20% (v/v) NChS and 5% (w/v) BSA in 1× TBS.
10. Incubate with primary antibodies (goat polyclonal anti-GFP and rabbit polyclonal anti-CREM primary antibodies) in TBS/20% (v/v) NChS/5% (w/v) BSA overnight at 4 °C.
11. Wash the sections twice in 1× TBS, 5 min each, and incubate HRP-conjugated chicken anti-rabbit secondary antibody diluted in TBS/20% (v/v) NChS/5% (w/v) BSA for 30 min followed by TSA™ Cy3 plus system for 3 min at room temperature in the dark.
12. Wash the sections twice in 1× TBS, 5 min each, at room temperature in the dark.
13. Incubate with donkey anti-goat 488 secondary antibody diluted in TBS for 1 h at room temperature in the dark.
14. When the acrosome needs to be visualized, incubate the sections with Alexa Fluor 594-conjugated PNA diluted in TBS for 1 h and 30 min at room temperature in the dark instead of the CREM antibody.
15. Mount the sections with DAPI and seal the coverslips with nail polish.

4 Notes

1. Filter-sterilize all solutions using sterile syringe filter units, pore size 0.22 μm, diameter 33 mm if not specified otherwise.
2. DMSO cannot be filtered through a syringe filter unit unless DMSO-resistant filters are used. Sterile DMSO is packaged in glass vials and aspirated through the rubber cap using a 1 mL syringe with 26 G × 0.375″ needle to become added to filtered-sterilized DMEM/F12/0.07 M sucrose.
3. All cell-processing methods, from tissue collection to digestion, must be performed quickly on ice and using ice-cold media to maximize tissue and cell integrity.
4. Filter-sterilized DMEM/F12/0.07 M sucrose medium can be stored for long term at −20 °C. When needed, complete the cryopreservation medium following the thawing of DMEM/F12/0.07 M sucrose with DMSO supplementation.
5. Do not overfill the "Mr. Frosty" freezing container with 100% isopropyl alcohol. Add to the fill line and replace every 5th use.
6. Size and pattern of scaffolds can be adapted, interesting for delimitating the area in which testicular cells can reorganize or bioprinting lower cell numbers when tissue availability is an issue.
7. Dilute the digestion enzymes in DMEM/F12 the day of use, otherwise enzyme activity will be lost.
8. DNase is used to inhibit cell clumping by eliminating free sticky DNA from broken cells.
9. Dissolve 0.7% (w/v) agarose in 1× PBS by heating in a microwave and sterilize by autoclaving at 121 °C for 20 min.
10. It is advisable to make volumes of 50 mL or larger because agarose tends to aggregate and fails to dissolve completely otherwise.
11. Agarose should always be kept heated to avoid undesired gelling.
12. When working with fluorescent conjugated antibodies, samples should be protected from light during all phases of the experiment.
13. The collection of the testicular tissues needs to be performed in aseptic conditions in a cleaned laminar air flow.
14. Testicular tissues of adult mice have to be cut in pieces of approximately 1–2 mm^3 because the cryopreservation solution should be able to enter the tissue.

15. Deformation of the structures can happen post-printing. Therefore, the best-looking scaffolds in terms of macropore diameter were selected for further cultivation.
16. Incubate for 15 min or longer in culture medium at 35 °C in a humidified atmosphere containing 5% CO_2 to equilibrate.
17. If small volumes are used, seminiferous tubules can be disrupted by pipetting up and down using a 1 mL tip
18. Additional incubations of 5 min can be performed in case of incomplete disruption.
19. During the digestion of the tissue, the tubes are placed horizontally to improve the process.
20. The volume of storage buffer should be adapted to the cell pellet size to avoid excess concentration of the cells during the counting.
21. A mold and subsequent formation of an "agarose socket" ensure proper cell seeding in the macropores of CLS.
22. Mix well using a vortex and centrifuge.

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Chapter 12

Histological and Cytological Techniques to Study Perinatal Mouse Ovaries and Oocytes

Nikoleta Nikou, Maria López Panadés, and Ignasi Roig

Abstract

The regulation of female fertility in mammals depends on critical processes during oocyte development and maturation. Therefore, it is crucial to use specific approaches when studying mammalian female fertility to preserve ovary and oocyte structures effectively. The methods of collecting and culturing ovaries and oocytes play an essential role in the study of mammalian follicle development and oocyte quality. This chapter presents a collection of protocols that focus on various methods for studying mammalian ovaries and oocytes, providing researchers with a variety of approaches to choose from.

Key words Mammalian oocytes, Mammalian ovaries, Culture, Histology, Immunofluorescence

1 Introduction

Successful sexual reproduction involves the fusion of a mature haploid oocyte and sperm during fertilization, which generates a totipotent zygote. The tissues and cell lines of the new individual will derive from this zygote. [1]. The mature haploid gametes are generated during gametogenesis from primordial germ cells (PGCs) [2], which in mice appear on day 6 of embryonic development (E6) from epiblast cells. These will proliferate mitotically as they migrate toward the genital ridges, where about 500 PGCs will arrive on day 10.5 of embryonic development (E10.5) and are called oogonia in females [1]. In the female fetal gonad, the oogonia further divides through mitosis in a synchronized manner to increase its number, but they do not complete cytokinesis. Thus, groups of cells joined by the cytoplasm and connected through cellular bridges are formed, known as cysts [3, 4]. At E13.5, oogonia enter meiosis while still in cysts and become oocytes [1]. Meiosis consists of a single DNA replication followed by two

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cycles of chromosome segregation, thus ensuring the production of haploid gametes [5, 6], genetically different from each other, as well as from the parental cell [7]. In the first meiotic division, homologous chromosomes separate, while the second meiotic division is more similar to mitosis, where sister chromatids separate [7–9].

In prophase I of meiosis, meiotic recombination occurs, a process by which double-strand breaks are generated in DNA [5, 6, 10]. Their repair will lead to the encounter of the homologous chromosomes [6, 10, 11]. The oocytes will arrest at the end of meiotic prophase I once meiotic recombination is complete, generating the germinal vesicle. At the same time, oocyte cysts will break down around birth, resulting in the death of about two-thirds of the oocyte population [3, 4, 12]. The remaining oocytes will be surrounded by a layer of flat pregranulosa cells, forming primordial follicles [1].

In mammals, primordial follicles are the follicular stock for adult life [1]. These primordial follicles start being recruited from their formation [13]. Then, follicular growth will begin, where the oocyte grows, and the follicular cells change their morphology to cubic cells, transforming into primary follicles. These primary follicles develop two or more layers of granulosa cells, becoming secondary follicles. Secondary follicles increase in size with high proliferation of granulosa cells. Furthermore, at this stage, an extra layer is formed on the external side of the follicle from theca cells, which structurally support the follicle [4]. Secondary follicles continue to grow until the antral follicle stage is reached (Fig. 1) [1]. From this point on, folliculogenesis depends on

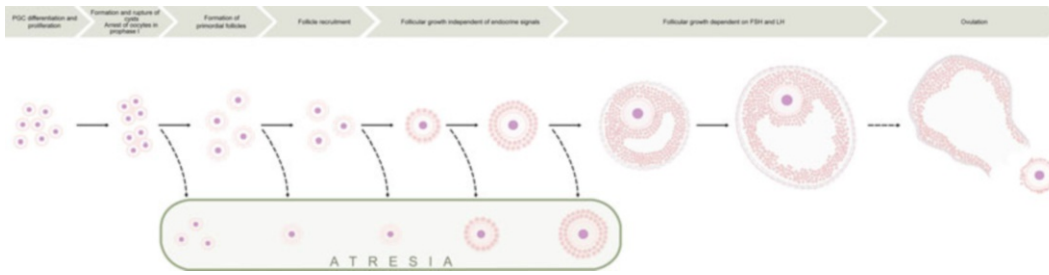

Fig. 1 Follicle development. PGCs reach the gonads and proliferate without a complete division of the cytoplasm, creating cysts. These fragments and the oocytes progress through meiosis until they arrest at the end of prophase I. Oocytes are surrounded by a layer of flat granulosa cells to generate primordial follicles, which remain quiescent until recruited. When the primordial follicles are recruited, granulosa cells become cube-shaped cells, the oocyte grows and the granulosa cells proliferate by mitosis, independently of gonadotropins. At this point, the growth becomes dependent on FSH and LH, the follicular fluid produces the antrum, and the follicle grows in size until ovulation when the oocyte exits the follicle. Atresia, or follicular death, occurs while oocytes and follicles go through the different stages of growth and maturation. Thus, only one or a few follicles are ovulated each time

gonadotropins. Thus, all follicles that reach the antral stage before puberty will die due to the lack of gonadotropins [4]. From puberty, the appropriate endocrine signals, FSH and LH, are provided, and the granulosa cells begin to secrete follicular fluid; the antrum is generated [1]. Eventually, the oocyte undergoes germinal vesicle breakdown (GVBD) and resumes meiosis until it arrests at metaphase II, at which point it is ovulated (Fig. 1), ready to finish meiosis if fertilized. Then, the granulosa and theca cells of the ovulated follicle transform to form the corpus luteum, which plays a crucial role in supporting gestation [14, 15]. Noteworthy, the vast majority of recruited follicles do not reach the preovulatory stage and are eliminated during the female's reproductive life by a process called atresia or follicular death (Fig. 1) [16].

Oocyte cytokinesis is asymmetric, generating a small polar body and a large oocyte at the end of both meiosis I and meiosis II. This generation of larger secondary oocytes is required to ensure that after fertilization, they will have enough cytoplasm and nutrients to thrive [14].

Here, we explain the most relevant methodologies used in the study of perinatal oogenesis: the culture and analysis of oocytes and follicles extracted from ovaries and the morphological analysis of ovaries using histological techniques.

2 Materials

2.1 Mouth Pipette Preparation

1. Glass Pasteur pipettes.
2. Filtered tips for 1000 μL Pipette.
3. Mouth pipetting aspirator tube.
4. Flame/gas.
5. Protecting gloves.

2.2 Collection of the Ovaries

1. M2 media at 37 °C: HEPES (5.42726 g/L), $NaHCO_3$ (0.35 g/L), glucose (1.0 g/L (Dextro)), phenol red (0.0106 g/L), sodium pyruvate (0.0363 g/L).
2. Ethanol 70%.
3. 10× PBS (phosphate buffered saline): Add 2 g KCl, 2 g KH_2PO_4, 14.4 g $Na_2HPO_4{\cdot}2H_2O$, 80 g NaCl to 1000 mL of distilled H_2O. Adjust the pH to 7.2.
4. Cold 1× PBS: dilute 100 mL of PBS 10× in 900 mL of distilled H_2O.
5. Sterilized fine scissors.
6. Sterilized fine pinchers.
7. Needle (21G 1½).

8. Cell culture dishes; 35 × 10 mm.
9. 5 mL tube.
10. Stereoscope microscope.

2.3 Oocyte Collection and Culture

1. M16 media: $NaHCO_3$ (2.101 g/L), phenol red (0.0106 g/L), sodium pyruvate (0.0363 g/L), glucose (1.0 g/L (Dextro)).
2. M2 media: HEPES (5.42726 g/L), $NaHCO_3$ (0.35 g/L), glucose (1.0 g/L (Dextro)), phenol red (0.0106 g/L), sodium pyruvate (0.0363 g/L).
3. Mineral oil (Sigma-Aldrich).
4. 35 × 10 mm cell culture dishes.
5. 5 mL tubes.
6. Hot block at 37 °C.
7. Needle (21G 1½).
8. Syringe filter unit: 0.22 μm.
9. 10 mL syringe.
10. Fine pinchers.
11. Mouth pipette aspiration device.
12. Incubator, set at 37 °C and 5% CO_2.
13. Stereoscope microscope.

2.4 Fixation and Immunofluorescence of Oocytes

1. 10× PBS (phosphate buffered saline): Add 2 g KCl, 2 g KH_2PO_4, 14.4 g $Na_2HPO_4{\cdot}2H_2O$, 80 g NaCl to 1000 mL of distilled H_2O. Adjust the pH to 7.2.
2. 1× PBS: dilute 100 mL of PBS 10× in 900 mL of distilled H_2O.
3. 4% paraformaldehyde in PBS: Add 4 g of paraformaldehyde to 50 mL of H_2O, then add 1 mL of 1 N NaOH and mix gently in a hot block at 60 °C approximately until paraformaldehyde is dissolved. Add 10 mL of 10× PBS and let the mixture cool down to room temperature. Adjust the pH to 7.4 with 1 N HCl (approximately 1 mL). Adjust the final volume to 100 mL with H_2O. Store at −20 °C.
4. Mineral oil.
5. Washing buffer: Add 0.25 g of BSA (0.5%) to 50 mL of 1× PBS and add 50 μL of $NaNH_2$.
6. Permeabilizing solution (0.5% Triton X-100 in 1× PBS): Add 50 μL of Triton X-100 to 10 mL of 1× PBS.
7. Blocking buffer (3% BSA, 0.2% glycine in 1× PBS): Add 0.3 g of bovine serum albumin (BSA) and 0.02 g of glycine to 10 mL of 1× PBS.
8. Primary antibody of choice.

9. Fluorescence-conjugated secondary antibody of choice.
10. DAPI (8 μg/mL): Add 100 μL of DAPI stock solution to 900 μL of H_2O Milli-Q. Add 400 μL of this first solution to 600 μL of H_2O Milli-Q. Finally, add 200 μL of this second solution to 800 μL of washing buffer.
11. Mouth pipette aspiration device.
12. Cell culture dishes: 35 × 10 mm.
13. Fridge.

2.5 Fixation and Processing of Ovaries

1. 10× PBS (phosphate buffered saline): Add 2 g KCl, 2 g KH_2PO_4, 14.4 g $Na_2HPO_4{\cdot}2H_2O$, 80 g NaCl to 1000 mL of distilled H_2O. Adjust the pH to 7.2.
2. 1× PBS (phosphate buffered saline 10× pH 7.2 in distilled water): dilute 100 mL of 10× PBS in 900 mL of distilled H_2O.
3. 4% paraformaldehyde in PBS: Add 4 g of paraformaldehyde to 50 mL of H_2O, then add 1 mL of 1 N NaOH and mix gently in a hot block at 60 °C approximately until paraformaldehyde is dissolved. Add 10 mL of 10× PBS and let the mixture cool down to room temperature. Adjust the pH to 7.4 with 1 N HCl (approximately 1 mL). Adjust the final volume to 100 mL with H_2O. Store at −20 °C.
4. Bouin's fixative: Combine 75 mL of saturated picric acid (*see* **Note 1**), 25 mL of formalin, and 5 mL of glacial acetic acid. Store at 4 °C.
5. 50% EtOH: Combine 50 mL of 100% EtOH and 50 mL of distilled H_2O.
6. 70% EtOH: Combine 70 mL of 100% EtOH and 30 mL of distilled H_2O.
7. 85% EtOH: Combine 85 mL of 100% EtOH and 15 mL of distilled H_2O.
8. 96% EtOH: Combine 96 mL of 100% EtOH and 4 mL of distilled H_2O.
9. 100% EtOH.
10. Histo-Clear II: histological Clearing Agent.
11. Paraffin plasticized pellets, melting point 56–58 °C: melt 335 g of paraffin to obtain 500 mL of liquid paraffin at 65 °C.
12. Paraffin:Histo-Clear II (1:1): combine 335 g of paraffin pellets with 250 mL of Histo-Clear II and melt at 65 °C.
13. Plastic cassettes.
14. Foam squares.
15. Paraffin embedding station.

16. Molds for paraffin block preparation.
17. Fridge.
18. Heater at 65 °C.

2.6 Sectioning

1. Microscope slides (76 × 26 mm gebrauchfertig, Geschliffen/Mattrand, Objektträger knittel GLASS).
2. Poly-L-lysine: diluted 10% with distilled water.
3. Water bath.
4. Distilled H_2O.
5. Microtome.
6. Blades.
7. Small paint brushes.
8. Heater at 37 °C.

2.7 Immunofluorescence of Ovarian Sections

2.7.1 Deparaffinization—Rehydration

1. Xylene.
2. 100% EtOH.
3. 96% EtOH: Combine 96 mL of 100% EtOH and 4 mL of distilled H_2O.
4. 70% EtOH: Combine 70 mL of 100% EtOH and 30 mL of distilled H_2O.
5. Distilled H_2O.

2.7.2 Antigen Retrieval

1. Sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20 in Milli-Q H_2O, pH 6.0): Add 2.94 g of trisodium citrate (dihydrate) to 1 L of Milli-Q H_2O and mix to dissolve. Adjust the pH to 6.0 with 1 N HCl. Finally, add 0.5 mL of Tween-20 and mix. Store at 4 °C for up to 3 months.
2. Microwave.
3. Microwave-safe glass lunch box.
4. Plastic slide rack.

2.7.3 Immunofluorescence Staining

1. 10× PBS (phosphate buffered saline): Add 2 g KCl, 2 g KH_2PO_4, 14.4 g $Na_2HPO_4{\cdot}2H_2O$, 80 g NaCl to 1000 mL of distilled H_2O. Adjust the pH to 7.2.
2. 1× PBS (phosphate buffered saline 10× pH 7.2 in distilled water): dilute 100 mL of 10× PBS in 900 mL of distilled H_2O.
3. Washing buffer (0.1% Tween-20 in 1× PBS): Add 1 mL of Tween-20 to 1000 mL of 1× PBS.
4. Permeabilizing solution: Add 1 mL of 20% (v/v) Triton X-100 to 200 mL of 1× PBS.
5. Goat Serum Blocking Solution: Prepare 10 μL of Tween-20, 0.3 g of BSA, 0.0252 of glycine, 1 mL of 10× PBS, 1 mL of

goat serum, 8 mL of Milli-Q water, 0.1 mL of 20% Triton X-100. Filter and add 90 mL of 1× PBS.

6. Primary antibody of choice.
7. Fluorescence-conjugated secondary antibody of choice.
8. DAPI (8 μg/mL): Add 100 μL of DAPI stock solution to 900 μL of H_2O Milli-Q. Add 400 μL of this first solution to 600 μL of H_2O Milli-Q. Finally, add 200 μL of this second solution to 800 μL of Vectashield mounting medium.
9. Plastic Coplin jar.
10. Shaking machine.
11. Parafilm.
12. Humid chamber: Prepare a plastic slide box with humidified tissue paper inside and cover it with aluminum foil so that light does not pass through.
13. Fridge.
14. Heater at 37 °C.
15. Coverslips.
16. Nail polish.
17. Epifluorescence microscope.

2.7.4 PAS-Hematoxylin Staining of Ovarian Sections

1. 1% periodic acid: Add 1 g of periodic acid to 100 mL of distilled H_2O (*see* **Note 2**).
2. 0.15 N hydrochloric acid (HCl): Prepare it in a fume hood. Mix 6 mL of 37% HCl with 444 mL of distilled H_2O.
3. Schiff reagent: Add 0.1 g of basic fuchsin and 7.6 g of sodium disulfite ($Na_2S_2O_5$) to 350 mL of 0.15 N HCl and mix for 2 h. Add 2 g of activated charcoal powder and mix for 1 h. Filter the solution using a filter paper humidified with 0.15 N HCl and fill up to 400 mL with 0.15 N HCl (*see* **Notes 3** and **4**).
4. Sulphurated water: For a final volume of 120 mL, prepare the following (*see* **Note 5**):
   - 10% potassium disulfite: Mix 1 g of potassium disulfite ($K_2S_2O_5$) with 10 mL of distilled H_2O.
   - 1 N HCl: Mix 0.862 mL of 37% HCl with 9.138 mL of distilled H_2O.
   - Add both solutions to 100 mL of distilled H_2O.
5. Mayer's Hematoxylin.
6. Coverslips.
7. DPX mounting medium.
8. Bright-field microscope.

3 Methods

3.1 Mouth Aspiration Unit Preparation

Preparation of the Pasteur pipettes:

1. Switch on the gas flame, being careful of flammable reagents nearby.
2. Get one glass Pasteur pipette and pass the narrow end of the Pipette through the flame.
3. Once it is hot, pull on both sides of the spot where the flame is touching the glass. This will create a thinner section of glass in between two narrow areas.
4. Break the glass off through the thinner part of the glass pipette to get a glass Pasteur pipette with a very thin end.

Preparation of the mouth aspiration unit:

1. Attach a 1000 μL filtered tip to each end of the aspiration tube.
2. Introduce the wider end of the glass Pasteur pipette to one filtered tip. This side will be used to collect oocytes or follicles.
3. The filtered tip on the other side will be put in your mouth to aspirate the liquid.

3.2 Collection of Ovaries

Collect ovaries from neonatal/young mice on the appropriate day under a stereoscope microscope. For adult mice, you can perform the collection without a stereoscope microscope.

Depending on the experiment, the ovaries could be fixed or cultured for ovarian culture and oocyte collection. Both of these protocols are described below.

3.2.1 Ovaries' Collection for Oocyte Culture

1. Prepare your reagents before the collection of ovaries:
   (a) M16 preparation:
      - Filter around 2.5–3 mL of M16 per mouse with a 0.22 μm filter and a 10 mL syringe.
      - Place it in a cell culture dish and put it in the incubator at least 1 h before the ovary collection.

   (b) M2 preparation:
      - Put 3 mL of M2 per mouse on small tubes on the hot plate at 37 °C at least 30 min to 1 h before the ovary collection.
      - By the time of the ovaries' collection, prepare the cell culture dishes with 1 mL of M2 media.
2. Sacrifice the animals (*see* **Note 6**).
3. Open the abdominal cavity of the mice using a pair of sterilized scissors and sterilized pinchers.

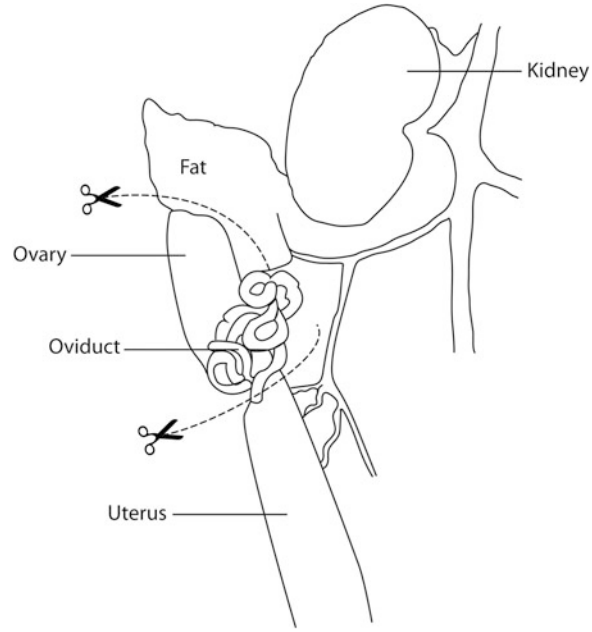

Fig. 2 Schematic representation of the location of the ovary in a mouse abdominal cavity. The ovaries should be located underneath the intestine, attached to the oviducts and fat, in turn attached to the kidneys

4. Remove the adipose tissue and the intestine to detect the ovaries. They should be covered by adipose tissue at the end of the oviducts (Fig. 2).
5. Hold the ovaries with the pinchers and cut with scissors where the oviduct starts (*see* **Note 7**).
6. Instantly transfer the ovaries to the M2 media plate at 37 °C.

3.2.2 Ovaries' Collection for Fixation

1. Prepare PFA or Bouin's fixative in a 1.5 mL tube before starting with the collection of ovaries.
2. Sacrifice the animals (*see* **Note 6**).
3. Open the abdominal cavity of the mice using a pair of sterilized scissors and sterilized pinchers.
4. Remove the adipose tissue and the intestine to detect the ovaries. They should be covered by adipose tissue at the end of the oviducts (Fig. 2).
5. Hold the ovaries with the pinchers and cut with scissors where the oviduct starts (*see* **Note 7**).
6. Wash the ovaries in cold 1× PBS.
7. With a needle, under the stereoscope microscope, carefully remove the adipose tissue without damaging the ovary.

3.3 Oocyte Collection and Culture

The collection and culture of oocytes from neonatal and adult ovaries should be performed under a stereoscope.

1. At least 1 h before the collection of the ovaries, prepare the following:
   (a) Add 5 mL of M2 media in a small tube and place it on the hot block at 37 °C. Prepare one for every 3–4 ovaries.
   (b) Place 1 mL of M2 media on a cell culture dish cap to process the ovaries.
   (c) Prepare a cell culture dish, add one elongated and three small drops of M2 media, and cover them with mineral oil. Place the cell culture dish in a hot block at 37 °C.
   (d) Filter the M16 media using the 10 mL syringe and the 0.22 μm filter. Add four drops to a cell culture dish, cover it in mineral oil, and put it in an incubator set at 5% CO_2 and 37 °C (*see* **Note 8**).
2. Place the ovaries in the cell culture dish cap with 1 mL of M2.
3. Using a needle and pinchers, puncture the ovaries into small pieces and release the cumulous cells–oocyte complexes (COCs).
4. After the ovary is thoroughly fragmented, use a mouth pipette aspirator device to collect the COCs.
5. Place them in the elongated M2 media drop (*see* **Note 9**).
6. Clean the oocytes from the debris of the ovarian tissue by moving them from the first drop of M2 media to the second and then to the third, and try to collect the fewer extra cells you can each time (*see* **Note 10**).
7. Collect the desired quantity and quality COCs in the last small M2 media drop (*see* **Note 11**).
8. Let them rest for 20–30 min in the dark (*see* **Note 12**).
9. Move the COCs to the first M16 drop.
10. Move them across the drops to clean them further.
11. Culture them in the last M16 drop in an incubator set at 5% CO_2 and 37 °C for the desired time until they reach GVBD or MI stages (*see* **Notes 13–15**).

3.4 Fixation and Immunofluorescence of Cultured Oocytes

1. Add two drops of 4% PFA to a cell culture dish and cover them with mineral oil.
2. Place the oocytes in the first drop, directly transfer them to the second one, and let them incubate for 20 min (*see* **Notes 16** and **17**).
3. Prepare another cell culture dish with three drops of washing buffer and cover them with mineral oil.
4. After the incubation in PFA, place the oocytes in the first drop of the washing buffer for 5 min.

5. Move the oocytes to the second drop for 5 min and then to the third drop for another 5 min.
6. Using a cap of a cell culture dish, draw with a marker one dot on the center of it and add 0.5% Triton X-100 until it covers the cell culture dish fully. As Triton is a detergent, it cannot be mixed with oil, so it is impossible to create dots and cover them with oil as previously. The use of drawing a dot is to determine the location of where the cells will be placed in the cap.
7. Take your oocytes from the washing buffer with the mouth Pipette and carefully release them in the dotted area (*see* **Note 18**).
8. Incubate the oocytes in the 0.5% Triton X-100 for 15 min.
9. Put a drop of washing buffer in a cell culture dish cap and carefully place the oocytes (*see* **Note 19**).
10. Put three drops of washing buffer in a cell culture dish cap and carefully place the oocytes in the first drop for 5 min.
11. Move the oocytes to the second drop for 5 min and then to the third drop for another 5 min.
12. Place two drops of blocking buffer into a cell culture dish and cover them with mineral oil.
13. Put the oocytes in the first blocking buffer drop and then move them to the second drop for 1 h at room temperature.
14. Dilute the primary antibody to the appropriate concentration in the blocking buffer. The concentration of the primary antibody depends on the antibody of choice. Put two drops of the primary antibody solution in a cell culture dish and cover them with mineral oil.
15. Place the oocytes in the first drop, pipetting them five times, and move them to the second drop.
16. Incubate them at 4 °C overnight.
17. Prepare a cell culture dish cap with three drops of washing buffer.
18. Place the oocytes in the first drop of the washing buffer for 5 min.
19. Move the oocytes to the second drop and then to the third drop for 5 min each.
20. Dilute the fluorescence-conjugated secondary antibody in blocking solution. Put two drops of the secondary antibody solution in a cell culture dish and cover them with mineral oil in the dark (*see* **Note 20**).
21. Place the oocytes in the first drop, pipetting them five times, and move them to the second drop in the dark.
22. Incubate them at room temperature for 2 h.

23. Wash the oocytes in three drops of washing buffer, as mentioned above, for 5 min in each drop.
24. In a cell culture dish, prepare two drops of 8 μg/mL DAPI, diluted in washing buffer, and cover them with mineral oil.
25. Place the oocytes in the first drop and move them to the second one after pipetting a bit. Incubate in the dark for 10 min.
26. Prepare a cell culture dish with three drops of washing buffer.
27. Place the oocytes in the dark in each drop of the washing buffer for 5 min. Keep cells in the last drop.
28. Visualize in an epifluorescence inverted microscope or store at 4 °C in the dark (Figs. 3 and 4).

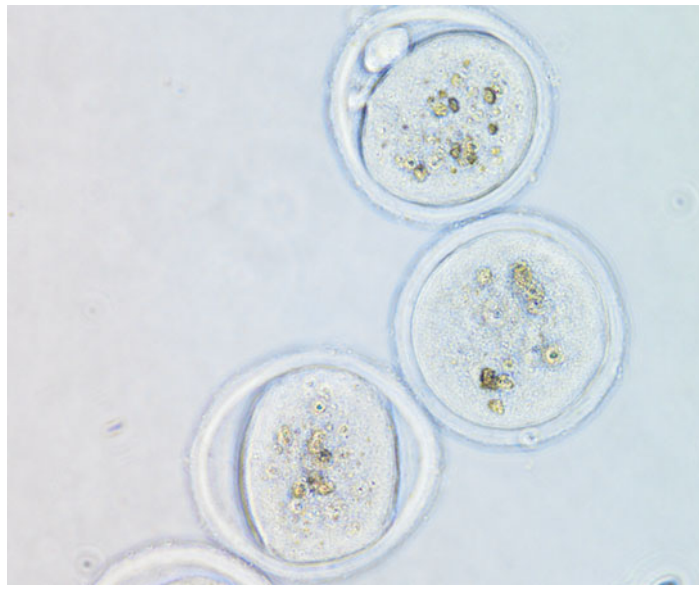

Fig. 3 Oocytes in GVBD and MI stage collected from 2-month-old female mice and cultured in M16 media in 5% CO_2 at 37 °C incubator. The cells were fixed in 4% PFA

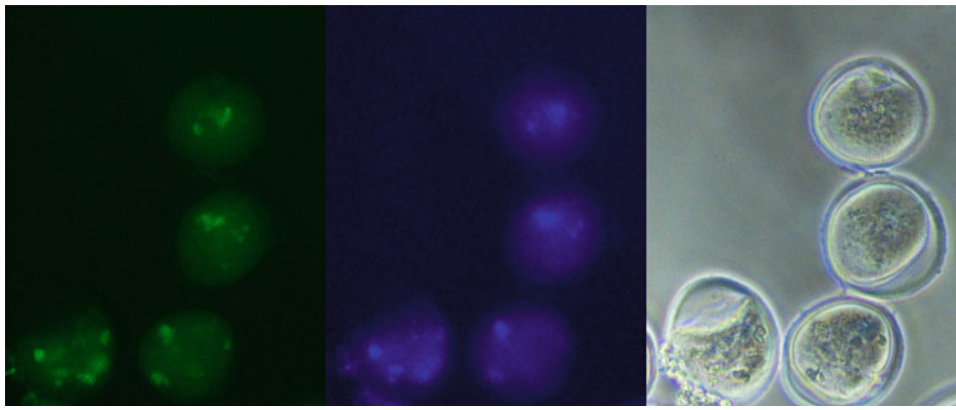

Fig. 4 Oocytes labeled for H2AZ and DAPI. GVBD oocytes were collected and immunostained against the histone H2AZ and counterstained with DAPI

3.5 Fixation and Processing of Ovaries

1. After collecting the ovaries (see Subheading 3.2.2), wash samples in cold 1× PBS.
2. Fix ovaries for 4–16 h with the following:
   (a) 4% PFA in PBS at 4 °C, if you want to perform immunofluorescence assays afterward.
   (b) Bouin's fixative at 4 °C, if later, you want to perform a morphological analysis through PAS-Hematoxylin staining.
3. Wash samples in cold 1× PBS twice for 30 min.
4. Dehydrate the sample:
   (a) 1× 30 min 50% EtOH at 4 °C.
   (b) 1× 30 min 70% EtOH at 4 °C (*see* **Note 21**).
   (c) 1× 30 min 85% EtOH.
   (d) 1× 30 min 96% EtOH.
   (e) 2× 30 min 100% EtOH.
5. Inclusion in paraffin:
   (a) Incubate the ovaries three times for 30 min each with Histo-Clear II.
   (b) Place the ovaries in cassettes with foam to avoid losing them in the following steps.
   (c) Place the cassettes in a beaker with Histoclear:Paraffin (1:1) for 45 min at 56 °C.
   (d) Place the cassettes in a beaker with paraffin for 2 h at 56 °C.
   (e) Place the cassettes in a beaker with paraffin overnight at 56 °C.
6. Prepare the paraffin blocks using a paraffin embedding station:
   (a) Place the appropriate molds and the cassettes inside the paraffin at 65 °C.
   (b) Take the cassette into the station's hot surface and open it using two pinchers.
   (c) Take the mold out of the hot paraffin and place it on the hot surface as well.
   (d) Add a small amount of paraffin to the mold using the paraffin tap.
   (e) Place the ovary in the proper orientation and place the mold on the small cold surface of the paraffin station.
   (f) Add the cassette backward and add more paraffin with the tap.
   (g) Place the mold in the cold station to solidify the paraffin.
   (h) Remove the mold after 30 min.
7. Store the blocks at 4 °C until the time to section them (*see* **Note 22**).

3.6 Sectioning

1. Coat slides with poly-L-lysine for 5 min and let them air dry.
2. Prepare a water bath and preheat water at 46 °C.
3. Place the block in the microtome, put the blade in its designated place, and cover it with the guard while not actively sectioning the sample (*see* **Notes 23** and **24**).
4. Orient the block to be at the same angle as the blade.
5. Section tissue at a desired thickness (5–7 μm thick sections) (*see* **Note 25**).
6. By using two brushes, move the section to the water bath.
7. Let the section lay flat on top of the water before catching it with a poly-L-lysine-coated slide.
8. Place the sections in a nonconsecutive way every five sections (e.g., in slide number 1, place sections number 1, 6, 11, 16, 21, etc.).
9. Put slides in a heater at 37 °C overnight to dry.
10. Store the slides at RT until use.

3.7 Immunofluorescence of Ovarian Sections

Immunofluorescence staining is commonly performed in PFA-fixed samples, although other fixation methods may also allow certain immunostainings.

3.7.1 Deparaffinization—Rehydration

1. Submerge the slides containing PFA-fixed ovarian sections into xylene for 5 min.
2. Repeat three times to completely remove the paraffin.
3. Rehydrate the slides by submerging them in a decreasing ethanol concentration gradient, starting with 100% ethanol for 3 min, two times.
4. Submerge the slides into 96% ethanol for 2 min, two times.
5. Place the slides into 70% ethanol for 2 min.
6. Finally, place the slides in distilled water for 2 min.

3.7.2 Antigen Retrieval

1. In a microwave-safe plastic box, place the slides in a plastic slide rack and fill the box up with sodium citrate buffer.
2. Place the box in the microwave and heat it for 5 min at 250 W (*see* **Note 26**).
3. Check the slides to see if they are still covered with sodium citrate buffer. If they are not, add some more buffer to cover them entirely.
4. Repeat **steps 2** and **3** three times.
5. Let the box cool down for 20 min.
6. Remove the slides from the box.

3.7.3 Immunofluorescence Staining

1. Put the slides into a Coplin jar and ensure that sections from different slides are not in contact between them.
2. Wash the slides with PBS-Tween for 2 min, three times.
3. Perform permeabilization by submerging the slides in the permeabilizing solution for 15 min, shaking at medium speed.
4. Wash the slides with PBS-Tween for 2 min, two times.
5. Submerge the slides in blocking solution, shaking at medium speed for 1 h.
6. Dilute the primary antibody to the appropriate concentration in the blocking buffer (*see* **Note 27**).
7. Add 10 μL of the diluted primary antibody solution on top of each section and cover them with parafilm (*see* **Note 28**).
8. Incubate the slides in a humid chamber at 4 °C overnight.
9. Wash the slides three times with PBS-Tween for 2 min.
10. Dilute the fluorescence-conjugated secondary antibody in the blocking solution (*see* **Note 29**).
11. Add 10 μL of the diluted secondary antibody per section and cover them with parafilm.
12. Incubate the slides in a humid chamber at 37 °C for 1 h.
13. Wash the slides with PBS-Tween three times for 2 min.
14. Remove the slides from the Coplin Jar and let them slightly dry.
15. Add 5 μL of 8 μg/mL DAPI diluted in Vectashield mounting medium to each section.
16. Cover the slides with a coverslip and remove the extra DAPI by slightly pressing the slide with a tissue paper.
17. Fix the coverslip by placing a few drops of nail polish on the edges of the coverslip.
18. Analyze the slides in an epifluorescence microscope.
19. Store the slides at −20 °C for long-term storage or at 4 °C for short periods.

3.8 PAS-Hematoxylin Staining of Ovarian Sections

1. Perform deparaffinization and rehydration as explained in Subheading 3.7.1 to the Bouin-fixed ovarian sections.
2. Incubate slides in 1% periodic acid for 10 min.
3. Wash slides twice in distilled water for 3 min.
4. Incubate in Schiff reagent in the dark for 10 min (*see* **Notes 30** and **31**).
5. Wash slides twice in sulphurated water for 3 min.
6. Wash slides in distilled water for 1 min.
7. Stain slides with Mayer's Hematoxylin for 1 min.

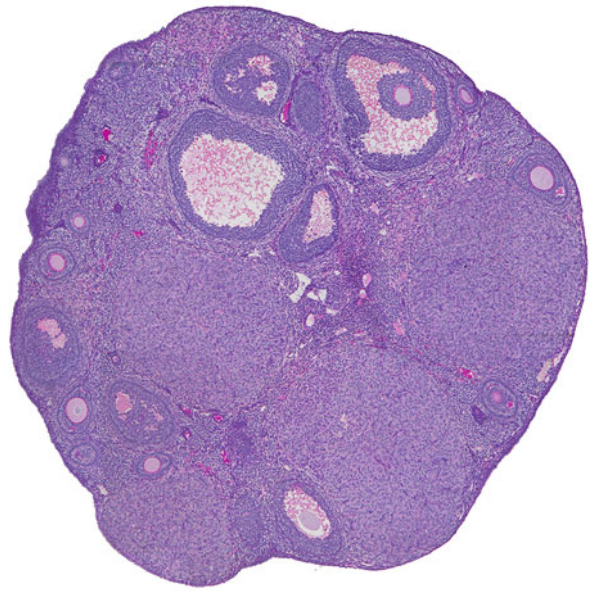

Fig. 5 Image of a mouse ovary stained with PAS-Hematoxylin. A 7 μm section of a 19-week-old mouse ovary stained with the previously mentioned protocol of the PAS-Hematoxylin staining and captured in a bright-field microscope

8. Wash slides under running water for 1 min to eliminate the excess staining (*see* **Note 32**).
9. Dehydrate and mount:
   (a) Wash slides in 70% EtOH for 2 min.
   (b) Wash slides in 96% EtOH twice for 2 min.
   (c) Wash slides in 100% EtOH twice for 3 min.
   (d) Wash slides in xylene three times for 5 min.
   (e) Let slides slightly dry.
   (f) Add a drop of DPX mounting medium and place a coverslip on top.
   (g) Slightly press down on the coverslip to spread the DPX all over the section.
   (h) Let slides air dry.
10. Visualize on a bright field microscope or store at RT (Fig. 5).

4 Notes

1. (2.5) The saturated picric acid in solid form is explosive. Take care when preparing the liquid solution.
2. (2.7.4) The 1% periodic acid should be prepared under a fume hood, and be careful, as it is highly explosive.

3. (2.7.4) The Schiff reagent preparation must be done with all the materials covered in aluminum foil and under a fume hood. Store it at 4 °C in the dark.
4. (2.7.4) The Schiff solution must be transparent and colorless. If it is slightly colored (pinkish), it is becoming spoiled, and a new one should be prepared.
5. (2.7.4) The sulphurated water should be prepared under a fume hood. Note that it has a very unpleasant smell.
6. (3.2) Removing the ovaries immediately after sacrificing the female mouse is important.
7. (3.2) The ovaries in neonatal mice are not easily detectable with the naked eye, so you should cut on the area on the top of the oviduct and next to the kidneys. Note that this area contains a lot of adipose tissue. The ovary should be there. To confirm that you collected them, check the sample in the stereoscope by removing the adipose tissue.
8. (3.3) It is essential to place the M16 media in the 5% CO_2 incubator, otherwise it will be oxidized.
9. (3.3) When transferring cells from one drop to another, taking a small amount of the "destination" drop's liquid with the mouth aspiration device before retrieving the oocytes is critical.
10. (3.3) It is recommended to count the oocytes at any step to ensure that they have been collected.
11. (3.3) If you need to denudate the cumulus–oocyte complex, you can use hyaluronidase to achieve it. Then, by pipetting the cells multiple times, you can eliminate the cumulus cells.
12. (3.3) The oocytes should rest at 37 °C hot plate and in the dark. Place the Petri dishes on the hot plate and cover them with a bottle cap to get the desired darkness.
13. (3.3) If the experiment requires drug treatment, the drug can be diluted into the media in the desired concentration.
14. (3.3) IBMX drug can be diluted in the M2 and M16 media to keep the oocytes arrested in the GV stage. To maintain the oocytes arrested, collecting the ovaries in the M2+IBMX media is important.
15. (3.3) In the case of IBMX treatment, to clean the oocytes from the drug and release them to develop further, prepare a cell culture dish with elongated drops (three to four) of M16 media in the 5% CO_2 incubator. Place the cells on the edge of the first drop and move the cells from the one edge of the drop to the other (so in total six to eight transfers) (Fig. 6).
16. (3.4) The PFA fixation makes the oocytes stickier, causing them to stick to the glass pipette when moving the cells from one drop to the next.

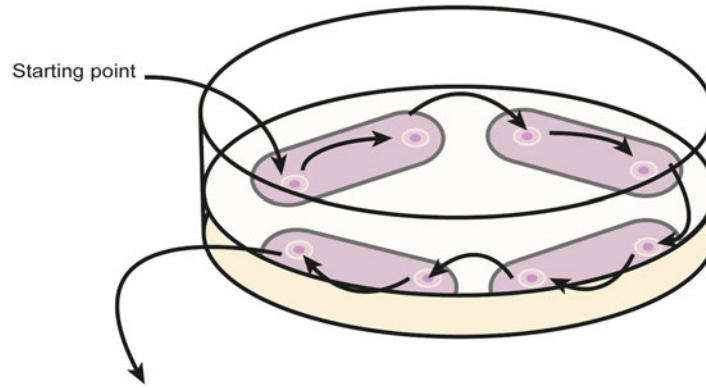

Fig. 6 Schematic representation of the cleaning of oocytes. Start by pipetting the oocytes on one edge of an elongated drop and pipette them to the other side of the drop. Move them like this in three to four drops to sufficiently dilute the drug in the washing buffer. When the cells are on the last cleaning transfer, they will be clean enough to follow with the next step of the protocol

17. (3.4) Use transition drops for the fixation solution (PFA), blocking solution, antibodies, and DNA staining, as with the transferring of the oocytes, apart from the oocytes, we move liquid as well, so it may reduce the desired concentration. With the addition of transition drops, this should be minimized.
18. (3.4) **CRITICAL STEP**: The cells may move because of their round shape, and there is a risk of losing them. Avoid moving the petri dish around; try to use the dot as a point of reference and place them near it to control their location.
19. (3.4) **CRITICAL STEP**: The Triton X-100 composition destroys the washing buffer's drop. Considering that this step is critical, because you may lose cells, the use of a "transition" drop, to wash the cells out of the Triton X-100, may minimize this risk.
20. (3.4) During and after the secondary antibody incubation, the cell culture dishes should be kept in the dark, using a bottle cap to cover the plate.
21. (3.5) If you do not want to perform the protocol in one day, you can stop when the samples are in 70% EtOH at 4 °C to continue during the following month.
22. (3.5) Cutting the blocks on the same day of their preparation is not recommended.
23. (3.6) It is imperative to cover the microtome blade with the guard to prevent accidents while placing, orientating, or removing the block from its holder.

24. (3.6) Sectioning should be performed in a cold room (around 15–22 °C), so the paraffin is hard enough and the blade easily cuts the block.
25. (3.6) If the sections are not good enough (e.g., they keep breaking while sectioning), gently touch the block surface with a damp paper tissue. This will cool down the block and will result in better quality sections.
26. (3.7.2) Considering that every microwave might differ, the critical step is to submerge the slides in boiling sodium citrate solution for 10–15 min for the protocol to work.
27. (3.7.3) The concentration of the primary antibody depends on the antibody of choice.
28. (3.7.3) Whenever you place the parafilm on top of the slides, ensure that there are no bubbles.
29. (3.7.3) During and after the secondary antibody treatment of the slides, ensure that you are working in a low-light/dark room if possible.
30. (3.8) Remove the Schiff reagent from the fridge and place it in RT for at least 1 h before using it in a dark or light protective bottle.
31. (3.8) The Schiff reagent stains pinkish extremely easily. So, wear gloves and a lab coat while using it.
32. (3.8) While washing slides under running water, use a plastic tray under the running tap water to remove the excess hematoxylin by moving the slides tray inside the plastic tray.

Acknowledgments

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Chapter 13

Method of Isolation and In Vitro Culture of Primordial Follicles in Bovine Animal Model

Pritha Dey, Noemi Monferini, Ludovica Donadini, Valentina Lodde, Federica Franciosi, and Alberto Maria Luciano

Abstract

The mammalian ovary is a substantial source of oocytes arranged into follicles at various stages of folliculogenesis, from the primordial to the ovulatory ones. Primordial follicles constitute the most abundant source of gametes inside the mammalian ovary at any given time.

The isolation of a high number of primordial follicles, together with the development of protocols for in vitro follicle growth, would provide a powerful tool to fully exploit the female reproductive potential and boost the rescue and restoration of fertility in assisted reproduction technologies in human medicine, animal breeding, and preservation of threatened species. However, the most significant limitation is the lack of efficient methods for isolating a healthy and homogeneous population of viable primordial follicles suitable for in vitro culture. Here, we provide a fast and high-yield strategy for the mechanical isolation of primordial follicles from limited portions of the ovarian cortex in the bovine animal model.

Key words Primordial follicle, Preantral follicle, Culture, Mechanical isolation, Viability, Folliculogenesis, In vitro growth, Oocyte, Ovary

1 Introduction

The mammalian ovary contains a fixed number of nongrowing primordial follicles established before birth representing the ovarian reserve that declines with age and culminates at the end of the reproductive lifespan [1]. The number of follicles in the ovaries of mammals is remarkably variable at birth, ranging, for example, from 350,000 to 1,100,000 in humans [2, 3] and approximately 14,000 to 250,000 in cattle [4, 5].

Folliculogenesis begins during fetal life and proceeds until the end of reproductive capacity. It starts with the recruitment of primordial follicles followed by the cyclic recruitment that brings to the development of the preovulatory follicle containing an oocyte, which is ovulated and is able to be fertilized and become

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an embryo [6]. Of the initial pool recruited to grow, only a few follicles reach the preovulatory stage, and less than 1% escape the process of atresia at various stages of development, particularly during the preantral to early antral transition, which is the most susceptible to this process [6, 7]. In women, of the original primordial follicle stockpile at birth, only approximately 400 will fully mature into secondary oocytes, being ovulated and ready to be fertilized during a woman's reproductive lifespan [8, 9], while the vast majority are destined to undergo atresia [10–12].

Primordial follicles represent the largest population of the ovarian reserve in mammals at any given time, thus constituting the most relevant repository of the female reproductive potential in mammals [13].

Efficient culture systems for the primordial follicles may enhance fertility preservation opportunities in women, expand genetically important livestock breeds and support conservation programs for endangered species [14]. In the bovine model, the current assisted reproductive technologies can rely only on a limited number of follicles, namely the population of fully grown oocytes isolated from the medium–large antral follicles (Table 1) [15–17] and with relative success from oocytes isolated from early antral follicles [18].

Recruiting preantral follicles (from primordial to secondary follicle stage), and particularly primordial follicles, for in vitro growth would enormously broaden the availability of gametes for a massive exploitation of the reproductive potential of a female individual. The development of in vitro follicle growth system would also deepen our knowledge of the processes that initiate mammalian folliculogenesis, allowing investigation of folliculogenesis and oogenesis in a tightly controlled environment.

In the last two decades, several attempts to develop follicle culture systems for preantral follicles in situ, i.e., in the intact ovary or fragments of the ovarian cortex, have been made in several species [19]. Although advances in current systems have enhanced oocyte growth and maturation to some extent, further optimization is required to improve oocyte competence with genetic integrity for proper embryonic development. Most of the attempts were ineffective, with little success regarding follicle development, and limited to mice [20] as proof of principle, while in large mammals, the techniques are still considered experimental [21, 22, 19].

The ability to rescue a high number of primordial follicles and the development of in vitro follicle 3D growth protocols, as recently suggested in mice [23], may provide a powerful means of fully exploiting the female reproductive potential. However, the most significant limitation in all the species considered so far is the lack of efficient methods for isolating a homogeneous population of primordial follicles.

Table 1
Extent of follicle reserve and follicle categories in 1–8 years old bovine ovaries

Follicle category	Primordial	Primary	Secondary	Early antral	Mid-large antral
Number/ovary (heifers)	84,000	21,000	5000	120	25
Number/ovary (cows)	64,000	23,000	1800	120	25
Incidence of atresia	<2%	<5%	8%	30%	60%

Data were based on the estimation from [5, 40, 42, 54–56]

The currently available techniques for isolating primordial follicles—or preantral follicles, in general—have been optimized in several labs to develop the most efficient method (mechanical, enzymatic, or a combination of both [24–26]) to increase follicle yield. In large mammals, despite considerable progress in the methodology for the isolation of preantral follicles, regardless of the isolation method, most of the studies have reported the recovery of primary and secondary follicles [27–32], very often from fetal ovaries [24, 33–35], occasionally in adult individuals [24, 36], and frequently processing a high number of ovaries [37, 26, 38]. Surprisingly, the yield rate is only occasionally declared, but when available, the number of isolated primordial follicles is lower than 5, while primary and secondary follicles vary from 2 to about 40.

Here, we provide a fast and effective strategy to maximize the isolation of primordial follicles in the bovine model from limited portions of the ovarian cortex. Specifically, starting from a 0.5 to 1 mm thick ovarian cortex slice of 2 cm^2 in size, the present methodology allows for recovering 166.5 ± 40.8 ($N = 10$) primordial follicles of 34.5 ± 3.8 in diameter ($N = 176$). After collection and 1 h of culture, 88% of the primordial follicles were viable. Furthermore, the entire mechanical isolation procedure lasts 30–40 min from the time of isolation of the 2 cm^2 fragment of the ovarian cortex.

Finally, we propose the bovine animal model because bovine and human reproductive biology share numerous characteristics [39]. For example, cows and women have similar folliculogenesis length [3, 40–42], are monovular, cycle continuously while not pregnant, have a gestation period of approximately 9 months, and their ovaries are similar in size (approximately 3 cm × 2 cm × 1.5 cm), morphology [43], and architecture [44, 45].

To conclude, considering the limited success of the in situ culture system in cattle and humans [46, 47], isolating a high number of primordial follicles to be used in suitable in vitro culture systems (2D and 3D) is extremely encouraging. The development of in vitro primordial follicles growth systems in the bovine model can provide a tool for deepening our knowledge of mammalian folliculogenesis, overcoming logistical and ethical limits in using

human ovarian samples, and studying tailored approaches, minimizing the invasiveness of the interventions to preserve female fertility.

2 Materials

Disposable sterile plasticware is from NUNC IVF Line, SARSTEDT Green line (for suspension cells), and Sterilin™ by ThermoScientific. Final filtration of all stock solutions and the preparation of working solutions are performed using sterile techniques under a biohazard laminar flow cabinet or a horizontal laminar flow hood to keep sterility. All glassware is exclusively dedicated to gamete and embryo culture and is high-pressure steam-sterilized by autoclaving at 121 °C for 20 min. After use, glassware is washed and rinsed with running tap water for 30 min, rinsed three times with 18.2 mΩ water, then dried thoroughly and covered with aluminum foil until sterilization. All the procedure's dedicated steel instruments (forceps, spatula, scalpel handle) are high-pressure steam-sterilized by autoclaving at 121 °C for 20 min.

All the procedures are conducted at room temperature (26 °C) unless otherwise specified.

2.1 Manipulation Solution and Media

1. Collection and washing solution: Prepare 0.9% saline solution by adding 9 g of NaCl in 1 L of sterile ultrapure 18.2 mΩ water. Supplement the saline solution with penicillin 100 U/mL and streptomycin 0.1 mg/mL.
2. Isolation Medium: Leibovitz's L-15 Medium supplemented with 0.3% Bovine Serum Albumin, 0.164 mM Penicillin, and 0.048 mM Streptomycin (*see* **Notes 1** and **2**).
3. For homogenization procedure, prepare aliquots of 15 mL isolation medium into 50 mL Falcon tubes.

2.2 Culture Medium

1. Culture Medium: αMEM supplemented with 0.1% Bovine Serum Albumin fatty acid-free, 1 mg/mL r-hInsulin, 0.55 mg/mL hTransferrin, 0.5 μg/mL Sodium Selenite, 10^{-4} IU/mL r-hFSH, 0.164 mM Penicillin, and 0.048 mM Streptomycin (*see* **Note 3**).
2. Prepare a 4-well plate filled with 500 μL of Culture Medium and equilibrate at 38.5 °C and 5% CO_2 in air, maximum humidity, for at least 4 h before use.

2.3 Dual-Fluorescence Viability Assay

1. Manipulation Buffer: Polyvinyl Alcohol dissolved in Phosphate Buffer Saline to a final concentration of 0.1%.

2. Dual staining solution: Fluorescein Diacetate (FDA) and Propidium Iodide (PI) diluted to a final concentration of 1 μg/mL each in the previously prepared Manipulation Buffer.

2.4 Equipment

1. Scalpel handle with a surgical blade no. 22.
2. Single-edge carbon steel razor blades 1.5″ with aluminum back.
3. High-density polyethylene cutting board.
4. IKA ULTRA-TURRAX® T25 Digital Advanced Homogenizer.
5. IKA Plastic Disperser Tool S25D-14G-KS (stator diameter: 14 mm, rotor diameter: 9.5 mm).
6. Cell strainer of 300, 100, 70, 40, and 30 μm mesh size.
7. Mouth pipette with pulled glass capillary (inner diameter about 100 μm).
8. Culture petri dish 35 and 60 mm for suspension cell culture.

3 Methods

3.1 Isolation and Culture of Primordial Follicles

The passages described below are illustrated in Fig. 1.

1. Collect bovine ovaries from Holstein Friesian cattle subjected to routine veterinary inspection and following the specific health requirements. Transport to the laboratory on ice within 1 h in a 50 mL tube with sterile collection saline solution.
2. Under a horizontal laminar flow hood, place one ovary on a sterile cutting board. Using surgical blade no. 22 mounted on a scalpel handle, cut a 0.5–1 mm thick ovarian cortex slice of 2 cm^2 in size (*see* **Note 4**). Chop the cortical slices into tiny fragments with 1.5″ single-edge razor blades and carefully mince them on the sterile cutting board.
3. Wash the minced ovarian cortex by transferring the fragments with a spatula into a sterile 60 mm Petri dish containing 3 mL of isolation medium (*see* **Note 5**).
4. Remove the isolation medium using a pipette and transfer the washed minced cortical pieces to a 50 mL Falcon tube containing 15 mL isolation medium (*see* **Note 6**).
5. Place the 50 mL Falcon tube containing the minced cortical pieces dispersed in 15 mL of isolation medium under the IKA ULTRA-TURRAX® T25 Homogenizer with the Disperser Tool S25D-14G-KS.
6. Homogenize the minced fragments in the 50 mL Falcon tube at 3000 rpm for 6 min (*see* **Note 7**).

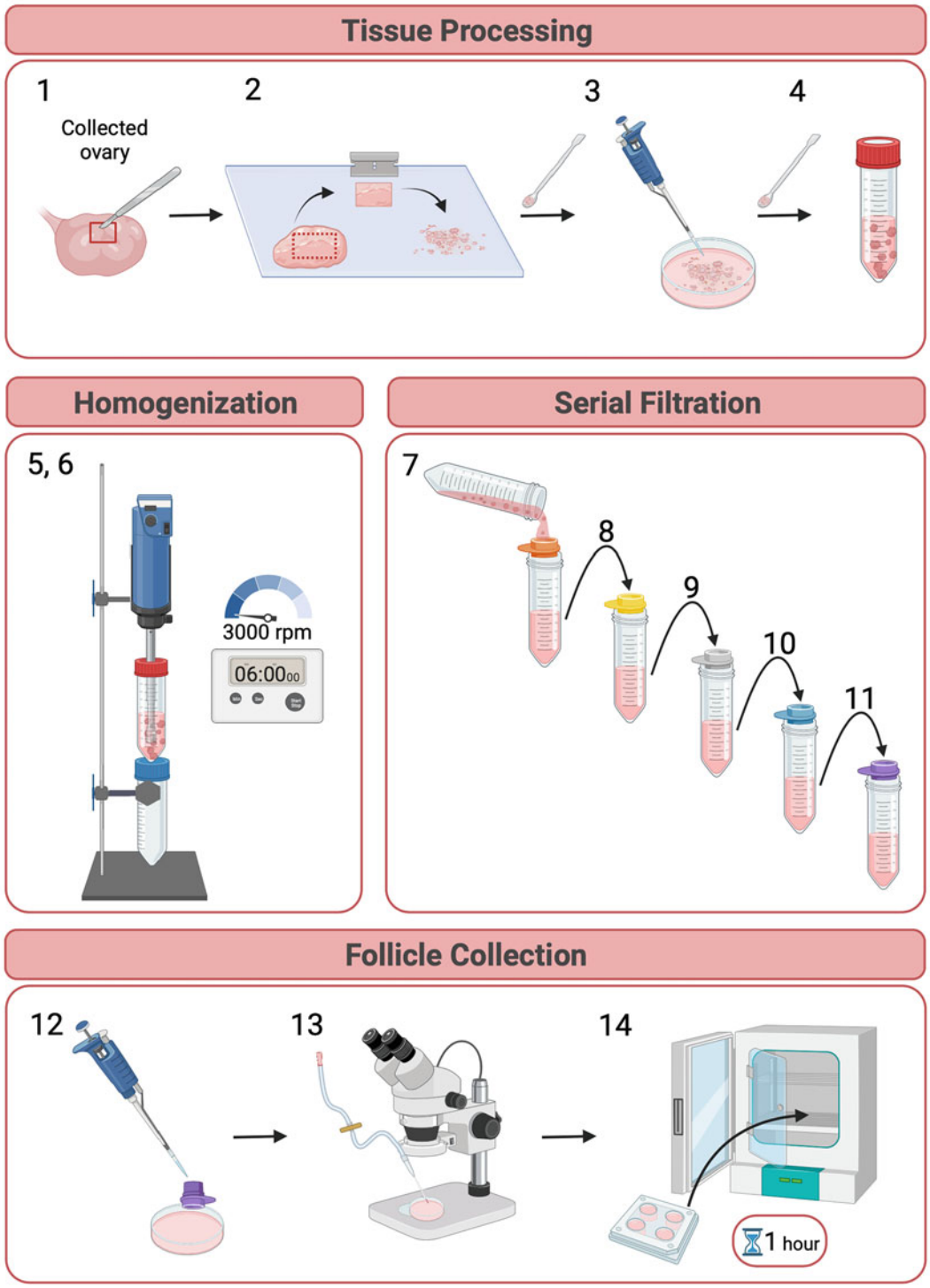

Fig. 1 Schematic representation of the workflow described in Subheading 3 (Methods). (Created with BioRender.com (December 7, 2023))

7. Filter the homogenate through a 300 μm strainer placed at the top of an open empty 50 mL Falcon tube. Wash the strainer by pipetting 1 mL of isolation medium five times.
8. Pour the filtrate through a 100 μm strainer placed atop an open empty Falcon tube. Wash the strainer by pipetting 1 mL of isolation medium five times.
9. Pour the filtrate through a 70 μm strainer placed atop a Falcon tube. Wash the strainer by pipetting 1 mL of isolation medium five times.
10. Pour the filtrate through a 40 μm strainer placed atop a Falcon tube. Wash the strainer by pipetting 1 mL of isolation medium five times (*see* **Note 8**).
11. Pour the filtrate through a 30 μm strainer placed atop a Falcon tube. Wash the strainer by pipetting 1 mL of isolation medium five times.
12. The 30 μm mesh traps the bovine primordial follicles. Flip the 30 μm strainer upside down and stably hover over a 60 mm Petri dish. Wash the strainers by pipetting 1 mL of isolation medium five times (*see* **Note 9**).
13. Under the stereomicroscope, select the primordial follicles from the resultant filtrate with a mouth pipette and transfer them into a 35 mm Petri dish with 2 mL of manipulation medium (*see* **Notes 5**, **8**, **9**, and **10**).
14. Collect groups of 20 primordial follicles and place them in a 4-well plate containing 500 μL of the previously prepared Culture Medium at 38.5 °C and 5% CO_2 in air, maximum humidity.

3.2 Viability Assessment

1. Assess primordial follicle viability after 1 h of incubation in the culture medium (*see* **Note 11**).
2. In a 35 mm Petri dish, make a 50 μL drop of the previously prepared manipulation buffer and dual staining solution.
3. Collect individual groups of primordial follicles cultured from the 4-well plate in maximum 5 μL of media and wash them in the droplet of manipulation buffer.
4. Transfer the primordial follicles in the dual stain droplet and observe them under a fluorescence microscope at appropriate wavelengths.
5. Count as live follicles those showing green fluorescence in all cells (intact) or <10% of dead (red) cells [48] (Fig. 2).

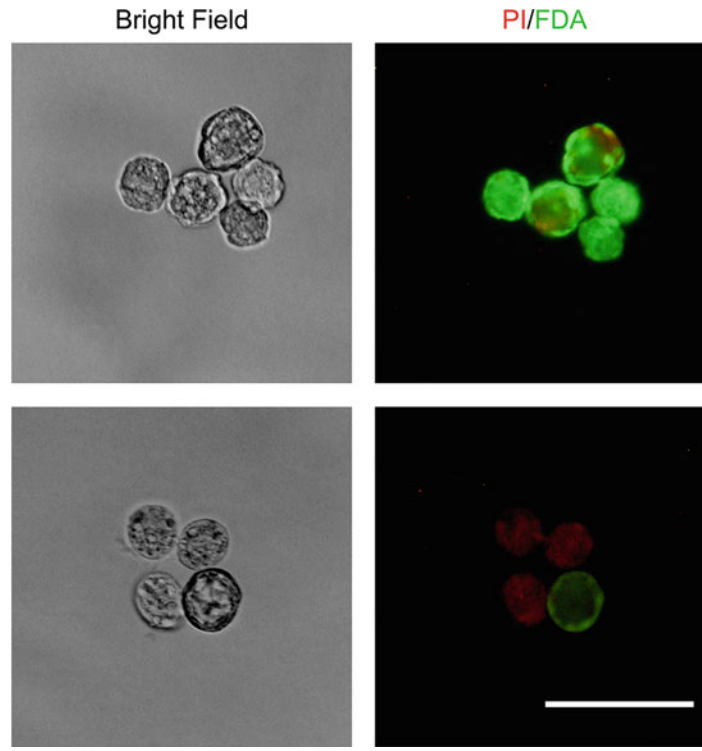

Fig. 2 Representative images of primordial follicles subjected to dual-fluorescence viability assay using Fluorescein Diacetate (FDA, green, live cells) and propidium iodide (PI, red, dead cells). Scale bar = 100 μm

4 Notes

1. Ensure that the isolation medium is at room temperature (26 °C) prior to use.
2. L-15 should be with phenol red, GlutaMAX™, sodium pyruvate, and galactose, and without glucose, HEPES, and sodium bicarbonate.
3. From previous reports in bovine and human species, it is recommended the use of Medium αMEM with specific nucleosides, nucleoside triphosphate, and ribonucleosides to preserve the morphology, morphometry, and ultrastructure of pre-antral follicles and ensure their survival and growth [49–52].
4. As described by Van Wezel and Rodgers [53], bovine ovaries have a major distinct polarity from the surface to the medulla, in histological sections, and is composed of at least five identifiable zones. The zones containing primordial follicles are substantially avascular and localized in the thickness between 0.5 and 1 mm of the depth of the ovarian cortex [53].

5. Ensure that the Petri dishes (60 and 35 mm) used during all the procedures are suspension cell culture dishes, not those for adherent cells. It will prevent the adhesion of follicles on the bottom of the plate, thereby avoiding damage due to applying mechanical force during the collection with glass or plastic tips.
6. **Steps 2–4** should be performed within 5 min.
7. Position the falcon tube under the homogenizer such that the level of isolation medium in the tube is above the minimum (indicated as "MIN") line on the IKA Plastic Disperser tool. Stabilize the Falcon tube by providing support to the walls and tip of the tube, allowing it to remain upright independently during the homogenization. This procedure will avoid foam formation during the homogenization process. Foam could entrap follicles that may subsequently be lost during filtration.
8. The serial filtrations with decreasing mesh size allow for isolating a homogeneous population of primordial follicles with minimum debris. Primordial follicles can also be found entrapped in the 40 μm strainer due to the prolate shape of bovine primordial follicles [53], which have three dimensions measured as length (45.4 ± 2.4 μm), breadth (26.8 ± 1.5 μm), and depth (30.4 ± 1.4 μm) (mean ± SEM). They can be recovered by washing the 40 μm strainer as described for the 30 μm one in **step 12**.
9. To optimize yield and viability, **steps 7–13** should be completed within 25–30 min, as also recently reported in mice [23] and bovine [38].
10. Use a pulled glass capillary with an inner diameter of approximately 100 μm to collect a clean population of PMF.
11. Before assessing the viability of the primordial follicles at the time of collection, incubate the follicles for at least 1 h to allow the cells to recover [23].

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Part III

Somatic Reprogramming to Studying Human Aneuploidy Features

Chapter 14

Generation of iPSC Cell Lines from Patients with Sex Chromosome Aneuploidies

Veronica Astro and Antonio Adamo

Abstract

Somatic cell reprogramming allows the generation of human induced pluripotent stem cells (iPSCs) from patient's cells. The derived iPSCs provide an unlimited source of patient-specific cells that can be virtually differentiated in any cell of the human body. The generation of iPSCs has important implications for all human medicine fields, as they can be used for drug discovery, regenerative medicine, and developmental studies. Klinefelter Syndrome (KS) is the most common chromosome aneuploidy in males. KS is typically characterized by a 47,XXY karyotype, representing 80–90% of KS patients. In rare cases, high-grade sex chromosome aneuploidies (SCAs), 48,XXXY; 48,XXYY; 49,XXXXY, are also observed in males. Since the advent of the reprogramming technique, a few KS-iPSCs have been described. Here, we detail the methodology for generating primary fibroblasts from patients' skin biopsies and the subsequent derivation of iPSCs using an efficient integrative-free mRNA-based somatic reprogramming approach.

Key words Fibroblasts, mRNA-based reprogramming, Klinefelter syndrome, Induced pluripotent stem cells, X chromosome aneuploidies

1 Introduction

1.1 Somatic Cell Reprogramming

Somatic cell reprogramming was first described in 2006 by a team of scientists led by Shinya Yamanaka. Yam\anaka and colleagues used a combination of four transcription factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*), also known as Yamanaka or OSKM factors, to convert mouse fibroblasts into induced pluripotent stem cells (iPSCs) [1]. The Yamanaka factors are master regulators of cell fate and can effectively reset the gene expression patterns of adult cells into an embryonic-like pluripotent state, allowing them to differentiate into virtually any cell type of the human body. This groundbreaking research opened new avenues for generating patient-derived cells suitable for drug discovery, regenerative medicine, and disease modeling studies.

Somatic cells successfully reprogrammed into iPSCs include fibroblasts, blood cells, adipose cells, and keratinocytes

Marco Barchi and Massimo De Felici (eds.), *Germ Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 2770, https://doi.org/10.1007/978-1-0716-3698-5_14,

[2]. Recently, scientists demonstrated that urine-derived cells (UDCs) can also be reprogrammed into iPSCs, providing an easily accessible and noninvasive patient' cell source [3–5]. iPSCs obtained from UDCs display a differentiation potential comparable to fibroblast-derived iPSCs [6]. However, UDCs may have limitations compared to other somatic cells, such as lower reprogramming efficiency associated with variable quality and quantity of the starting material.

Somatic cell reprogramming could be achieved using traditional lentivirus- or retrovirus-based integrative systems or non-integrative mRNA-based protocols. The virus-free and integration-free methods are preferable over the viral and integrative approaches for multiple reasons. Some of the critical limitations of the integrative approaches are: (1) the stable integration of exogenous DNA into the genome of the target cells; (2) the risk of insertional mutagenesis and genomic aberrations; and (3) the selection of partially reprogrammed false positive clones whose pluripotency is sustained by the continuous expression of the transgenes rather than the reactivation of the endogenous pluripotency circuitry.

In conclusion, integrative approaches are exposed to a high risk of harmful and unpredictable genomic rearrangements and require extra validation steps. On the other hand, mRNA-based reprogramming methods are virus-free and integration-free and have been successfully applied to generate iPSCs from control subjects and patients carrying sex chromosome aneuploidies [7–13].

1.2 Klinefelter Syndrome and High-Grade Sex Chromosome Aneuploidies

Klinefelter syndrome (KS) is the most common human sex chromosome disorder (prevalence 1:600 born males) and is characterized by a supernumerary X chromosome (47,XXY karyotype) in patients' cells [14, 15]. The karyotype 47,XXY is present in 80–90% of cases, while mosaicism 46,XY/47,XXY, high-grade sex aneuploidies (SCAs) 48,XXXY; 48,XXYY; 49,XXXXY, or structurally abnormal X chromosomes 47,iXq,Y account for the remaining cases [16, 17]. KS patients display a broad spectrum of clinical manifestations, including infertility, intellectual disability, tall stature, cardiac abnormalities, metabolic disorders, and cancer [18–22]. Notably, patients with 46,XY/47,XXY mosaicism exhibit milder phenotypes and symptomatology [23]. On the other hand, patients with high-grade SCAs display more severe clinical aspects, including aberrant neurodevelopmental, cognitive delays, and psychological disorders [24]. The molecular basis of Klinefelter syndrome lies in the abnormal number of sex chromosomes, which leads to altered gene expression patterns and developmental abnormalities. However, gene-to-phenotype correlation studies linking the supernumerary X chromosomes to the clinical symptoms are still missing. Intriguingly, each supernumerary X chromosome is epigenetically inactivated through a XIST-dependent mechanism [25]. A few genes,

called escape, elude X inactivation and their expression is sensitive to X dosage, resulting in aberrant gene expression impacting the whole transcriptome [12, 26].

Overall, the molecular basis of Klinefelter syndrome and high-grade SCAs are complex and multifaceted, and the impact of supernumerary X chromosomes on human development and physiology is a field of study largely unexplored. The ongoing research is focused on understanding the specific genetic and molecular mechanisms underlying these conditions.

1.3 Disease Modeling of Klinefelter Syndrome and SCAs

The iPSC-based approach to model Klinefelter syndrome and SCAs offers a unique opportunity to investigate the impact of extra X chromosomes on the genome in the first stages of development. It is plausible that patients affected by these conditions show an altered transcriptomic profile, which can affect many developmental processes, including brain, heart, and skeletal muscle development. iPSCs differentiated into disease-relevant cell types, such as neurons, cardiomyocytes, or muscle cells, can be used to study in vitro the molecular and cellular mechanisms underlying sex chromosome disorders. Moreover, the use of cell reprogramming to model Klinefelter syndrome and high-grade SCAs may also lead to the identification of novel therapeutic targets for treating such conditions.

Several studies reported the establishment of iPSCs from somatic cells of individuals with Klinefelter syndrome (Table 1).

In 2022, Adamo's group derived the largest iPSC cohort of Klinefelter syndrome patients and reported the first successful reprogramming of high-grade SCAs patients with 49,XXXXY karyotype using a non-integrative mRNA-based reprogramming technology [12]. The spontaneous supernumerary chromosomal loss, a phenomenon occurring during reprogramming [33], also allowed the derivation of isogenic 46,XY and 48,XXXY iPSC clones from non-mosaic patients (Table 2). Importantly, isogenic cells provide the unique opportunity to model the transcriptional impact of increasing X dosage in a virtually identical genomic background [12]. In 2023, the same research group generated KS-iPSCs from KS Saudi patients demonstrating the existence of a unique transcriptomic dysregulation signature, specific for KS patients, regardless of their geographical origins and genetic makeup [13].

Overall, these studies demonstrated the value of the human iPSC models in studying the transcriptional and epigenomic impact of supernumerary X chromosomes in pluripotency and during differentiation into disease-relevant lineages. However, KS modeling in vitro is still a relatively new area of research. Further studies are needed to explore the potential of this approach for understanding the molecular and cellular mechanisms underlying sex chromosome aneuploidies.

Table 1
List of generated KS-iPSCs reporting the reprogramming technique and cell source

iPSC line	Karyotypes	Sources	Reprogramming method	References
ES-like cells	47,XXY	Testicular tissues	Transduction of lentiviral vectors coding for the transcription factors, OCT4, SOX2, KLF4, and C-MYC	Kobayashi et al. 2011 [27]
KS-iPSC-1, KS-iPSC-4, KS-iPSC-8, KS-iPSC-11	47,XXY	Foreskin fibroblasts	Retrovirus transduction of OCT4, SOX2, KLF4, and C-MYC	Ma et al. 2012 [28]
KS-iPSC	47,XXY	Testicular tissues	Sendai viral vectors encoding four transcription factors, OCT4, SOX2, KLF4, and C-MYC (CytoTuneR-iPS 2.0)	Shimizu et al. 2016 [29]
iPS-KS-1, iPS-KS-2	47,XXY	Skin fibroblasts	Non-integrative episomal plasmids encoding for the OCT4, SOX2, KLF4, and C-MYC factors	Panula et al. 2019 [30]
iPSCs #11, iPSCs #16	47,XXY	Skin fibroblasts	Polycistronic lentiviral vector, carrying the pluripotent genes OCT4, KLF4, SOX2, and c-MY	Botman et al. 2020 [31]
2 XXY iPSCs, 3 XY iPSCs, 3 XX iPSCs, 1 XO iPSCs	47,XXY, 46,XY, 46,XX, 45,XO	EBV-immortalized B cell line	Nucleofection with plasmids overexpressing SOX2, POU5F1 (OCT4), KLF4, LIN28A, MYCL, SV40LT, and shTP53	Waldhorn et al. 2022 [32]

In the next chapter, we provide a detailed stepwise protocol for generating primary fibroblasts from patients' skin biopsies and deriving iPSCs using an efficient integrative-free mRNA-based somatic cell reprogramming approach.

2 Materials

2.1 Fibroblast Culturing, Passaging, and Freezing

1. Fibroblast complete media: DMEM (+D-Glucose 4,5 g/L; -Pyruvate, Gibco), 20% FBS (HI) + GlutaMAX, NEA (100×) and P/S (100×).
2. Fibroblast freezing media: 90% Fibroblast complete media, 10%DMSO.

2.2 Somatic Cell Reprogramming

1. Preparation of Fibroblast Expansion Medium:

Add 5 mL human serum (Sigma) and 0.5 mL Glutamax Supplement to 44.5 mL A-DMEM (10% human serum final concentration). The media can be stored at 4 °C for up to 2 weeks.

Table 2
List of hiPSCs derived from KS and High-grade SCA patients in Adamo's laboratory using a virus-free, non-integrative approach

iPSC lines	Karyotype	Sources	Notes	Patient origin	References
KAUSTi008-A,-B, KAUSTi008-C,-D, KAUSTi008-E,-F,-G	47,XXY, iso46,XY	Skin fibroblasts	Non-mosaic KS patient	Europe, North America	Fiacco et al. 2020a [10]
KAUSTi003-A, KAUSTi004-A,-B, KAUSTi005-A,-B,-C	49,XXXXY, 46,XY, 46, XX	Skin fibroblasts	High-grade SCAs and healthy relatives. Source: NIGMS Cell Repository	Europe, North America	Alowaysi et al. 2020a [9]
KAUSTi001-A, KAUSTi002-A	49,XXXXY, iso48, XXXY	Skin fibroblasts	Source: NIGMS Cell Repository	Europe, North America	Alowaysi et al. 2020b; Astro et al. 2022 [11, 12]
KAUSTi006-A, KAUSTi006-B	47,XXY, iso46,XY	Skin fibroblasts	Mosaic KS patient. Source: Galliera Genetic Bank	Europe, North America	Fiacco et al. 2020b [7]
KAUSTi007-A,-B; KAUSTi009-A,-B; KAUSTi010-A,-B	47,XXY	Skin fibroblasts	Unrelated KS patients. Source: Telethon Cell Repository	Europe, North America	Alowaysi et al. 2020c [8]
KS#1, KS#2, KS#3, KS#4, KS#5	47,XXY, 46, XY	Skin fibroblasts	Isolated in Adamo's lab from biopsies	Saudi Arabia	Astro et al. 2023 [13]

2.3 iPSC Picking and Replating

1. Preparation of Essential 8 (E8) Medium:

Thaw one bottle of E8 Supplement (Thermo Fisher Scientific) overnight at 4 °C. Mix the bottle of E8 Supplement (10 mL) in 500 mL of E8 media. Supplement the media with 5 mL (1%) of P/S antibiotics. The media can be stored at 4 °C for up to 2 weeks.

2. Preparation of Essential 8 (E8) Medium supplemented with RevitaCell supplement (Thermo Fisher Scientific, 100×) on the splitting day. Store the Medium for up to a week at 4 °C.
3. Preparation of iPSC Freezing Medium:

Add 10% DMSO to 90% E8+ RevitaCell (100×). Prepare it fresh on the day of cryopreservation.

3 Methods

3.1 Isolation of Fibroblasts from Skin Biopsies (Fig. 1)

1. Skin punch biopsies (about 4 mm^2) were collected from the patient's forearms (*see* **Note 1**).
2. Use the lid of a sterile 10 cm tissue culture dish and add 1.5 mL of DMEM 20% FBS media.
3. Using sterile forceps, move the skin punch biopsies in the DMEM complemented with 20%FBS for transportation to the lab (45–60 min).
4. Place an inverted microscope (e.g., EVOS FL, Thermos Fisher) inside a laminar flow hood.
5. Place the 10 cm dish under the microscope and start dissecting the biopsies using two sterile scalpels: one will hold the tissue and the other will cut the biopsies in equal halves, about 12–15 pieces.
6. Move two to three pieces of the dissected tissues into each well of a 0.1% Gelatin-coated 6-well plate in the presence of 1 mL DMEM containing 20% FBS. Gelatin coating should be prepared as follow:
   (a) Add 1 mL of 0.1% Gelatin to each well of a 6-well plate.
   (b) Set the plate aside for 30–60 min.
   (c) Aspirate the gelatin solution and add 1 mL of DMEM/20% FBS media.
7. Incubate the 6-well plates at 37 °C in the presence of 5% CO_2 and 5% O_2.
8. For the first week, monitor the cells and add about 200 μL of DMEM containing 20% FBS every 2 days to ensure a film of media correctly covers the dish.
9. During the second week, increase the volume of DMEM/20% FBS media to 2 mL and change media every 2–3 days.

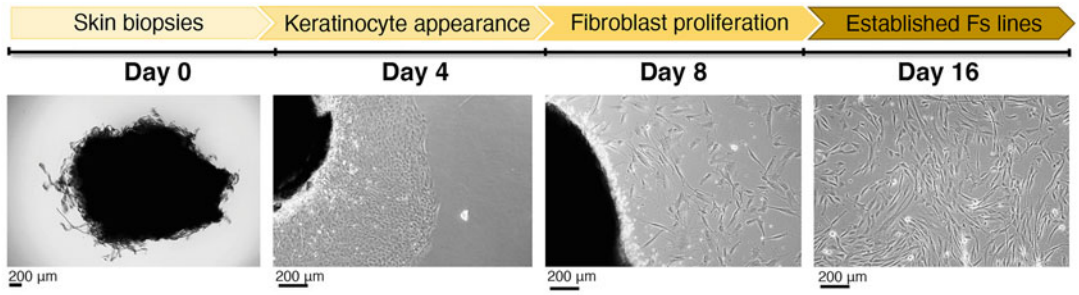

Fig. 1 Derivation of fibroblasts from skin punch biopsies. Upper panel: Timeline of the fibroblast derivation from skin biopsies. Lower panel: Brightfield images of a representative biopsy and fibroblast culture establishment. Fs fibroblasts. Scale bar, 200 μm

10. Three weeks after tissue plating, a confluent monolayer of fibroblasts covering the entire well area of the 6-well plates should be visible.
11. When confluent fibroblasts are detached with 0.05% trypsin/EDTA and seeded into T25 flasks in the presence of DMEM/10% FBS.

3.2 Culture of Primary Fibroblasts

1. Primary fibroblasts are cultured at 37 °C, 5% CO_2 and 5% O_2 in T25 ventilated flasks in the presence of Fibroblast complete media (*see* Subheading 2.1).
2. Fibroblasts are detached and reseeded when they reach about 90% confluency, approximately every 3–4 days, when diluted at a 1:3 ratio (*see* **Note 2**).
3. Fibroblasts are detached as follows:
   (a) Wash cells twice using 5 mL of DPBS.
   (b) Detach the cells in 1 mL of 0.05% Trypsin/EDTA for 3–5 min at 37 °C. Check the plate after 3 min. If cells are still attached, wait 2 more minutes.
   (c) Inactivate the Trypsin/EDTA by adding 4 mL of complete media.
   (d) Carefully resuspend the cells in the flask and transfer them to a 50 mL falcon tube containing 10 mL of media (15 mL in a total of cell suspension).
   (e) Aliquot 5 mL of cell suspension in three T25 vent flasks to obtain a dilution of 1:3.
4. Fibroblasts are cryopreserved as follows:
   (a) Follow **steps** from **3.2.3** (**a**) to **3.2.3** (**c**), carefully resuspend the cells in 5 mL of complete media, and transfer them to a 15 mL falcon tube.
   (b) Centrifuge the tube at 850 × *g* for 3 min at room temperature (RT).
   (c) Resuspend the cell pellet at 1×10^6 cells/mL per vial in 1 mL of fibroblast freezing media (*see* Subheading 2.2) and transfer to cryovials for freezing.

3.3 Somatic Cell Reprogramming of Fibroblasts (Fig. 2)

1. The day before the transfection (Day 0):
   (a) Coat a 6-well plate using 1 mL of 2.4 mg/mL iMatrix for each well of a 6-well plate as follow:
      (i) On the day of fibroblast plating for reprogramming, dilute iMatrix-511 in cold DPBS at the final concentration of 2.4 μg/mL. Coat every well of a 6-well plate using 1 mL of the coating solution.
      (ii) Incubate at 37 °C for 1 h.

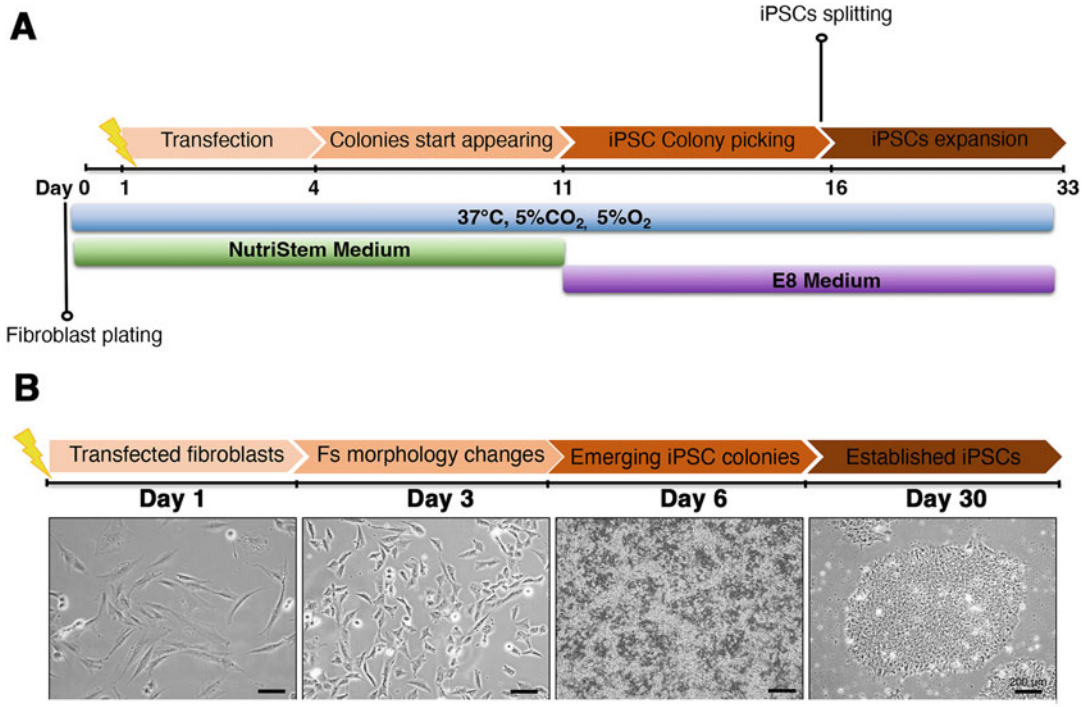

Fig. 2 Fibroblast reprogramming using NM-RNA-based reprogramming. (**a**) Timeline of the experiment. (**b**) Brightfield images showing the morphology change of the reprogrammed fibroblast. Fs fibroblasts. Scale bar, 200 μm

(iii) Remove the coating solution and add 1 mL of Fibroblast Expansion media.

(iv) Store the coated plates at room temperature until fibroblasts are ready for plating.

(b) Remove the culture media and wash the fibroblasts with 5 mL of DPBS.

(c) Remove the DPBS and detach the fibroblasts using 1 mL 0.05% Trypsin/EDTA.

(d) Incubate for 4 min at 37 °C, 5% CO_2, 5% O_2.

(e) Tap the flask to detach the cells from the flask completely.

(f) Add 3 mL Fibroblast Expansion Medium to the flask to neutralize the Trypsin/EDTA.

(g) Transfer the harvested cell suspension from the flask to a 15 mL conical tube. Pipette up and down gently to disrupt the cell aggregates.

(h) Centrifuge the cells for 3 min at 180× *g*.

(i) Remove the supernatant and resuspend the pellet in 1 mL Fibroblast Expansion Medium.

(j) Count the cells and seed about 5×10^4–8×10^4 fibroblasts/well on iMatrix511-precoated 6-well plates (Stemgent; *see* **Note 3**) containing 1 mL of Fibroblast Expansion Medium. The final volume in each well is 2 mL. To prepare the coating proceed as follow:

1. On the day of fibroblast plating for reprogramming, dilute iMatrix-511 in cold DPBS at the final concentration of 2.4 μg/mL. Coat every well of a 6-well plate using 1 mL of the coating solution.
2. Incubate at 37 °C for 1 h.
3. Remove the coating solution and add 1 mL of Fibroblast Expansion media.
4. Store the coated plates at room temperature until fibroblasts are ready for plating.

(k) Incubate the cells at 37 °C, 5% CO_2, and 5% O_2.

2. Twenty-four hours after seeding, proceed with the first transfection (Day 1):

(a) Recommended: Wipe down all working surfaces (*see* **Note 4**).

(b) Warm up NutriStem Medium in a 37 °C water bath (*see* **Note 5**).

(c) Remove the old Medium from the fibroblasts seeded for reprogramming and add 2 mL NutriStem Medium to each well (*see* **Note 6**).

(d) Incubate in hypoxic incubator at 37 °C, 5% CO_2, 5% O_2.

(e) Preparation of NM-RNA Reprogramming Cocktail aliquots:

(i) Thaw three vials of NM-RNAs provided in the kit (Stemgent® StemRNA-NM Reprogramming Kit, Stemgent; OSKMNL NM-RNA, EKB NM-RNA, and NM-microRNAs) on ice. Once thawed, keep the vials on the ice and aliquot each vial's content.

(ii) Briefly centrifuge the vials to collect the contents at the bottom of the tube.

(iii) Use Table 3, showing the calculated amounts of NM-RNAs for four consecutive transfections of one, two, or three wells of fibroblasts in a 6-well plate format. Use the appropriate volume for your experiment design (e.g., different densities or fibroblast lines).

(iv) Divide the mixture of the three vials into four single-use aliquots (*see* Table 3) in sterile, RNase-free microcentrifuge tubes. Store the aliquots at −80 °C for up to 3 months. Avoid additional freeze–thaw cycles.

Table 3
Calculations for the preparation of the transfection cocktail of NM-RNAs

	One well	Two wells	Three wells	Four wells
OSKMNL NM-RNA (μL)	8 (0.8 μg)	16	24	32
EKB NM-RNA (μL)	6 (0.6 μg)	12	18	24
NM-microRNAs (μL)	1.4 (0.4 μg)	2.8	4.2	5.6
NM-RNA reprogramming cocktail (μL)	15.4	30.8	46.2	61.6
Number of transfections	36	18	12	9

(f) Thaw an NM-RNA Reprogramming Cocktail single-use aliquot at room temperature, then immediately place it on ice. Label as tube "A (RNA + Opti-MEM)."

(g) Label a sterile, RNase-free 1.5 mL microcentrifuge as tube "B (RNAiMAX+ Opti-MEM)." The provided calculations are for one well format. Scale appropriately for larger experiments:

(i) To tube A, add 234.6 μL Opti-MEM (tube A already contains 15.4 μL NM-RNA Reprogramming Cocktail).

(ii) Add 6 μL RNAiMAX transfection reagent to tube B containing 244 μL of Opti-MEM.

(h) Pipette gently three to five times to mix.

(i) Using a P1000, transfer the entire contents of tube B to tube A dropwise at the meniscus level. Mix by tapping the bottom of the tube. Incubate at room temperature for 15 min.

(j) Add 500 μL NM-RNA transfection complex solution to the well in the reprogramming plate by tilting the plate and pipetting dropwise into Medium. Mix by rocking in the X- and Y-directions.

(k) Incubate the reprogramming plate in hypoxic conditions (37 °C, 5% O_2, 5% CO_2) overnight.

3. Three additional rounds of transfection (Days 2–4):

(a) Recommended: Wipe down all working surfaces (gloves, reagent bottles, biosafety cabinet surfaces, pipettors, etc.) with RNase Zap.

(b) At the beginning of the day (9 AM), warm NutriStem Medium in a 37 °C water bath.

(c) Remove the old Medium from the wells in the reprogramming plate. Add 2 mL NutriStem Medium to each well.

(d) Incubate in a hypoxic incubator at 37 °C, 5% CO_2, and 5% O_2 for at least 6 h (*see* **Note 7**).

(e) At around 3 pm, thaw the NM-RNA Reprogramming Cocktail single-use aliquot at room temperature, then immediately place it on ice.

(f) Proceed as described in **Methods steps 3.3.2** (**f**) to **3.3.2** (**k**).

4. Change media daily using NutriStem Medium (Days 5–10):

(d) Remove the medium from each well in the reprogramming plate and exchange it with 2 mL fresh NutriStem Medium containing 1% P/S (*see* **Note 8**).

(e) Return the reprogramming plate to a hypoxic incubator (5% O_2) overnight.

3.4 iPSC Picking and Expansion

1. We recommend performing colony picking using a bright-field microscope (e.g., EVOS FL, Thermo Scientific) in sterile conditions, optimally in a horizontal flow hood (*see* **Note 9**).
2. Coat the appropriate number of wells (maximum 16 wells) of a 96-well plate with hESC-qualified Matrigel (Corning) before picking as follow:

(a) Thaw a bottle of Matrigel on ice in the cold room overnight.

(b) Aliquot 500 μL of Matrigel in prechilled 1.5 mL Eppendorf tubes and store the aliquots at −20 °C for up to 3 months.

(c) Dilute 500 μL of Matrigel in 19.5 mL of cold DMEM/F12 (1:40 dilution).

(d) Coat the 96-wells using 100 μL of diluted Matrigel and incubate for 1 h at 37 °C. Use 1 mL of diluted Matrigel to coat a well of a 6-well plate. Alternatively, the coating can be performed overnight at 4 °C.

(e) Remove the Matrigel solution and add 100 μL in each well of a 96-well plate or 1 mL in each well of a 6-well plate of Essential 8 (E8) Medium supplemented with RevitaCell supplement (100×).

(f) Store the plate at 37 °C and use it the same day.

3. Aspirate the Medium from the wells of the Fibroblast Reprogramming Plate and add 2 mL of pre-warmed NutriStem Medium.
4. Using a microscope, identify the iPSC colonies based on morphology and using a needle, clean the area around the iPSC colony from the attached fibroblasts.
5. Using a P200 pipette (set to 40 μL), gently separate, aspirate, and transfer the colony into one Matrigel pre-coated well of a

96-well plate. These wells already contain 100 μL of E8+ RevitaCell (*see* **Note 10**).

6. Repeat the picking and replating process for each iPSC colony. Pick one colony at a time and transfer the cell aggregates of each colony to a different well of the prepared 96-well plate. After six iPSC colonies have been picked and replated, place both 96-well and Fibroblast Reprogramming plates in the hypoxic incubator (5% O_2) for re-equilibration.
7. Continue to culture the 6-well Fibroblast Reprogramming Plate until the picked colonies are established.
8. The day after picking, replace the E8+ RevitaCell Medium with 100 μL of fresh E8 Medium in each well of the 96-well plate.
9. Change E8 Medium in the 6-well Fibroblast Reprogramming plate and in the 96-well plate containing the single iPSC clones every day thereafter.
10. Once colonies are large enough (it takes approximately 3–4 days depending on the original size of the colony), incubate iPSC colonies in Versene for 4 min at 37 °C to dissociate the colony and transfer it into 48-well plates (*see* **Note 11**).
11. Repeat a similar dissociation method to expand the colonies gradually from the 48-well plate into 24-well, 12-well, and 6-well plates (*see* **Note 12**).
12. Once the iPSC colonies are expanded in 6-well plates, it is possible to cryopreserve them using iPSC freezing media (see Subheading 2.3, **item 3**). We recommend freezing one well of a 6-well plate containing 70–80% confluent iPSCs in 1 mL of iPSC freezing media per cryovial.

4 Notes

1. Once the biopsies have been collected, it is crucial to transfer the sample to the laboratory and process the biopsies as soon as possible.
2. After isolation from the skin biopsies, fibroblasts should be cultured and expanded for at least three passages in Fibroblast complete media before cell reprogramming. Reprogramming efficiencies depend on fibroblast passage. The optimal passage number is between P2 and P5. After passage seven, the reprogramming efficiency can drop below 1%.
3. The optimal starting number of cells could vary depending on the specific fibroblast cell line. We recommend seeding and transfecting at least three cell quantities in independent wells with a range of 5×10^4– 8×10^4 cells/well. The best seeding condition will be the one where cells survive the four

consecutive transfections and produce viable iPSC colonies by Days 11 and 12 post-transfection.

4. Wipe down all working surfaces (gloves, reagent bottles, biosafety cabinet surfaces, pipettors, etc.) with RNase Zap.
5. NutriStem medium can be successfully substituted with E8 medium**.** In our experience, using E8 medium does not affect the fibroblasts' reprogramming efficiency significantly.
6. The day after seeding, the optimal confluency of the fibroblasts for NM-RNA transfection is about 30%. Choose the wells containing a number of cells closer to this density from the different dilutions of seeded cells.
7. Increasing the recovery time before adding the next transfection complex decreases cell toxicity.
8. NutriStem medium can be substituted with E8 medium (2 mL) at every media change. At Day 5, fibroblasts should have reached 90–100% confluency. Monitor the cells daily for the appearance of iPSC clones within the fibroblast carpet. Mature iPSCs should appear as compact colonies with distinct borders and well-defined edges and a high nucleus-to-cytoplasm ratio. When the colony reaches a minimum size of 100 μm, they are ready to be picked.
9. Pick no more than 16 colonies at once to avoid keeping the cells out of the incubator for extended periods (30 min max).
10. To maintain clonal iPSC lines, transfer each colony pieces into a separate well of a 96-well plate. Change needle and pipet tips with each new colony to be transferred to avoid cross-contamination of clonal lines.
11. For the first few passages after picking, iPSCs colonies should be passaged using Versene or similar EDTA passaging methods at low split ratios (1:2–1:4) to build dense cultures. Once an iPSC colony has been passaged at least five times and a cryopreserved stock of the colony has been established, it is possible detaching iPSCs using a single-cell based enzymatic method and seeding them in the presence of RevitaCell or Rock Inhibitor to increase cell viability. We suggest performing further applications (e.g., iPSC colony characterization for pluripotency markers, teratoma formation assay, and differentiation) not earlier than 10 passages after colony picking. We recommend validating the established cell lines by checking the expression of the pluripotency markers *OCT4*, *NANOG*, *SOX2*, and *SSEA4* at mRNA and protein levels by qPCR and immunofluorescence, respectively. An additional pluripotency validation test is the formation of teratoma in vivo that reflects

the ability of the iPSCs to give rise to tumor cells with endodermal, ectodermal, and mesodermal origin [7–11].

12. Human iPSC cultures should be carefully monitored every day, as the overall quality of the culture can change rapidly. iPSCs are generally passaged every 3–5 days in culture. The split ratio for each passage will vary depending on the cell culture's quality and growth rate and can be iPSC line specific. Splitting the cells before the cultures become overgrown is vital to avoid unwanted spontaneous differentiation. We recommend detaching iPSCs when they reach 75–80% confluency and adopting clump passaging, using Versene or similar EDTA passaging methods instead of enzymatic methods, unless needed for single cell-based applications.

Acknowledgments

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Part IV

Informatic Tools for Studying Germ Cells Development and Recombination

Chapter 15

Data Analysis Pipeline for scRNA-seq Experiments to Study Early Oogenesis

Wei Ge, Teng Zhang, Yang Zhou, and Wei Shen

Abstract

Germ cells as the means for the transmission of genetic information between generations have been a hot topic of research for decades. The analysis of the transcriptomes, that is of the RNA transcripts produced by the genotype at a given time, of germ cells and the surrounding somatic cells, is essential to unravel the cellular and molecular processes regulating gametogenesis. However, the asynchronized differentiation of germ cells and high cellular heterogeneity in the developing ovary or testis represent two unsurmountable challenges for delineating the transcription regulation mechanism of germ cells using traditional bulk RNA sequencing. By performing single-cell RNA sequencing (scRNA-seq), it is now possible to dissect the transcriptome of germ cell development at single-cell resolution, and apply powerful bioinformatics methods to translate raw sequencing data into meaningful information. Here, using the 10× Genomic platform and the most widely cited bioinformatics tools, we describe how to analyze early female germ cell development using scRNA-seq data generated from mouse E11.5 to E14.5 ovaries. This pipeline will provide a guide for exploring the processes of early germ cell development at single-cell resolution.

Key words Single-cell transcriptome, Meiotic transcriptome, Meiosis, scRNA-seq, Bulk RNA-seq

1 Introduction

In the present chapter, as a practical example of the possibility offered by scRNA-seq data and related bioinformatics, we describe the application of this new technology to the study of the beginning of meiosis in female germ cells in the developing mouse ovary. One of the major topics of our laboratory.

Meiosis is a fundamental biological process, through which haploid gametes are generated from diploid germ cell precursors [1]. For decades, many studies have focused on this fascinating biological event since besides meiosis is an essential source of genetic variability for species, and defects in this complicated process can be a source of congenital malformations and in some cases infertility [2, 3]. In mice, it is known that female germ cells enter meiosis in the fetal ovaries between E12.5 and E14.5 in an

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asynchronous manner, thus making this process difficult to explore using traditional genetic methods [4]. Moreover, the scenario is complicated by the fact that several factors intrinsic and extrinsic to germ cells cooperate in triggering such a process and its progression and that it occurs in a microenvironment in which heterogeneous cell populations interact among them and with germ cells in a dynamic manner [1].

Using bulk RNA sequencing, the transcriptional profiles of germ cells in the developing ovary have been systematically investigated, and the core transcriptome profiles involved in meiotic initiation have been identified [5]. However, it remains to unveil the complete molecular networks regulating the mitotic to meiotic switch in female germ cells due to the lack of stage-specific markers. The reason that bulk RNA-seq cannot accurately describe meiotic beginning and progression is the fact that it requires a large number of cells as input, and the generated gene expression matrices are intrinsically averaged from different cell types [6]. Therefore, using bulk RNAseq for heterogeneous samples is suboptimal and requires more advanced methods.

Advances in single-cell technologies enable us to gain an unparalleled opportunity to explore cellular heterogeneity at single-cell resolution, and in a single experiment, it is possible to analyze the transcriptome of thousands of single cell using just a single cDNA library [7, 8]. Different from bulk RNA sequencing, which generated "averaged" gene expression matrices, scRNA-seq can generate "real" gene expression matrices at a single-cell scale and can be applied to highly heterogeneous samples. Using such a technology, gene expression profiles underlying meiotic progression in mouse germ cells in the developing ovary have been recently reported [9, 10], which provides a valuable resource for exploring the meiotic program.

Here, using scRNA-seq data generated from E11.5 to E14.5 mouse ovaries by the 10× Genomic platform [9], we will describe a detailed workflow to characterize the transcriptome of female germ cells from a mitotic proliferative state (primordial germ cells or oogonia) to pre-, early-, and late meiotic prophase I using CellRanger and Seurat software. Starting from original FASTQ files generated by the platform, we illustrate how to perform gene expression matrices generation using CellRanger, basic quality control (QC), removal of doublets, data integration, removal batch correlation, and cell type characterization. The aim is to provide a workflow for an easy-to-use guide for scientists without a background in bioinformatics to explore the transcriptome of heterogeneous germ cell populations engaged in dynamic biological processes at single-cell resolution.

2 Materials

The procedure to obtain single-cell populations from embryonic gonads is described in detail in [9, 11] and summarized in Fig. 1. Briefly, isolated E11.5 gonadal ridges and E12.5–14.5 ovaries are cut into small pieces and incubated in 0.25% trypsin and collagenase (2 mg/mL), for 6–8 min at 37 °C. Tissues are then disaggregated with a pipette to generate single cells, and the solution is filtered through 40-μm cell strainers and washed two times with PBS containing 0.04% BSA. Single-cell samples (1000 cells/μL, Trypan Blue viability above 80%) are loaded onto a 10× Chromium chip, and single-cell gel beads in emulsion are generated by using Single Cell 3' Library and Gel Bead Kit V2 (10× Genomics Inc., 120237). Finally, single-cell RNA-seq libraries are constructed and pair-end 150 bp sequencing is performed to produce high-quality data on an Illumina HiSeq X Ten (Illumina, San Diego, CA, USA).

2.1 Required Resources

The 10× Genomics scRNA-seq data analysis pipeline can be divided into two main parts: (1) the use of CellRanger to process FASTQ files into gene expression matrices; (2) downstream quality control (QC) of data and their visualization. For the first part, a Linux system is required for the successful running of the CellRanger pipeline. For the second part, mainstream analysis workflows include R-based frameworks (Seurat, https://satijalab.org/seurat/) and Python-based frameworks (Scanpy, https://scanpy.readthedocs.io/en/stable/) (Fig. 2).

2.2 Equipment

A computer or server running a recent Linux distribution is essential for performing CellRanger pipelines. The recommended configuration includes: (1) 8-core Intel or AMD processor (16 cores recommended); (2) 64 GB RAM (128 GB recommended); (3) 1 TB free disk space; (4) System: 64-bit CentOS/RedHat 7.0 or Ubuntu 14.04.

For the downstream visualization, a recent version of R (https://www.r-project.org/) is required either on Linux or Windows systems. For Windows users, a personal computer (PC) with at least 16 GB RAM is required for successfully running this chapter.

2.3 Equipment Setup

2.3.1 Software Requirements

The following software and tools should be installed before performing downstream analysis:

Cell Ranger7.0.1. A collection of pipelines for processing Chromium single-cell data to align readings, produce feature-barcode matrices, cluster data, and carry out various secondary analyses; https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/installation

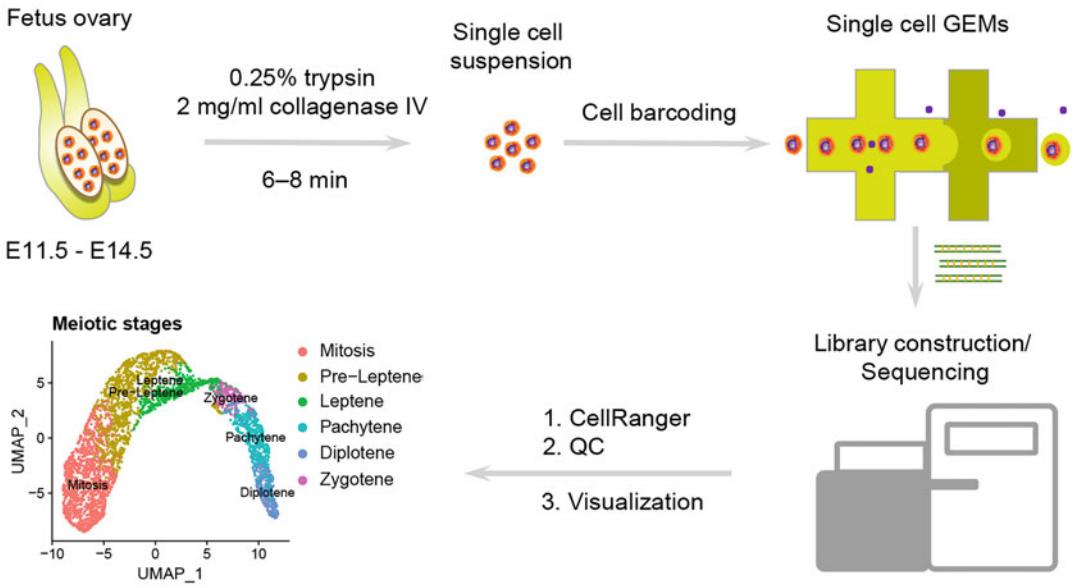

Fig. 1 Schematic diagram of the scRNA-seq analysis procedure. Female embryonic gonads were isolated and disaggregated into single-cell suspensions; cells were barcoded and used for library construction onto the 10× Genomics platform, and the data produced after sequencing were analyzed by dedicated software

R 3.6.3. A software environment for statistical computing and graphics; https://www.r-project.org/

RStudio. A set of integrated tools designed to be more productive with R (a free software environment for statistical computing) and Python (a programming language that can be used on a server to create web applications); https://posit.co/downloads/

Seurat. Seurat is designed for QC, analysis, and exploration of single-cell RNA-seq data. Seurat intends to enable users to combine various types of single-cell data, as well as discover and evaluate causes of heterogeneity from single-cell transcriptome data; https://satijalab.org/seurat/

Ggplot2. A software used for creating publication-ready scientific graphics; https://cran.r-project.org/web/packages/ggplot2/index.html

Dplyr. A grammar of data manipulation; https://cran.r-project.org/web/packages/dplyr/index.html

Cowplot. A software providing various features that help with creating publication-quality figures; https://cran.r-project.org/web/packages/cowplot/index.html

DoubletFinder. A widely used tool for identifying cell doublets in scRNA-seq data; https://github.com/chris-mcginnis-ucsf/DoubletFinder

Harmony. An algorithm for robust, scalable, and flexible multi-dataset integration; https://github.com/immunogenomics/harmony

2.3.2 Software Installation

Installing CellRanger

CellRanger is easy to be redeployed in a Linux machine because it does not require centralized installation. Directly download the self-contained, relocatable tar file from the official website (https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest) into your working directory using the *wget* or *curl* download tools:

```
mkdir ~/cellranger
cd cellranger
curl -o cellranger-7.0.1.tar.gz "https://cf.10xge-
nomics.com/releases/cell-exp/cellranger-7.0.1.
tar.gz?Expires=1669056610&Policy=eyJTdGF0ZW1lbn-
Q iO l t 7 I l J l c 2 9 1 c m N l I j o i a H R 0 c H M 6 L y 9 j -
Zi4xMHhnZW5vbWljcy5jb20vcmVsZWFzZXMvY2VsbC1leHA-
vY2VsbHJhbmdlci03LjAuMS50YXIuZ3oiLCJDb25kaXRp-
b24iOnsiRGF0ZUxlc3NUaGFuIjp7IkFXUzpFcG9jaFRpb-
WUiOjE2NjkwNTY2MTB9fX1dfQ__&Signature=NaqVSV22Q-
h~UBrrywq1tgNz~wP-0BDRq9Q4X3Oli6FExfyEmJQC-
mlcPwY5b-i~3CZve9mzn8McZ4qovopSjZ0FcqCre0-
T72uUyOxmGi4zwNAOubg179Zbrjte5dzgSs5tS2gSC-
dauEUDzv8nVfKEq36E875JynIM1Cu6gd5Bd2T8zHM0-
nORfqHw2lBx~ZS7vRT8rwfeWylcsL2xKO6jnNhMMyolT2r5B-
SutqKv7zPAOiGjyUtT25XOpkgaTzH1fGpbBKkNq3mChMAB-
xixWEQDjdZJpRHJx7OsFYjWuhjvirelwFlwUNR-
laq0GMwfHLzLng5ZPzKN847JSvfE6SPA__&Key-Pair-
Id=APKAI7S6A5RYOXBWRPDA"
```

Then, unpack the *cellranger-7.0.1.tar.gz* into your target directory using the following command:

```
tar -zxvf./cellranger-7.0.1.tar.gz
cd cellranger-7.0.1/bin
./cellranger
```

Alternatively, add CellRanger to environment variables for easier use in the future:

```
export PATH=/home/XXX/XXX/cellranger-7.0.1:$PATH
```

After that, CellRanger can be called in the terminal by directly inputting the *cellranger* command.

Installing R
Directly download the latest version of R from the official website (https://vps.fmvz.usp.br/CRAN/), then run *R-4.2.2-win.exe* on your PC and install them into your target directory (*see* **Note 1**).

Installing RStudio
For the installation of RStudio, download *RStudio-2022.07.2-576.exe* file from the official website (https://posit.co/downloads/) and double-click the executable file will guide you to install RStudio on your PC.

Installing Essential R Packages
Open RStudio on the PC and install essential packages using *install.packages()*, *remotes:install_github()*, or *BiocManager:install* function. To install the required packages and their dependencies used in this chapter, run the following command:

```
# install from CRAN:
install.packages("Seurat")
install.packages("dplyr")
install.packages("cowplot")
install.packages("ggplot2")
install.packages("RColorBrewer")
```

The CRAN network of FTP and web servers house identical, up-to-date versions of code and documentation for R (https://cran.r-project.org/web/packages/). Setting the closest CRAN mirror before installing the packages can greatly speed up the installation process. When used in RStudio, it can be set in the *Global Options* option under the *Tools* menu. Restart your Rstudio for changes to take effect.

```
## install from Github repository
remotes:install_github('chris-mcginnis-ucsf/DoubletFinder')
```

GitHub (https://github.com/) is a website and cloud-based service that enables developers to store and manage their code, as well as track and control changes to their code. Packages deposited in the GitHub repository can be installed via the *remotes: install_github* command.

```
## install from Bioconductor
if(!requireNamespace("BiocManager", quietly = TRUE)
install.packages("BiocManager")
BiocManager:install("harmony", version = "3.8")
```

Bioconductor (https://www.bioconductor.org/) provides widespread access to a broad range of powerful statistical and graphical packages compiled by the R language. Packages deposited in the Bioconductor repository can be installed via the BiocManager tool.

Preparation of Reference Genome

For human and mouse datasets, 10× Genomics provides ready-to-use reference genome files that can be directly downloaded into a target directory using the following command:

```
mkdir ~/ref_genome
cd ref_genome
curl -O https://cf.10xgenomics.com/supp/cell-exp/
refdata-gex-mm10-2020-A.tar.gz
tar -zxvf./refdata-gex-mm10-2020-A.tar.gz
```

2.4 Datasets

2.4.1 Preparation of scRNA-seq Dataset

As reported above, here, we will focus on a dataset obtained from germ cells of ovaries of E11.5, E12.5, E13.5, and E14.5 C57/BL6J mouse embryo by Ge et al. [9]. The dataset is publicly available at NCBI's gene expression omnibus under accession number GSE128553. The raw FASTQ files can also be downloaded from the ENA (European Nucleotide Archive, https://www.ebi.ac.uk/ena/browser/view/PRJNA528089) website using the following command (before downloading the datasets, setting a working directory named *scRNAseq_ovary*):

```
mkdir ~/scRNAseq_ovary
cd ~/scRNAseq_ovary
wget ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR875/
008/SRR8753678/SRR8753678_1.fastq.gz
wget ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR875/
008/SRR8753678/SRR8753678_2.fastq.gz
wget ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR875/
009/SRR8753679/SRR8753679_1.fastq.gz
wget ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR875/
009/SRR8753679/SRR8753679_2.fastq.gz
wget ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR875/
000/SRR8753680/SRR8753680_1.fastq.gz
wget ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR875/
000/SRR8753680/SRR8753680_2.fastq.gz
wget ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR875/
001/SRR8753681/SRR8753681_1.fastq.gz
wget ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR875/
001/SRR8753681/SRR8753681_2.fastq.gz
```

Besides, the *CellRanger* processed output files have been uploaded to the Figshare server, and it can be used for downstream visualization directly; we can download them using the following link: https://figshare.com/articles/dataset/scRNA_seq_of_developing_ovary/21614778.

3 Methods

Currently, Seurat and Scanpy are the most widely used analytics tools. Although Seurat is compiled based on R and Scanpy is compiled using Python, their main workflow remains similar, including quality control (QC), normalization, batch effect correction, dimensionality reduction, unsupervised clustering, and differential expression analysis. Noteworthy, in a real scenario, it is common to combine these robust tools to elevate the accuracy of analysis and this provides transformation functions to elevate the interoperability among different pipelines. In this chapter, we will focus on how to process 10× Genomic-based scRNA-seq data and demonstrate basic workflows to explore early germ cell development.

3.1 Overview of the scRNA-seq Pipeline

We first process the raw FASTQ files using the standard CellRanger pipeline (https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/using/tutorials). This step will generate a single pipeline output directory containing a series of output files. In the outs directory, the *web_summary.html* file contains summary metrics and automated secondary analysis results, which can be used to evaluate the quality of sequencing files generated. Particularly, in the *web_summary.html* of abnormal data, alerts (including WARNING and ERROR) will be printed (*see* **Note 2**). While for the downstream Seurat pipeline, the input files include *barcodes.tsv.gz*, *features.tsv.gz*, and *matrix.mtx.gz* in the *outs/filtered_feature_bc_matrix/* directory, and these files can be directly read into Seurat using the *Read10X* function (these files can also be obtained at FigShare using the following link: https://figshare.com/articles/dataset/scRNA_seq_of_developing_ovary/21614778). Then, we can transfer these files to a local PC and follow the standard Seurat pipeline to explore the scRNA-seq data (Fig. 2).

3.2 Generation of Gene Expression Matrices Using CellRanger

First, we should rename the sample names according to the 10× platform naming convention (e.g., *Sample_S1_L00X_R1_001.fastq.gz*) using the following command:

```
cd ~/scRNAseq_ovary
mv ./SRR8753678_1.fastq.gz ./E11.5_S1_L001_R1_001.fastq.gz
mv ./SRR8753678_2.fastq.gz ./E11.5_S1_L001_R2_001.fastq.gz
```

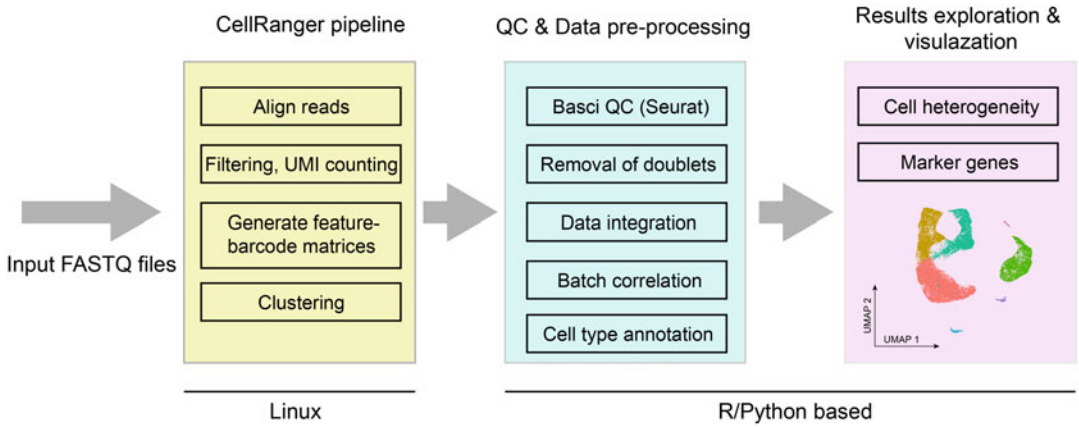

Fig. 2 An overview of scRNA-seq workflow

```
mv ./SRR8753679_1.fastq.gz ./E12.5_S1_L001_R1_001.fastq.gz
mv ./SRR8753679_2.fastq.gz ./E12.5_S1_L001_R2_001.fastq.gz
mv ./SRR8753680_1.fastq.gz ./E13.5_S1_L001_R1_001.fastq.gz
mv ./SRR8753680_2.fastq.gz ./E13.5_S1_L001_R2_001.fastq.gz
mv ./SRR8753681_1.fastq.gz ./E14.5_S1_L001_R1_001.fastq.gz
mv ./SRR8753681_2.fastq.gz ./E14.5_S1_L001_R2_001.fastq.gz
```

Then, we perform the CellRanger pipeline using the following command:

```
--cellranger count --id=E11.5 \
--localcores 40 \
--transcriptome=/home/XXX/XXX/ref_genome/refdata-
gex-mm10-2020-A \
--fastqs=/home/XXX/XXX/scRNAseq_ovary \
--sample=E11.5 \
--chemistry=threeprime

--cellranger count --id=E12.5 \
--localcores 40 \
--transcriptome=/home/XXX/XXX/ref_genome/refdata-
gex-mm10-2020-A \
--fastqs=/home/XXX/XXX/scRNAseq_ovary \
--sample=E12.5 \
--chemistry=threeprime

--cellranger count --id=E13.5 \
--localcores 40 \
--transcriptome=/home/XXX/XXX/ref_genome/refdata-
gex-mm10-2020-A \
--fastqs=/home/XXX/XXX/scRNAseq_ovary \
--sample=E13.5 \
--chemistry=threeprime
```

```
--cellranger count --id=E14.5 \
--localcores 40 \
--transcriptome=/home/XXX/XXX/ref_genome/refdata-gex-mm10-2020-A \
--fastqs=/home/XXX/XXX/scRNAseq_ovary \
--sample=E14.5 \
--chemistry=threeprime
```

CellRanger output gene expression matrices (in the *outs/filtered_feature_bc_matrix/* directory) can then be easily imported into Seurat for downstream visualization.

3.3 Constructing Seurat Object from CellRanger Output Files and Basic QC

The Seurat package is one of the most widely used tools for analyzing scRNA-seq data and includes a resourceful tool for the preparation of publication-ready plots for the non-bioinformatics scientist. Besides, the Seurat object is compatible with other popular scRNA-seq analyzing tools, and we can transform the Seurat object into other objects, such as *SinlgeCellExperiment* and *anndata* object, using the predefined transformation function (for details see: https://satijalab.org/seurat/archive/v2.4/conversion_vignette.html).

To import scRNA-seq data-based 10× platform, Seurat provides *Read10X* function to load gene expression matrices from the CellRanger output files. We can then generate a sparse matrix, with rows indicating genes and columns indicating cells.

If we would like to perform downstream visualization on a PC, we need to download all files in the "outs/filtered_feature_bc_matrix" into your target directory and rename the *filtered_feature_bc_matrix* directory with their group information (e.g., E11.4, E12.5, E13.5, and E14.5).

First, we need to load the essential packages before visualization.

```
### load required packages
library(Seurat)
library(dplyr)
library(cowplot)
library(DoubletFinder)
library(ggplot2)
library(harmony)
```

Next, use the *Read10X* function to import the gene expression matrix.

```
### read inputdata
E11.5.data<-Read10X(data.dir="E:/XXX/XXX/cellranger_output/E11.5/filtered_feature_bc_matrix")
E12.5.data<-Read10X(data.dir="E:/XXX/XXX/cellran-
```

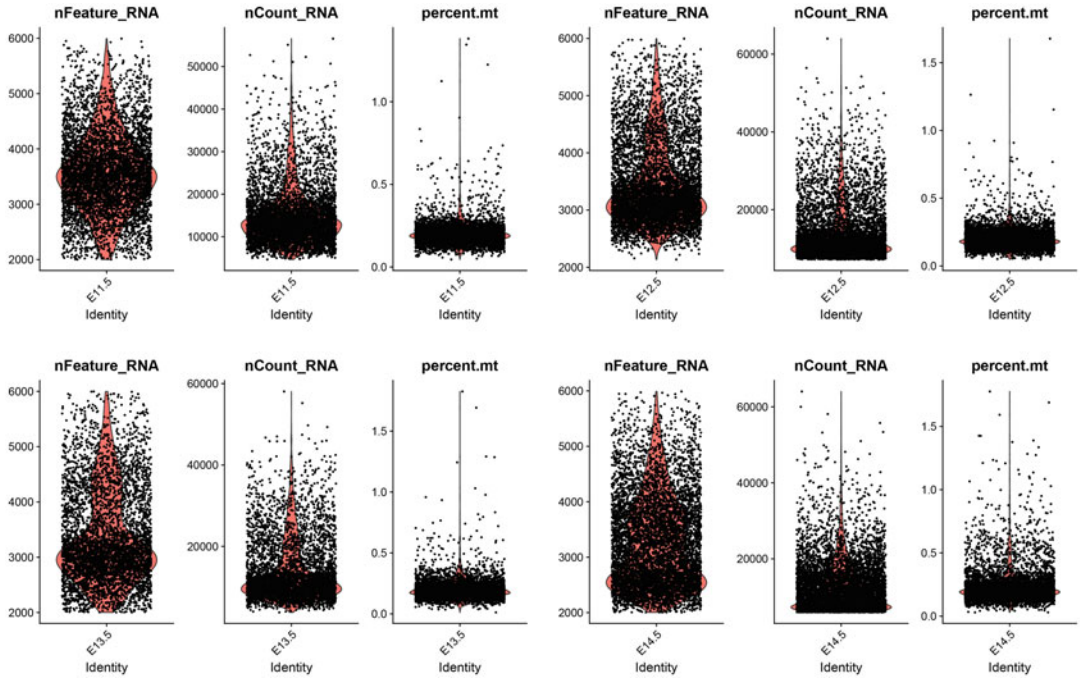

Fig. 3 Basic QC matrices of E11.5, E12.5, E13.5, and E14.5 scRNA-seq datasets. *nFeature_RNA* indicates the number of genes detected per cell, *nCount_RNA* indicates the number of UMIs per cell, and *percent.mt* indicates the percentage of mitochondrial genes

```
ger_output/E12.5/filtered_feature_bc_matrix")
E13.5.data<-Read10X(data.dir="E:/XXX/XXX/cellran-
ger_output/E13.5/filtered_feature_bc_matrix")
E14.5.data<-Read10X(data.dir="E:/XXX/XXX/cellran-
ger_output/E14.5/filtered_feature_bc_matrix")
### add cell annotation
colnames(x = E11.5.data) <- paste('E11.5', colnames
(x = E11.5.data), sep = '_')
colnames(x = E12.5.data) <- paste('E12.5', colnames
(x = E12.5.data), sep = '_')
colnames(x = E13.5.data) <- paste('E13.5', colnames
(x = E13.5.data), sep = '_')
colnames(x = E14.5.data) <- paste('E14.5', colnames
(x = E14.5.data), sep = '_')
```

After importing the gene expression matrix, the next step is to construct the Seurat object through the *CreateSeuratObject* function using the following command (*see* **Note 3**):

```
## Construct Seurat object
E11.5 <- CreateSeuratObject(counts = E11.5.data,
project = "E11.5", min.cells = 3, min.features = 200)
```

```
E12.5 <- CreateSeuratObject(counts = E12.5.data,
project = "E12.5", min.cells = 3, min.features = 200)
E13.5 <- CreateSeuratObject(counts = E13.5.data,
project = "E13.5", min.cells = 3, min.features = 200)
E14.5 <- CreateSeuratObject(counts = E14.5.data,
project = "E14.5", min.cells = 3, min.features = 200)
```

Generally, severe mitochondrial contamination is one of the most common characteristics of low-quality or dying cells, it is of significance to calculate the percentage of mitochondrial genes [12]. In the standard Seurat workflow, mitochondrial QC metrics can be easily obtained using the *PercentageFeatureSet* function, which calculates the percentage of counts originating from a set of features (Fig. 3). Besides, low-quality cells or empty droplets will often have very few genes, and cell doublets or multiplets may exhibit an aberrantly high gene count; it is therefore essential to remove these undesired cells using the *subset* function. Here, according to the QC metrics of each sample (Fig. 3), we filter cells that have unique feature counts over 6000 or less than 200, and cells that have >10% mitochondrial counts.

```
E11.5[["percent.mt"]]<-PercentageFeatureSet
(E11.5,pattern = "^Mt")
VlnPlot(E11.5, features = c("nFeature_RNA",
"nCount_RNA","percent.mt"), ncol = 3, pt.size = 0.5)
E11.5 <- subset(E11.5, subset = nFeature_RNA > 2000 &
nFeature_RNA < 6000 & percent.mt < 10 & nCount_RNA >200)

E12.5[["percent.mt"]]<-PercentageFeatureSet
(E12.5,pattern = "^Mt")
VlnPlot(E12.5, features = c("nFeature_RNA",
"nCount_RNA","percent.mt"), ncol = 3, pt.size = 0.5)
E12.5 <- subset(E12.5, subset = nFeature_RNA > 2000 &
nFeature_RNA < 6000 & percent.mt < 10 & nCount_RNA >200)

E13.5[["percent.mt"]]<-PercentageFeatureSet
(E13.5,pattern = "^Mt")
VlnPlot(E13.5, features = c("nFeature_RNA",
"nCount_RNA","percent.mt"), ncol = 3, pt.size = 0.5)
E13.5 <- subset(E13.5, subset = nFeature_RNA > 2000 &
nFeature_RNA < 6000 & percent.mt < 10 & nCount_RNA >200)

E14.5[["percent.mt"]]<-PercentageFeatureSet
(E14.5,pattern = "^Mt")
VlnPlot(E14.5, features = c("nFeature_RNA",
"nCount_RNA","percent.mt"), ncol = 3, pt.size = 0.5)
E14.5 <- subset(E14.5, subset = nFeature_RNA > 2000 &
nFeature_RNA < 6000 & percent.mt < 10 & nCount_RNA >200)
```

Next, we can proceed with the normalization, feature selection, and scaling steps for each dataset using the following command:

```
E11.5 <- NormalizeData(E11.5, normalization.method
= "LogNormalize", scale.factor = 10000)
E11.5 <- FindVariableFeatures(E11.5, selection.
method = "vst", nfeatures = 2000)
E11.5$group<-"E11.5"
all.genes <- rownames(E11.5)
E11.5 <- ScaleData(E11.5, features = all.genes)
E11.5@meta.data$time <- "E11.5"

E12.5 <- NormalizeData(E12.5, normalization.method
= "LogNormalize", scale.factor = 10000)
E12.5 <- FindVariableFeatures(E12.5, selection.
method = "vst", nfeatures = 2000)
E12.5$group<-"E12.5"
all.genes <- rownames(E12.5)
E12.5 <- ScaleData(E12.5, features = all.genes)
E12.5@meta.data$time <- "E12.5"

E13.5 <- NormalizeData(E13.5, normalization.method
= "LogNormalize", scale.factor = 10000)
E13.5 <- FindVariableFeatures(E13.5, selection.
method = "vst", nfeatures = 2000)
E13.5$group<-"E13.5"
all.genes <- rownames(E13.5)
E13.5 <- ScaleData(E13.5, features = all.genes)
E13.5@meta.data$time <- "E13.5"

E14.5 <- NormalizeData(E14.5, normalization.method
= "LogNormalize", scale.factor = 10000)
E14.5 <- FindVariableFeatures(E14.5, selection.
method = "vst", nfeatures = 2000)
E14.5$group<-"E14.5"
all.genes <- rownames(E14.5)
E14.5 <- ScaleData(E14.5, features = all.genes)
E14.5@meta.data$time <- "E14.5"
```

3.4 Removing Doublets Using DoubleFinder

Seurat filters doublets or multiplets simply using the QC matrices, and assuming that doublets have aberrantly high gene count, but if two low-quality cells are encapsulated into the same droplet, they may show "normal" QC matrices [13]. To further remove potential doublets, we will use the *DoubletFinder* package (https://github.com/chris-mcginnis-ucsf/DoubletFinder) in this chapter to further remove remaining low-quality cells. Different from the Seurat QC strategy, DoubletFinder predicts doublets in scRNA-seq

data by creating artificial doublets from input scRNA-seq data, and it can be easily adapted to the Seurat analysis pipeline. Next, we will use this package to remove potential doublets in our four datasets independently.

```
E11.5<-RunPCA(E11.5)
E11.5<-RunUMAP(E11.5,dims = 1:10)
sweep.res.list_E11.5 <- paramSweep_v3(E11.5, PCs =
1:20, sct = FALSE)
sweep.stats_E11.5 <- summarizeSweep(sweep.res.
list_E11.5, GT = FALSE)
bcmvn_E11.5 <- find.pK(sweep.stats_E11.5)
E11.5<-FindNeighbors(E11.5,reduction ="pca",dims =
1:20)
E11.5<-FindClusters(E11.5,resolution = 0.5)
annotationscon_E11.5<-E11.5@meta.data$seur-
at_clusters
homotypic.prop <- modelHomotypic(annotation-
scon_E11.5)
nExp_poi <- round(0.09*(length(E11.5@active.
ident)))
nExp_poi.adj <- round(nExp_poi*(1-homotypic.prop))
seu_con_E11.5 <- doubletFinder_v3(E11.5, PCs = 1:
10, pN = 0.25, pK = 0.09, nExp = nExp_poi, reuse.
pANN = FALSE, sct = FALSE)
seu_con_E11.5@meta.data$cellfilter <- seu_co-
n_E11.5@meta.data$DF.classifica-
tions_0.25_0.09_501

E12.5<-RunPCA(E12.5)
E12.5<-RunUMAP(E12.5,dims = 1:10)
sweep.res.list_E12.5 <- paramSweep_v3(E12.5, PCs =
1:20, sct = FALSE)
sweep.stats_E12.5 <- summarizeSweep(sweep.res.
list_E12.5, GT = FALSE)
bcmvn_E12.5 <- find.pK(sweep.stats_E12.5)
E12.5<-FindNeighbors(E12.5,reduction ="pca",dims =
1:20)
E12.5<-FindClusters(E12.5,resolution = 0.5)
annotationscon_E12.5<-E12.5@meta.data$seur-
at_clusters
homotypic.prop <- modelHomotypic(annotation-
scon_E12.5)
nExp_poi <- round(0.09*(length(E12.5@active.
ident)))
nExp_poi.adj <- round(nExp_poi*(1-homotypic.prop))
seu_con_E12.5 <- doubletFinder_v3(E12.5, PCs = 1:
10, pN = 0.25, pK = 0.09, nExp = nExp_poi, reuse.
```

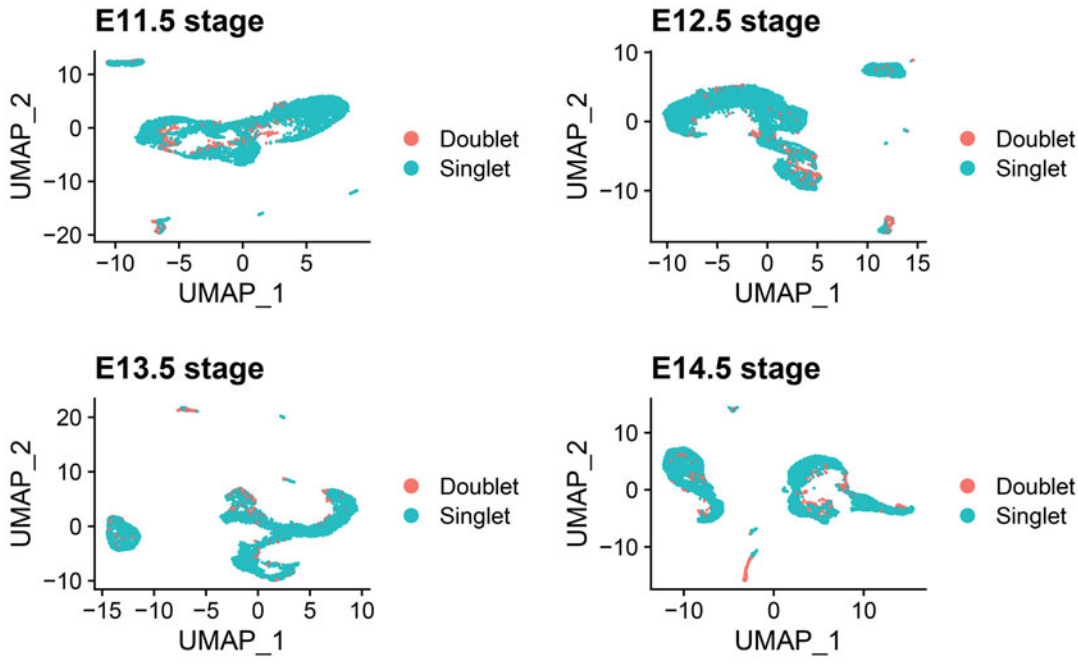

Fig. 4 UMAP displays the singlet and doublet identified by *DoubletFinder* in each dataset. Singlet indicates high-quality cells used for downstream visualization, while doublet indicates droplets with two cells, which are known to confound downstream visualization

```
pANN = FALSE, sct = FALSE)
seu_con_E12.5@meta.data$cellfilter <- seu_co-
n_E12.5@meta.data$DF.classifica-
tions_0.25_0.09_612

E13.5<-RunPCA(E13.5)
E13.5<-RunUMAP(E13.5,dims = 1:10)
sweep.res.list_E13.5 <- paramSweep_v3(E13.5, PCs =
1:20, sct = FALSE)
sweep.stats_E13.5 <- summarizeSweep(sweep.res.
list_E13.5, GT = FALSE)
bcmvn_E13.5 <- find.pK(sweep.stats_E13.5)
E13.5<-FindNeighbors(E13.5,reduction ="pca",dims =
1:20)
E13.5<-FindClusters(E13.5,resolution = 0.5)
annotationscon_E13.5<-E13.5@meta.data$seur-
at_clusters
homotypic.prop <- modelHomotypic(annotation-
scon_E13.5)
nExp_poi <- round(0.09*(length(E13.5@active.
ident)))
nExp_poi.adj <- round(nExp_poi*(1-homotypic.prop))
seu_con_E13.5 <- doubletFinder_v3(E13.5, PCs = 1:
```

```
10, pN = 0.25, pK = 0.09, nExp = nExp_poi, reuse.
pANN = FALSE, sct = FALSE)
seu_con_E13.5@meta.data$cellfilter <- seu_co-
n_E13.5@meta.data$DF.classifica-
tions_0.25_0.09_436

E14.5<-RunPCA(E14.5)
E14.5<-RunUMAP(E14.5,dims = 1:10)
sweep.res.list_E14.5 <- paramSweep_v3(E14.5, PCs =
1:20, sct = FALSE)
sweep.stats_E14.5 <- summarizeSweep(sweep.res.
list_E14.5, GT = FALSE)
bcmvn_E14.5 <- find.pK(sweep.stats_E14.5)
E14.5<-FindNeighbors(E14.5,reduction ="pca",dims =
1:20)
E14.5<-FindClusters(E14.5,resolution = 0.5)
annotationscon_E14.5<-E14.5@meta.data$seur-
at_clusters
homotypic.prop <- modelHomotypic(annotation-
scon_E14.5)
nExp_poi <- round(0.09*(length(E14.5@active.
ident)))
nExp_poi.adj <- round(nExp_poi*(1-homotypic.prop))
seu_con_E14.5 <- doubletFinder_v3(E14.5, PCs = 1:
10, pN = 0.25, pK = 0.09, nExp = nExp_poi, reuse.
pANN = FALSE, sct = FALSE)
seu_con_E14.5@meta.data$cellfilter <- seu_co-
n_E14.5@meta.data$ DF.classifica-
tions_0.25_0.09_590
```

Next, we can evaluate how many cells are potential doublets, and this information has been stored in the *seu@meta.data$cellfilter* slot (Fig. 4). We can filter out these cells identified by *DoubletFinder* using the following command:

```
options(repr.plot.height = 5, repr.plot.width = 8)
p1<-DimPlot(object = seu_con_E11.5, reduction =
"umap", pt.size = .1,
 group.by = "cellfilter") + ggtitle('E11.5 stage')
p2<-DimPlot(object = seu_con_E12.5, reduction =
"umap", pt.size = .1,
 group.by = "cellfilter") + ggtitle('E12.5 stage')
p3<-DimPlot(object = seu_con_E13.5, reduction =
"umap", pt.size = .1,
 group.by = "cellfilter") + ggtitle('E13.5 stage')
p4<-DimPlot(object = seu_con_E14.5, reduction =
"umap", pt.size = .1,
 group.by = "cellfilter") + ggtitle('E14.5 stage')
```

```
plot_grid(p1,p2,p3,p4)

E11.5.singlet <- subset(seu_con_E11.5, subset =
cellfilter == 'Singlet')
E12.5.singlet <- subset(seu_con_E12.5, subset =
cellfilter == 'Singlet')
E13.5.singlet <- subset(seu_con_E13.5, subset =
cellfilter == 'Singlet')
E14.5.singlet <- subset(seu_con_E14.5, subset =
cellfilter == 'Singlet')
```

3.5 Data Integration, Removal of the Batch Effect, and Nonlinear Dimensional Reduction Using UMAP

The batch effect is a common issue when analyzing scRNA-seq data generated from multiple time points or groups [14]. Due to the high dimensional, noisy, and sparse nature of scRNA-seq data, improper data integration can confound biological variations of interest and are therefore of significance to remove batch effect before differential analysis. To address these challenges, robust tools have been developed to perform batch correlation of multiple scRNA-seq data. Among these tools developed, such as *Harmony* [15], *Seurat* [16], *fastMNN* [17], and *BBKNN* [18], a recent benchmarking study demonstrated that the *Harmony* algorithm outperforms other batch correlation tools [19]; here, we will use *Harmony* to remove batch effect in our scRNA-seq datasets.

```
seurat_list <- list(E11.5.singlet,E12.5.singlet,
E13.5.singlet,E14.5.singlet)
seurat_obj <- merge(x=seurat_list[[1]], y=seurat_-
list[2:length(seurat_list)])
seurat_obj <- NormalizeData(seurat_obj, normaliza-
tion.method = "LogNormalize", scale.factor = 10000)
seurat_obj <- FindVariableFeatures(seurat_obj, se-
lection.method = "vst", nfeatures = 2000) ## 2000
features in default
all.genes <- rownames(seurat_obj)
seurat_obj <- ScaleData(seurat_obj, features = all.
genes)
seurat_obj <- RunPCA(seurat_obj)
```

First, we can visualize the batch effect before batch correction using the following command:

```
options(repr.plot.height = 5, repr.plot.width = 12)
p1 <- DimPlot(object = seurat_obj, reduction =
"pca", pt.size = .1, group.by = "group")
p2 <- VlnPlot(object = seurat_obj, features =
"PC_1", group.by = "group", pt.size = .1)
plot_grid(p1,p2)
```

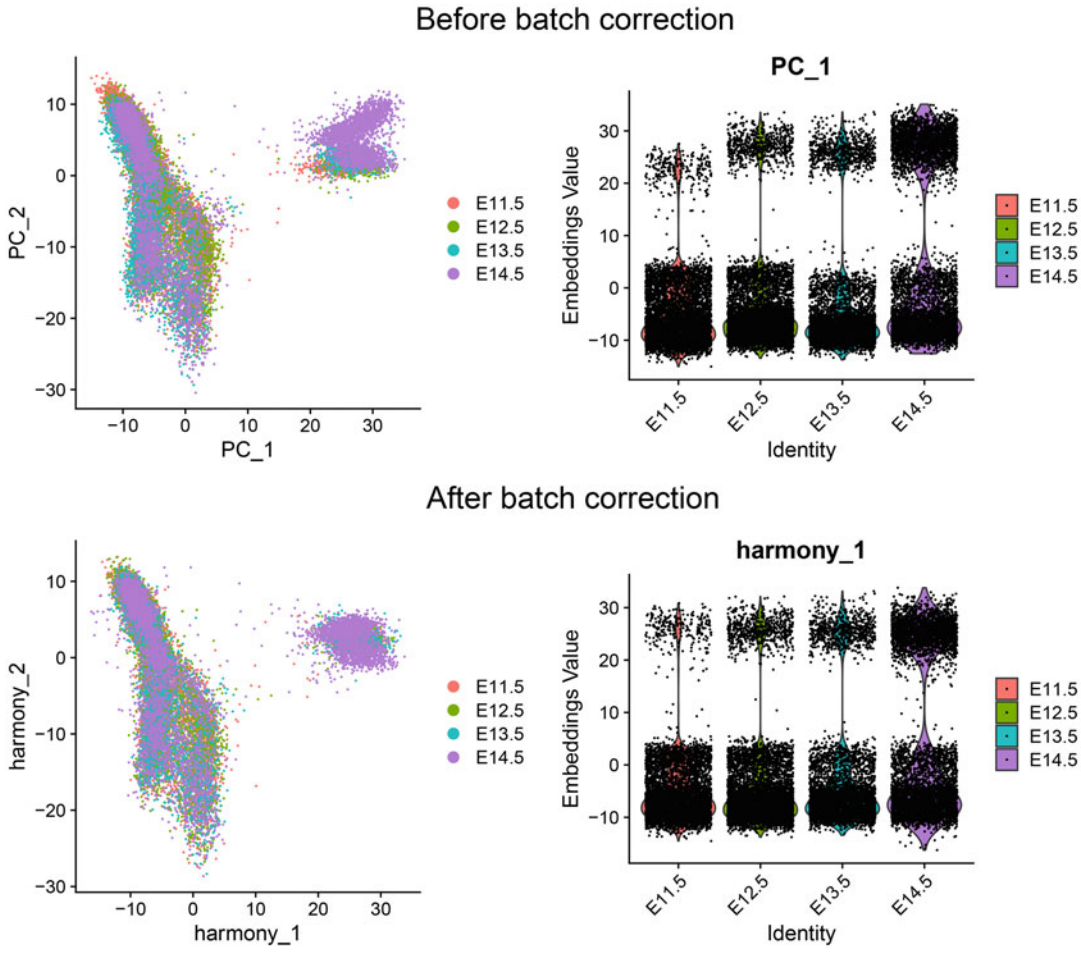

Fig. 5 PCA plot of all cells and violin plot displaying embedding values. The top panel shows results from the uncorrected dataset, and we can observe an obvious batch effect in the integrated Seurat object. The bottom panel shows harmony corrected dataset and we can find that harmony removes the dataset-specific variation

Then, we can remove the batch effect in our four groups using the *RunHarmony* function (Fig. 5).

```
seurat_obj <- seurat_obj %>% RunHarmony("group",
plot_convergence = TRUE)
harmony_embeddings <- Embeddings(seurat_obj, 'har-
mony')
options(repr.plot.height = 5, repr.plot.width = 12)
p1 <- DimPlot(object = seurat_obj, reduction =
"harmony", pt.size = .1, group.by = "group")
p2 <- VlnPlot(object = seurat_obj, features = "har-
mony_1", group.by = "group", pt.size = .1)
plot_grid(p1,p2)
```

Next, we can perform nonlinear dimensional reduction using UMAP (uniform manifold approximation and projection) to explore cellular heterogeneity in the integrated dataset.

```
seurat_obj <- RunUMAP(seurat_obj, reduction='harmony', dims = 1:10)
seurat_obj <- FindNeighbors(seurat_obj, reduction='harmony')
seurat_obj <- FindClusters(seurat_obj, resolution = 0.4)
options(repr.plot.height = 5, repr.plot.width = 12)
p1<-DimPlot(seurat_obj, group.by='seurat_clusters', reduction='umap', label = T) + ggtitle('seurat clusters')
p2<-DimPlot(seurat_obj, group.by='time', reduction='umap') + ggtitle('Sequencing Batch')
plot_grid(p1,p2)
```

3.6 Cell-Type Characterization

After clustering using UMAP, we successfully identified several cell populations in the developing ovary. However, what are these cells remains to be explored. Seurat can help user to find cell-cluster-specific expressed genes by comparing the particular cluster to the remaining cells, and these marker genes can be used for cell type characterization (Fig. 6). Here, we defined different cell types using the following markers (Table 1) [9]:

```
FeaturePlot(seurat_obj, features = c("Dazl", "Stra8", "Wnt4", "Wnt6", "Krt19", "Upk3b", "Nr2f2", "Col1a1","Kdr", "Pecam1", "Alad", "Alas2", "Cd52", "Fcer1g"))
seurat_obj <- RenameIdents(seurat_obj, '0' = "Pregranulosa", '1' = "Pregranulosa", '2' = "Epithelial", '3' = "Germ", '4' = "Mesenchymal", '5' = "Mesenchymal", '6' = "Epithelial", '7' = "Germ", '8' = "Pregranulosa", '9' = "Endothelial", '10' = "Erythriod", '11' = "Immune")
DimPlot(seurat_obj, reduction='umap', label = T) + ggtitle('Cell types')
```

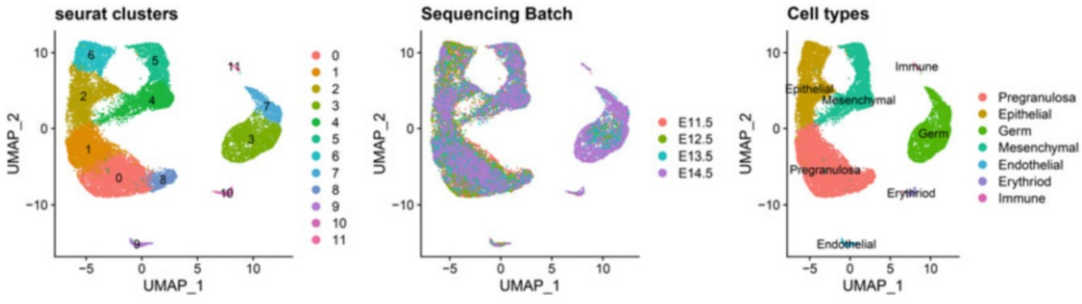

Fig. 6 UMAP plot showing the identified cell clusters, sequencing batch, and different cell types

Table 1
Markers used for cell type characterization

Cell type	Markers	Cluster ID
Germ	*Dazl*, *Stra8*	3,7
Pregranulosa	*Wnt4*, *Wnt6*	0,1,8
Epithelial	*Krt19*, *Upk3b*	2,6
Mesenchymal	*Nr2f2*, *Col1a1*	4,5
Endothelial	*Kdr*, *Pecam1*	9
Erythriod	*Alad*, *Alas2*	10
Immune	*Cd52*, *Fcer1g*	11

We can then use the *FindAllMarkers* function to identify cell type-specific markers, and the top 10 markers can be visualized using the *DoHeatmap* function using the following command:

```
cell.markers <- FindAllMarkers(seurat_obj, only.
pos = TRUE, min.pct = 0.25, logfc.threshold = 0.25)
top10 <- cell.markers %>% group_by(cluster) %>%
top_n(n = 10, wt = avg_logFC)
DoHeatmap(seurat_obj, features = top10$gene) + No-
Legend()
```

3.7 Subsetting Germ Cell Populations to Identify Fine-Scale Germ Cell Meiotic Transcriptome

To provide in-depth insight into the meiotic transcriptome, we can further subset the germ cell populations in the aforementioned Seurat object and rerun the standard Seurat pipeline to dissect germ cell heterogeneity.

```
germsub <- subset(seurat_obj, idents = c("Germ"))
germsub <- FindNeighbors(germsub, dims = 1:10)
germsub <- FindClusters(germsub, resolution = 0.6)
germsub <- RunUMAP(germsub, dims = 1:5)
options(repr.plot.height = 5, repr.plot.width = 12)
p1<-DimPlot(germsub, reduction = 'umap',label = T)
p2<-DimPlot(germsub, reduction = 'umap',group.by =
"time")
plot_grid(p1,p2)
```

In the UMAP plot, we can find that the overall distribution is linear from left to right, and projection of their developmental time in the UMAP plot also showed that germ cells at different time points showed continuous distribution on time scales, thus

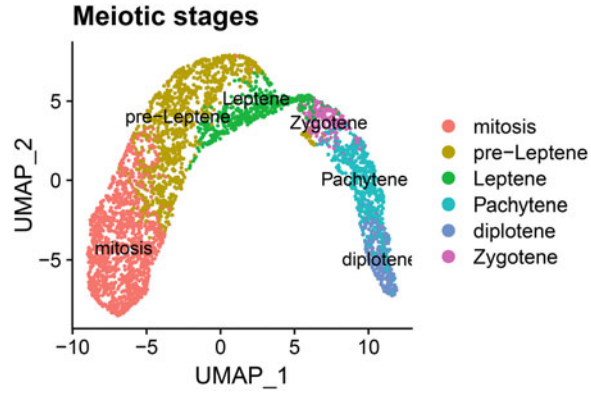

Fig. 7 Fine-scale visualization of meiotic cell clusters in the UMAP plot

indicating the UMAP projection successfully recapitulated meiotic program. Similarly, we next explore the meiotic stages of these cell clusters according to known stage-specific markers (Fig. 7) [10].

```
features <- c("Utf1","Pou5f1","Stra8","Rec8","Sp-
dya","Spo11","Dmc1","Sycp2","Ybx2")
germsub@active.ident <- factor(germsub@active.
ident, levels=c("0","2","5","1","3","7","4","6"))
DotPlot(germsub, features = features,cols = c
("#F5F5F5", "#4B0082")) + RotatedAxis()
```

Using the following command, we can annotate the germ cells at different stages, and we can further explore meiotic stage-specific markers using the *FindAllMarkers* function.

```
germsub <- RenameIdents(germsub, '0' = "mitosis",
'1' = "pre-Leptene", '2' = "mitosis", '3' = "Lep-
tene", '4' = "Pachytene", '5' = "pre-Leptene", '6' =
"diplotene", '7' = "Zygotene")
DimPlot(germsub, reduction='umap', label = T) +
ggtitle('Meiotic stages')
germ.markers <- FindAllMarkers(germsub, only.pos =
TRUE, min.pct = 0.25, logfc.threshold = 0.25)
top10 <- germ.markers %>% group_by(cluster) %>%
top_n(n = 10, wt = avg_logFC)
DoHeatmap(germsub, features = top10$gene) + NoLe-
gend()
```

4 Notes

1. *Installing R*. Here, we assume that you are working with a Windows system, and for other operating systems, download the corresponding version of R and install it according to the official guideline. It should be noted that the installation path should not contain special characters or non-English characters, otherwise problems may occur during the installation.
2. *Overview of the scRNA-seq pipeline*. WARNING indicates that some parameters may be optimized, but, generally, we can proceed with the downstream analysis. Including low fraction of reads confidently mapped to the transcriptome, and the fraction of cell barcode bases with Q-score, while ERROR indicates that the generated data is wrong, and special attention should be paid to the parameters or original data. These include no cells detected, an extremely low fraction of valid unique molecular identifiers (UMIs), and so on.
3. *Constructing Seurat object from CellRanger output files and basic QC*. The *min.cells* parameter sets the threshold for genes to only take the genes that are present in at least a specified number of cells, while *min.features* parameter sets the thresholds for cells that express at least a specified number of genes. These values are data specific and can be modified according to your research question.

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Chapter 16

Mapping Meiotic DNA Breaks: Two Fully-Automated Pipelines to Analyze Single-Strand DNA Sequencing Data, hotSSDS and hotSSDS-extra

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Abstract

Molecular approaches are required to detect DNA double-strand break (DSB) events and to map and quantify them at high resolution. One of the most popular molecular methods in the field of meiotic recombination is the ChIP-SSDS (Chromatin immuno-precipitation and single-strand DNA sequencing). Here, we present two fully-automated Nextflow-based pipelines to analyze the sequencing data generated by this method. The first one identifies highly reproducible DSB sites, while the second provides a characterization of recovered DSB sites, including the description of the hotspot distribution and intensity along the genome and the overlap with specific regions such as gene features or known DSB hotspots. Finally, we discuss limitations/advantages and key points to consider when applying this method to specific genotypes or unconventional species.

Key words Double-strand break mapping, ChIP single-strand DNA sequencing, Computational pipelines, Meiotic hotspot

1 Introduction

DNA breaks can occur spontaneously in a cell nucleus (i.e., TopoII breaks) from exogenous sources (e.g., X-rays, oxidation, restriction enzyme) or by developmentally regulated programs (e.g., VDJ recombination, replication-fork stalling, meiosis). All of them potentially threaten the integrity of the genome and therefore must be tightly regulated and repaired by coordinated cellular processes.

Understanding the regulatory mechanisms that cause or result in break formation involves detecting and mapping these events at high resolution (down to the base pair level) and quantifying them. Since the first published work that has mapped meiotic DSBs using

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RAD51 ChIP-seq [1], several molecular strategies have been developed to capture broken DNA, to generate libraries and perform their sequencing: BLISS [2, 3], i-BLESS [4], END-seq [5, 6], S1-seq [7], Spo11-oligo seq [8]. Chromatin immunoprecipitation followed by single-strand DNA sequencing (ChIP-SSDS) is another methodology developed for the sensitive mapping of meiotic hotspots [9]. It was originally developed for mapping DMC1-bound single-strand DNA fragments [9, 10], but has been successfully applied to map the binding sites of other proteins (RPA and RAD51 [11]) that are bound to ssDNA. Briefly, this method relies on a combination of both a specific ChIP library preparation procedure and a computational framework that allows the enrichment for and detection of ssDNA-bound fragments.

Here, we describe two fully-automated computational pipelines: the first detects reliable and reproducible binding regions of the targeted protein (i.e., meiotic DSB hotspots) and is derived from previous pipelines developed by K. Brick (*see* Subheading 2); the second provides a descriptive analysis of hotspot distribution and intensity across the genome and at selected regions (e.g., control hotspots, promoters). We designed both pipelines to be applied to any species with an available and annotated genome. They are both freely available on github, interoperable, and ensure the reproducibility of analysis. Here, we explain step by step how to use and configure the pipelines and how to control the quality of the results.

2 Materials

The hotSSDS pipeline is based on two previously published pipelines (SSDSnextflowPipeline [12] and callSSDSpeaks [13]) both developed by Kevin Brick. The method was described in the initial paper [9] and then in the technical paper [10].

We merged these two initial pipelines into a single one, and added some key features such as IDR statistical procedure for assessing peak robustness, FRiP statistics (Fraction of Reads in Peaks) computation, and the possibility of using other references than the *Mus musculus* genome. We also greatly improved the portability of the pipeline by integrating many of the Nextflow language options [14, 15]. Indeed, Nextflow is a powerful and scalable workflow language called DSL (domain specific language), which makes it easier to deploy complex parallel and reactive workflows on computing clusters. In fact, it offers built-in compatibility with reproducibility tools, such as software containers and a variety of computing environment managers.

We also developed a second pipeline called hotSSDS-extra. It produces general statistics and a descriptive analysis of identified

hotspots. It provides useful plots such as heatmaps and annotations from the signal data obtained after successfully running the hotSSDS pipeline.

We developed hotSSDS and hotSSDS-extra pipelines using DSL1, which is the first version of Nextflow DSL (*see* **Notes 1** and **2**).

2.1 Computing Resources

The hotSSDS and hotSSDS-extra pipelines are optimized to run on a high-performance computing (HPC) cluster. HPC clusters are usually managed by a scheduling and resource management software. Nextflow offers compatibility with a whole range of softwares, including PBS Pro, Slurm, PBS/Torque [16]. Nextflow pipelines can also be run on cloud computing solutions. A configuration file suited for the system used should also be provided (*see* Subheading 3.1.3).

2.2 Software Requirements

The hotSSDS and hotSSDS-extra pipelines require Nextflow [15] version 20.10.0, which can be easily installed using Conda package manager [17]. Pipelines also require at least one of the following programs: Conda [18], Mamba [19], Docker [20], or Singularity [21] (version 3.4.1 recommended). To download the pipelines, it is preferable to use git [22], otherwise the pipelines can be downloaded using command-line programs for retrieving files from the Internet such as wget [23].

The pipelines rely on the pre-existence of genome references on the system that is used. If the genome is not available and needs to be downloaded (*see* Subheading 3.1.3), then bwa and samtools programs will be required as well.

2.3 Data

The hotSSDS pipeline will process only paired-end data, in fastq(.gz) format, obtained from Illumina sequencing technology. Sequencing data should have been generated from libraries prepared following the specific protocol provided earlier [10]. The experimental design can include input control samples or not, and one or more biological replicates per sample.

The hotSSDS-extra pipeline will process output files from the hotSSDS pipeline, such as peaks/hotspots in bed format, mapped fragments in bam format along with their indexes (bai format), and profiles in bigwig format. An external hotspot set can also be included as a comparison reference.

All examples provided in this chapter have been obtained from the analysis of previously published mouse data [24] (https://www.ebi.ac.uk/ena/browser/view/PRJEB43730). Briefly, the dataset was obtained for a DMC1-SSDS experiment carried out in four samples consisting of two replicates of a mutant mouse genotype (*Top6bl*$^{\Delta 17Ct/\Delta 17Ct}$) and two replicates of the corresponding control (i.e. wild-type) mouse genotype.

3 Methods

3.1 hotSSDS Pipeline

In a nutshell, the main steps of the hotSSDS pipeline include preprocessing of raw reads, mapping of trimmed reads to the reference genome, ssDNA-derived fragment detection in mapped reads based on inverted terminal repeats (ITR) and microhomology lengths, peak-calling and normalization, optional IDR analysis (irreproducible discovery rate), and various quality checks (Fig. 1). A more detailed description of the workflow totaling 26 steps can be found in Table 1.

3.1.1 Download the Pipeline

Using git (recommended):

```
git clone https://github.com/jajclement/hotSSDS.
git
cd hotSSDS
```

Or download zip file using wget, then unzip file:

```
wget
https://github.com/jajclement/hotSSDS/archive/
refs/heads/master.zip
unzip hotSSDS-master.zip
mv hotSSDS-master hotSSDS
cd hotSSDS
```

3.1.2 Download Singularity Images

Singularity images are used to encapsulate all required softwares and dependencies for the different steps of the pipeline. They make the pipeline portable on different systems. As they can be voluminous, they are not included in the pipeline git repository. Prior to run the pipeline using Singularity execution profile, it is necessary to download the images from Zenodo "hotSSDS and hotSSDS-extra Pipelines Singularity Images" open repository (https://zenodo.org/record/7783473). To do this, two options are available depending on whether the computing environment on which the pipeline is executed has access to the Internet (Subheading 3.1.2, **step 1**) or not (Subheading 3.1.2, **step 2**).

– **Run the pipeline using the option --get_sif**

The option --get_sif allows to launch a "dry run" that will check the existence of Singularity images in the pipeline directory. If not present, the pipeline will download them. Once download is completed, the pipeline stops. It can then be run again without the option --get_sif to perform SSDS analyses (*see* Subheadings 3.1.6 and 3.1.7). In the following command-line, depending on which environment manager is present on the system, select either singularity, mamba, conda, or docker in the -profile parameter (*see* Subheading 2.2):

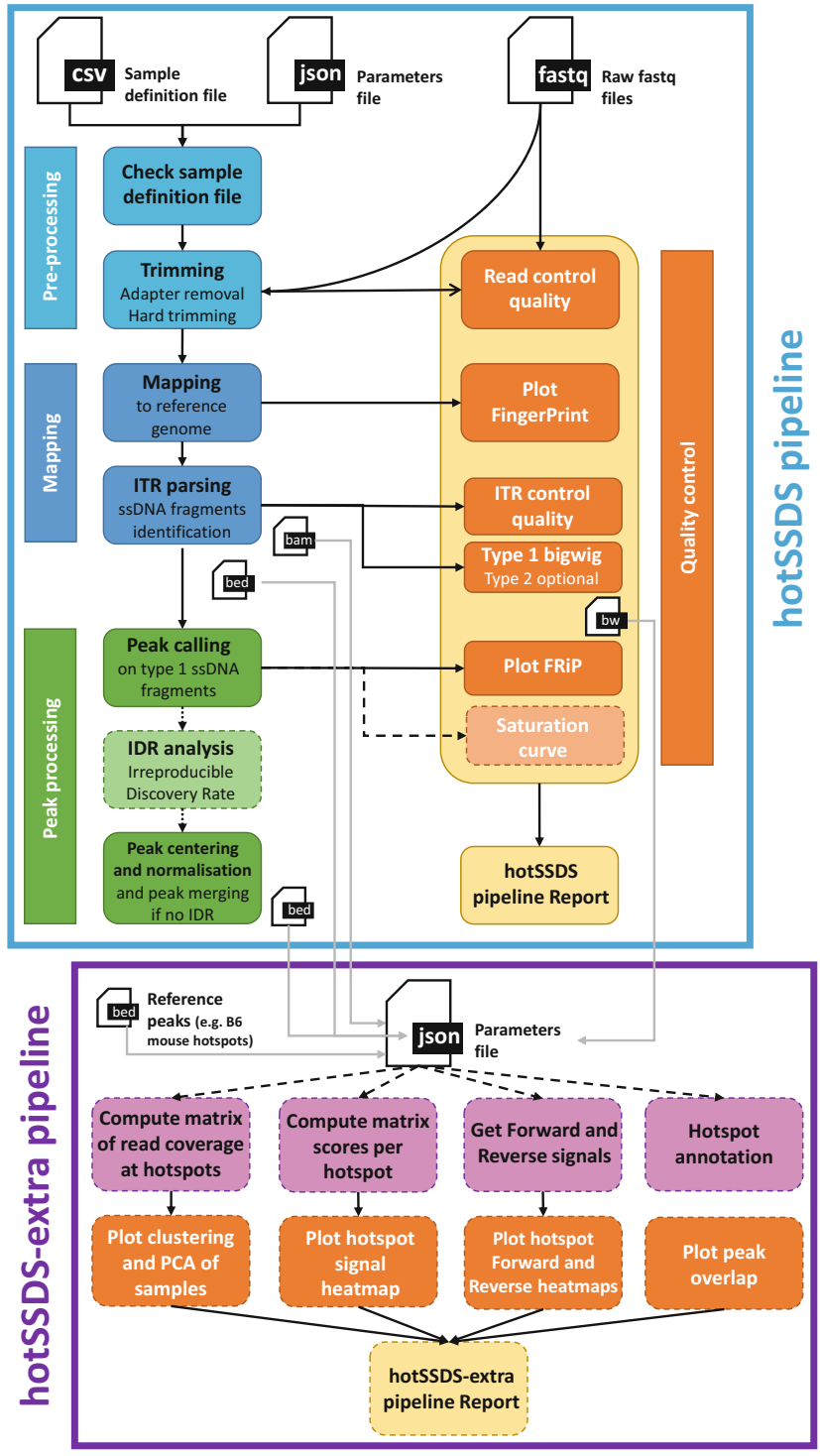

Fig. 1 hotSSDS and hotSSDS-extra analysis workflow. The blue box contains the schematic view of the hotSSDS pipeline and the purple box represents the hotSSDS-extra pipeline. Dotted lines represent optional steps. Input and output file types are mentioned in black. Gray arrows link the input files requested by hotSSDS-extra, most of which are generated by the hotSSDS pipeline

Table 1
Summary and description of the hotSSDS pipeline steps

	Step	Description	Output file(s)
Preprocessing	Input files checking	Check the integrity and format of the csv input file (user provided) Prepare configuration file for fastq-screen according to user parameters	.csv reformatted files .txt fastq-screen config file
	Raw reads trimming and qc	Hard-trimming of raw reads to 50 bp Adapters trimming (two options available for trimming tool) Screen user-defined contaminants in raw reads Quality check of raw and trimmed reads	.fastq.gz trimmed reads .html report
Mapping	Mapping to reference genome	Three-step mapping of trimmed R1 and R2 reads to reference genome: 1. aligns R1 reads (fully complementary to the genome) with bwa aln 2. aligns R2 reads (potentially contain fill-in ITR part at the end of the 5′) with bwa-ra aln (custom version of bwa that search for the longest mappable suffix in the query) 3. merge with bwa sampe Sort and index resulting bam files Optional: remove multimapper reads	.bam files
	Bam files filtering	Bam files filtering for duplicates, unmapped, and unpaired reads	Filtered .bam files
	Parsing of filtered bam	Parsing of filtered bam files into five main categories: **type 1 reads (T1)**: high confidence single-stranded DNA (ITR > 5 bp AND microHomology >0 bp AND fill-in >2 bp) **type 2 reads (T2)**: low confidence single-stranded DNA (ITR > 5 bp AND microHomology >0 bp AND fill-in <3 bp) **dsDNA**: low confidence double-stranded DNA (ITR < 3 bp) **dsDNA strict**: higher confidence double-stranded DNA (ITR < 1 bp AND microHomology <1 bp) **unclassified**: everything that remains unclassified	.bam files .bed files
Bigwigs	Bigwig files generation	Generation of normalized bigwig files from T1 and T2 fragments, for visualization through a genome browser. Options available: normalized bigwig files for merged replicates T1 and T1 + T2 bed files generate coverage and cumulative coverage plots generate forward/reverse only bigwig files	.bw files .bedgraph files

(continued)

Table 1
(continued)

	Step	Description	Output file(s)
Peak processing	Peak-calling	Peak-calling from shuffled T1 bed files, using input control samples or not Optional: peak filtering based on user-provided blacklist	.bed files .narrowPeak files .xls files
	IDR analysis (optional)	IDR (irreproducible discovery rate) analysis to assess replicate consistency (only available for $n = 2$ replicates) following ENCODE procedure, *see* https://github.com/ENCODE-DCC/chip-seq-pipeline2 (https://www.researchgate.net/publication/51946926_Measuring_Reproducibility_of_High-Throughput_Experiments)	.bed files of IDR passing peak
	Peak centering and normalization	Peak centering using Kevin Brick's method: 1. recenters the peaks by the median of the F/R distances 2. calculate in-peak background 3. output recentered peaks with strength Strength is normalized by the number of wrong-direction fragments (i.e., REV to left, FWD to right) and expressed as RPKM, using the total normalized in-hotspot tag count as a denominator	.bedgraph and .tab files of normalized peaks
	FRIP and fingerprint	Generation of fingerprint plots, i.e., profile of cumulative read coverages (quality control to assess ChIP signal) for all samples Optional: Generation of FRIP plots (Fraction of Reads In Peaks) as an indication of the signal-to-noise ratio	.png files
	Saturation curve (optional)	Samples saturation curve generation, computed from peak-calling in progressively down-sampled samples. The saturation curve plots the number of called peaks as a function of the number of reads in the samples	.png file
Report	Pipeline report	Quality control report generation from a range of statistics such as read quality, mapping rates, ssDNA-derived fragments proportion and distribution	.html file

```
nextflow run main.nf -c conf/cluster.config
-params-file conf/test.json -profile test,<singu-
larity|mamba|conda|docker> --get_sif >& get_sif_-
main_log.txt 2>&1
>& redirects stdout to file get_sif_main_log.txt
2>&1 redirects stderr to stdout and thus to file get_sif_-
main_log.txt
```

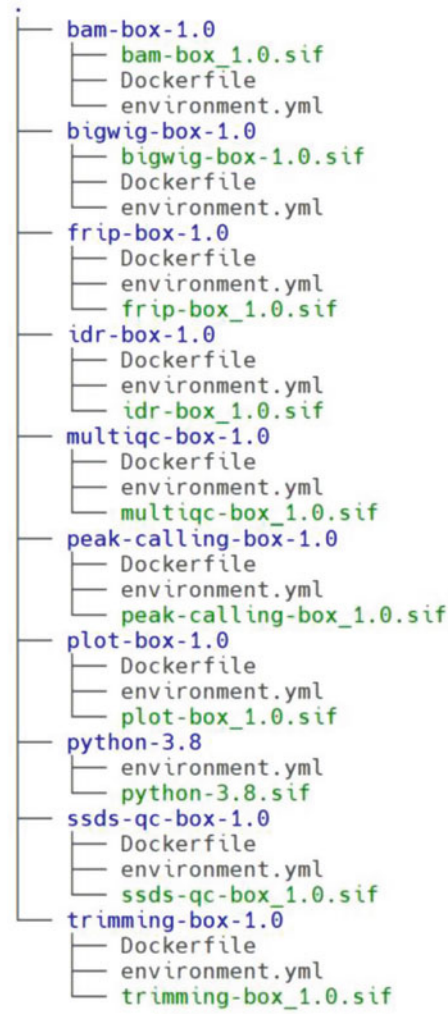

Fig. 2 Final containers repository file structure for the hotSSDS pipeline

– **Download all singularity images independently**

Download all the 10 .sif files from Zenodo open repository at https://zenodo.org/record/7783473 and place them in hotSSDS/containers folder so that the final container repository structure is consistent with the one shown in Fig. 2.

3.1.3 Configure Computing Parameter Files

Edit hotSSDS/conf/cluster.config file to adjust the parameters to a computing cluster. The following sections are expected to be overwritten:

1. "DEFAULT CLUSTER CONFIGURATION" section

"Cluster description" subsection (optional): fill-in cluster description, mail, and url; "Executor (cluster scheduler) parameters" subsection (mandatory): in "name" field, specify "slurm"

or "pbspro" for instance, depending on the system scheduling program; "Computing queues parameters" (mandatory): in "queue" field, specify the computing queues name(s) on which jobs will be submitted; "Max resources" (mandatory): adapt max_memory, max_cpus, and max_time fields if needed.

2. "PROFILES SPECIFIC PARAMETERS" section

   Adapt custom launching commands for Conda, Mamba, Singularity, and Docker programs.

3. "GENOMES LOCATION" section

   Write the absolute paths to reference genome(s):

   genome_fasta: absolute path to genome fasta file. If needed, download the fasta file of the reference genome (you can use the golden path https://hgdownload.soe.ucsc.edu/downloads.html), the best way to get all the required data for a genome if it is published on UCSC; genomedir: absolute path to directory containing genome fasta file AND associated bwa index files (to create them, use bwa-index following command: bwa index ref.fa); genome_name: genome name; fai: absolute path to genome fai index (to create with faidx tool: http://www.htslib.org/doc/samtools-faidx.html: `samtools faidx <ref.fasta> -o <ref.fai>`)

   If needed, edit hotSSDS/conf/resources.config to adjust resources of process. To do so, edit cpus/memory/time in "PROCESSES SPECIFIC RESSOURCES REQUIREMENTS" section. Specific computing queues can also be added to some categories.

   It is important to note that many institutes have one such configuration file referenced in nf-core/configs repository (https://github.com/nf-core/configs) that can be downloaded and adapt to hotSSDS pipeline.

3.1.4 Prepare Input File

The hotSSDS pipeline takes as input a csv formatted file (comma separated values) containing raw fastq file paths and minimal metadata about the samples: antibody used, replicate, and control identification. The csv file must contain the following six fields:

group: name of the sample group. Replicate samples must have the same group name.

replicate: replicate number, e.g., 1, 2, 3... If no replicate, write 1.

fastq_1: absolute path to R1 fastq(.gz) file

fastq_2: absolute path to R2 fastq(.gz) file

antibody: antibody used; leave empty if none. Replicate samples must have the same antibody.

control: group name of the corresponding control samples, if applicable.

If the samples do not have an associated input control sample, leave the last fields empty.

Control (input) samples must have the two last fields ("antibody" and "control") empty.

There must be no empty line at the end of the csv file.

Example

```
group,replicate,fastq_1,fastq_2,antibody,control
DMC1-chip-WT,1,/path/to/SRR1035576_R1.fastq.gz,/
path/to/SRR1035576_R2.fastq.gz,antiDMC1,Input-WT
DMC1-chip-WT,2,/path/to/SRR1035577_R1.fastq.gz,/
path/to/SRR1035577_R2.fastq.gz,antiDMC1,Input-WT
DMC1-chip-KO,1,/path/to/SRR1035578_R1.fastq.gz,/
path/to/SRR1035578_R2.fastq.gz,antiDMC1,Input-KO
DMC1-chip-KO,2,/path/to/SRR1035579_R1.fastq.gz,/
path/to/SRR1035579_R2.fastq.gz,antiDMC1,Input-KO
Input-WT,1,/path/to/SRR1035580_R1.fastq.gz,/path/
to/SRR1035580_R2.fastq.gz,,
Input-KO,1,/path/to/SRR1035581_R1.fastq.gz,/path/
to/SRR1035581_R2.fastq.gz,,
```

This input file format is based on nf-core chipseq pipeline (https://nf-co.re/chipseq).

3.1.5 Edit/Create Parameter File

Pipeline parameters can be set in two different ways: via parameter file or directly in the Nextflow command-line. Parameters passed through the command-line will overwrite those in the parameter file.

For *Mus musculus*-based analysis, the parameter file hotSSDS/conf/mm10.json contains default parameters that can be overwritten and changed, for example, when analyzing data from other species (*see* **Note 7**). You can list all parameters using the following command:

```
nextflow run main.nf --help
```

A detailed description of the available parameters can be found in Table 2.

3.1.6 Launch the Pipeline

Once you have set computing config file (*see* Subheading 3.1.3), input file (*see* Subheading 3.1.4), and customized parameter file (*see* Subheading 3.1.5), you can launch the pipeline using the following command-line (*see* **Note 3**):

Table 2
List of available parameters in the hotSSDS pipeline

	Parameter name	Type	Default value	Description
Input data	Inputcsv	FILE	NULL	Path to input csv file (*see* Subheading 3.1.4)
	params_file	FILE	hotSSDS/conf/mm10.json	Path to parameters json file
	Genomebase	DIRECTORY	NULL	Path to genome base folder
	Genomedir	DIRECTORY	NULL	Path to reference genome directory
	genome_name	STRING	mm10	Reference genome name
	genome_fasta	FILE	NULL	Path to genome fasta file (bwa index files must exist in the same folder)
	Fai	FILE	NULL	Path to genome .fai index file
	genome2screen	STRING	["mm10", "hg38"]	List of genomes to screen for contamination screening using fastq-screen (format: comma separated list of genomes to screen for contamination, names must correspond to existing genomes in your cluster config file)
	Chrsize	FILE	hotSSDS/data/mm10/mm10.chrom.sizes	Chromosome sizes file for reference genome (Default file is for mm10 reference genome, downloaded from https://hgdownload.soe.ucsc.edu/goldenPath/mm10/bigZips/mm10.chrom.sizes 2021-01-11)
	hotspots	DIRECTORY	hotSSDS/data/hotspots/mm10/hotspots	Path to reference hotspots files directory (set to "none" if none provided)
	Blacklist	FILE	hotSSDS/data/blacklist/mm10/blackList.bed	Path to blacklist bed file for peak-calling and idr (set to "none" if none provided)
General output parameters	Name	STRING	hotSSDS_pipeline	Analysis run name
	Outdir	DIRECTORY	hotSSDS/name.outdir/02_results	Path to output directory
	publishdir_mode	STRING	"copy"	Mode for exporting each process output files to output directory (must be "symlink," "relink," "link," "copy," "copynofollow," "move," *see* https://www.nextflow.io/docs/latest/process.html)

(continued)

Table 2
(continued)

	Parameter name	Type	Default value	Description
Trimming parameters	with_trimgalore	BOOLEAN	False	Use trim-galore instead of trimmomatic for quality trimming process
	trimgalore_adapters	FILE	None	trim-galore: path to adapters file
	trimg_quality	INTEGER	10	trim-galore: minimum quality threshold (phred score)
	trimg_stringency	INTEGER	6	trim-galore: trimming stringency, i.e., overlap with adapter sequence required to trim a sequence
	trim_minlen	INTEGER	25	trimmomatic: minimum length of reads after trimming
	trim_cropR1	INTEGER	50	fastx: cut r1 reads to that specified length
	trim_cropR2	INTEGER	50	fastx: cut r2 reads to that specified length
	trim_slidingwin	STRING	"4:15"	trimmomatic: perform a sliding window trimming, cutting once the average quality within the window falls below a threshold
	trim_illumina_clip	STRING	"2:20:10"	trimmomatic: cut adapter and other illumina-specific sequences from the read
	trimmomatic_adapters	FILE	hotSSDS/data/TruSeq2-PE.fa	Path to adapters file for trimmomatic (special formatting, *see* http://www.usadellab.org/cms/?page=trimmomatic)
Mapping parameters	with_multimap	BOOLEAN	False	If true, keep multimapping reads in mapped bam files
	bamPGline	STRING	"@PG\tID:ssDNAPipeline2.0_PAUFFRET"	Custom bam file header
	filtering_flag	INTEGER	2052	sam flag for filtering bam files (*see* https://broadinstitute.github.io/picard/explain-flags.html)
	picard_min_distance	INTEGER	−1	picard parameter for marking duplicates (−-minimum_distance): width of the window to search for duplicates of a given alignment, default: −1 (twice the first read's read length)
	picard_optdup_distance	INTEGER	100	picard parameter for marking duplicates (−-optical_duplicate_pixel_distance): the maximum offset between two duplicate clusters in order to consider them optical duplicates. The default is appropriate for unpatterned versions of the illumina platform (hiseq2500). For the patterned flowcell models (novaseq 6000), 2500 is more appropriate
	get_supp	BOOLEAN	False	If true, publish bam files of supplementary alignments

Bigwig parameters	bigwig_profile	STRING	T1	Bigwig profile using bedtools (normalization by total library size): "T1" will produce bigwig for Type 1 bed files only, one per replicates; "T12" will also produce bigwig for merged Type 1 + Type 2, one per replicate; "T1rep" will also produce Type 1 bigwig for merged replicates; "T12rep" will also produce Type 1 + Type 2 bigwig for merged replicates
	kbrick_bigwig	BOOLEAN	False	If true, compute bigwig files; fr bigwig files and coverage plots as in original pipeline by Kevin Brick using deeptools with fpkm normalization
	binsize	INTEGER	50	Deeptools binsize parameter (used only if kbrick_bigwig is true)
Peak-calling parameters	with_control	BOOLEAN	False	If true, use input control files for peak-calling analysis
	satcurve	BOOLEAN	False	If true, plot saturation curve
	sctype	STRING	Standard	For saturation curve generation: saturation curve type (either "minimal," "standard," or "expanded," reflecting the number of downsamples created from a given sample to compute saturation curve)
	reps	INTEGER	3	For saturation curve generation: number of times peak-calling will be computed on a given sample
	bed_trimqual	INTEGER	30	Mapping quality threshold for bed filtering
	macs_bw	INTEGER	1000	macs2 callpeak bandwidth parameter (i.e., the bandwidth that is used to scan the genome only for model building; can be set to the expected sonication fragment size)
	macs_slocal	INTEGER	5000	macs2 callpeak slocal parameter (i.e., small local regions size)
	macs_extsize	INTEGER	800	macs2 callpeak extsize parameter (i.e., while --nomodel is set, macs uses this parameter to extend reads in 5′- > 3′ direction to fix-sized fragments)
	macs_qv	FLOAT	0.1	macs2 callpeak *q*-value parameter (the *q*-value (minimum fdr) cutoff to call significant regions. Macs default is 0.05. *q*-values are calculated from *p*-values using the Benjamini–Hochberg procedure)
	macs_pv	FLOAT	−1	macs2 callpeak *p*-value parameter (i.e., the *p*-value cutoff), if not −1, will overrule macs_qv
	no_chrY	BOOLEAN	True	If true, filter out chromosome Y peaks from final peak bed files (i.e., filter out peaks located in "chrY" named chromosome)

(continued)

Table 2
(continued)

	Parameter name	Type	Default value	Description
IDR parameters	with_idr	BOOLEAN	False	If true, perform idr analysis, only possible if nb_replicates = 2
	nb_replicates	INTEGER	2	Number of replicates per sample
	idr_peaktype	STRING	"regionPeak"	Peak file format for idr (narrowpeak, regionpeak, or broadpeak)
	idr_setup	STRING	auto	Threshold profile for idr. This will define the thresholds for true replicates, pool replicates, self-replicates r1, and self-replicates r2. Profile "auto" is based on encode guidelines and profile "custom" allows to set custom thresholds (*see* parameters --idr_threshold_r1, —-idr_threshold_r2, —-idr_threshold_truerep, and --idr_threshold_poolrep)
	idr_threshold_r1	FLOAT	0.05	idr threshold for self-replicates r1 (used if --idr_setup is "custom" only)
	idr_threshold_r2	FLOAT	0.05	idr threshold for self-replicates r2 (used if --idr_setup is "custom" only)
	idr_threshold_truerep	FLOAT	0.05	idr threshold for true replicates (used if --idr_setup is "custom" only)
	idr_threshold_poolrep	FLOAT	0.01	idr threshold for pooled replicates (used if --idr_setup is "custom" only)
	idr_rank	STRING	p.value	Statistical value used for IDR ranking, possible values are "p.value" for p-value and "q.value" for q-value
	idr_filtering_pattern	STRING	"chr[1-9X]+"	regex for filtering bed files (default: "chr[1-9X]+" for mouse; set ".*" to keep everything)
	idr_macs_qv	FLOAT	−1	macs2 callpeak *q*-value parameter
	idr_macs_pv	FLOAT	0.1	macs2 callpeak *p*-value parameter, if not −1, will overrule macs_qv
QC parameters	with_ssds_multiqc	BOOLEAN	True	If true, run ssds multiqc to compute ssds-specific quality control
	multiqc_configfile	FILE	hotSSDS/conf/multiqc_config.yaml	Path to multiqc custom config file

Nextflow tower parameter	with-tower	BOOLEAN	False	If true, enable job monitoring with Nextflow tower (https://tower.nf/); if true, you must generate an access token on tower website then export tower_access_token environment variable in your computing environment. Requires internet access
Pipeline dependencies	src	DIRECTORY	hotSSDS/bin	Path to source and binaries directory
	custom_bwa	EXECUTABLE	hotSSDS/bin/bwa_0.7.12	Path to custom bwa executable
	custom_bwa_ra	EXECUTABLE	hotSSDS/bin/bwa_ra_0.7.12	Path to custom bwa_sra executable
Singularity parameters	sif_url	URL	https://zenodo.org/record/7783473/files	URL to Zenodo open repository hosting pipeline singularity images to download
	get_sif	BOOLEAN	False	If true, check the existence of singularity images in containers/ directory and download them if needed (requires internet access)

```
nextflow run main.nf -c conf/cluster.config
-params-file conf/mm10.json --inputcsv /path/to/
input.csv -profile <singularity|mamba|conda|dock-
er> --name "My_workflow_name" >& main_log.txt 2>&1
```

Select either singularity, mamba, conda, or docker in the -profile parameter (*see* Subheading 2.2) depending on which environment manager is present on the system.

It is highly recommended to launch the command in a batch job on the computing cluster, as its execution will take time and computing resources. It is also recommended to redirect the output of this main Nextflow command-line to an identified log file, which will be useful to monitor the pipeline execution.

3.1.7 Run a Short Test

A small dataset can be used to test if the pipeline is correctly running on your system. To do so, run:

```
nextflow run main.nf -c conf/cluster.config
-params-file conf/test.json -profile test,<singu-
larity|mamba|conda|docker> >& test_main_log.txt
2>&1
```

This test run should approximately take 5 min to complete.

On completion, the end of main log test_main_log.txt should look like:

```
executor >  pbspro (25)
[aa/ff63f4] process > check_design (input.csv)
     [100%] 1 of 1 ✔
[1f/c81a5f] process > makeScreenConfigFile (TEST_SSDS)
     [100%] 1 of 1 ✔
[f1/341fad] process > crop (TEST_IP_R1_T1)
     [100%] 1 of 1 ✔
[fe/67d328] process > trimming (TEST_IP_R1_T1)
     [100%] 1 of 1 ✔
[cb/a77283] process > bwaAlign (TEST_IP_R1_T1)
     [100%] 1 of 1 ✔
[38/e2e8c6] process > filterBam (TEST_IP_R1_T1)
     [100%] 1 of 1 ✔
[af/05397c] process > parseITRs (TEST_IP_R1_T1)
     [100%] 1 of 1 ✔
```

```
[d1/b74fbc] process > makeBigwig (TEST_IP_R1_T1)
     [100%] 1 of 1 ✔
[1f/256f21] process > shufBEDs (TEST_IP_R1)
     [100%] 1 of 1 ✔
[3c/f2b936] process > callPeaks (TEST_IP_R1)
     [100%] 5 of 5 ✔
[43/655990] process > samStats (TEST_IP_R1_T1)
     [100%] 5 of 5 ✔
[18/200309] process > makeSSreport (TEST_IP_R1_T1)
          [100%] 1 of 1 ✔
[8f/19ef51] process > makeFingerPrint (TEST_SSDS)
     [100%] 1 of 1 ✔
[c8/a77ede] process > ssds_multiqc (TEST_IP_R1_T1)
          [100%] 1 of 1 ✔
[85/6daa72] process > normalizePeaks (TEST_IP_R1)
     [100%] 1 of 1 ✔
[6a/122837] process > makeSatCurve (TEST_SSDS)
     [100%] 1 of 1 ✔
[a7/a00c9c] process > general_multiqc (TEST_SSDS)
     [100%] 1 of 1 ✔
Completed at: 10-Mar-2023 13:37:48
Duration    : 3m 57s
CPU hours   : 0.3
Succeeded   : 25
```

3.1.8 *Monitor the Pipeline*

To monitor the pipeline execution after it is launched as described in Subheading 3.1.6, the main log file can be checked (main_log.txt in the example command-line in Subheading 3.1.6) using the following command: `tail -f main_log.txt`. This file should look like:

```
executor >  pbspro (1)
[21/3786f7]  process  >  check_design  (Nore_input_fi...
[100%] 1 of 1 ✔
[57/bc36de]  process  >  makeScreenConfigFile  (SSDS_...
[100%] 1 of 1 ✔
[d3/c42056] process > crop (WT_R2_T1)             [100%]
4 of 4 ✔
[7d/c06fe8] process > trimming (WT_R2_T1)         [100%]
4 of 4 ✔
[ca/74adfd] process > bwaAlign (WT_R2_T1)         [100%]
4 of 4 ✔
[f0/dec2f6]    process    >    filterBam    (WT_R2_T1)
[100%] 4 of 4 ✔
[aa/39f5f4]    process    >    parseITRs    (WT_R2_T1)
[100%] 4 of 4 ✔
[79/c5fd62]    process    >    makeBigwig    (WT_R2_T1)
[100%] 4 of 4 ✔
[22/a02566]  process  >  makeDeeptoolsBigWig  (WT_R2_T1)
[100%] 20 of 20 ✔
[1b/218fa3]    process    >    toFRBigWig    (WT_R2_T1)
[100%] 20 of 20 ✔
[24/35b6ac] process > shufBEDs (WT_R1)            [100%]
4 of 4 ✔
[78/1c307b] process > callPeaks (MUT_R1)          [100%]
9 of 9 ✔
[18/b8edd6] process > samStats (WT_R2_T1)         [100%]
20 of 20 ✔
[e4/4461d1]   process    >    makeSSreport    (WT_R2_T1)
[100%] 4 of 4  ✔
[4b/e769b4]  process  >  makeFingerPrint  (SSDS_pipel...
[100%] 1 of 1  ✔
[58/02be5a]   process    >    ssds_multiqc    (WT_R2_T1)
[100%] 4 of 4  ✔
[5b/be1b53] process > createPseudoReplicates (MUT)    [
50%] 1 of 2
[37/0acb1c]    process    >    callPeaksForIDR    (WT)
[100%] 1 of 1 ✔
[-        ] process > IDRanalysis                   -
[-        ] process > IDRpostprocess                -
[-        ] process > normalizePeaks_idr            -
[-        ] process > makeSatCurve                  -
[-        ] process > general_multiqc               -
```

This main log file will give many information: it indicates the course of processes and how many of them are finished, running, programmed, or crashed. In the example above, one process is currently running (*createPseudoReplicates (MUT)*), 110 processes are finished, and five steps are yet to run (*IDRanalysis*, *IDRpostprocess*, *normalizePeaks_idr*, *makeSatCurve*, and *general_multiqc*). For each process, Nextflow creates a dedicated working directory in the main pipeline working directory (named *work/* by default). The hash key preceding the process name in the main log file corresponds to the process working directory name. For example, for process *check_design*, the associated key is **21/3786f7**, meaning that in the Nextflow *work/* directory, there will be a subfolder named 21, itself containing a log subfolder whose name begins with 3786f7. In this log subfolder, all detailed log files for process *check_design* can be found, along with input/output and temporary files. In every such log subfolder, all Nextflow log files listed in Table 3 can be checked (*see* **Note 4**).

There is also a .nexflow.log file created each time the pipeline is run (the latest is named .nexflow.log, the second latest is renamed .nexflow.log.2, and so on). This log file can give insights of Nextflow—scheduler communication during the pipeline run, such as jobs ID, run time, and so on. This file is located in the folder from where the pipeline is launched.

Usually, the main causes of pipeline crashes are:

- wrong parameters selection: check hotSSDS/conf/mm10.json file;
- input files not found: check --inputcsv parameter;
- time or memory resources exceeded (exit code is usually 143 in this case): adjust hotSSDS/conf/resources.config file;
- Conda conflicts (we recommend using -profile conda only as a last resort).

Table 3
List of log files created in each process working directory during pipeline execution

File name	Content
.command.out	Output of the process
.command.err	Error returned by the process, if any
.command.log	Log file of the process
.command.sh	Bash script executed for the process
.command.run	Nextflow script executed for the process
.command.trace	Resources used by the process
.exitcode	Exit code of the process (0 if process succeeded)
Process specific log (optional, *.log file)	Specific log file of the process

Nextflow also provides tools to manage pipeline through an online interface (*see* **Note 5**).

On success, the pipeline main log will output a completion message, such as:

```
Completed at: 29-Mar-2023 21:07:38
Duration : 56m 47s
CPU hours : 498.5 (99.4% cached)
Succeeded : 8
Cached : 123
```

3.1.9 Inspection of Results

On completion, three main folders are generated in the main output directory (specified with outdir parameter in command-line):

- **00_reports**: contains report files created by Nextflow each time the pipeline is run (*see* Table 4);
- **01_logs**: contains log files for all processes;
- **02_results**: contains results files, including:
  (a) **trimming** with trimmed fastq files;
  (b) **bwa** with mapped reads, filtered mapped reads and parsed fragments in bam and bed formats;
  (c) **bigwig** with bigwig and bedgraph files;
  (d) **peaks** with raw, filtered, centered peaks in bed format and with saturation curve and IDR files if options are selected;
  (e) **qc** with quality controls files and reports for sequencing, mapping, peak-calling, and parsing steps;
  (f) **idr** with peak set files for IDR statistical testing;

work: is the working directory for Nextflow. More details about output files can be found in Table 5.

Table 4
List of Nextflow report files generated during pipeline execution

Result folder name	File name	Description
outdir/00_reports	dag.png	png file containing direct acyclic graph (DAG) representation of the pipeline processes chain
	report.html	HTML pipeline execution report that includes metrics about resources usage and pipeline summary
	timeline.html	HTML report including pipeline execution timeline
	trace.txt	Text file containing detailed resources usages, working directory hash name (*see* Subheading 3.1.8), job ID, execution time, status, etc. for each process

Table 5
List of output files generated by the hotSSDS pipeline

Result subfolder names in 02_results		Description		
trimming	**trim_fastq**	Trimmed and cropped fastq files		
bwa	**bam**	Raw mapped files (bam format), indexes (bai format), and mapping stats files (flagstat)		
	filterbam/flag_XX	**Bam**	Filtered mapped files with samtools flag XX (*see* parameters, Table 2) in bam format with indexes (bai format)	
		Bed	Filtered mapped files with samtools flag XX (*see* filtering_flag parameter, Table 2) in bed format	
		Parseitr	**Type1**	Bam and bed files for ssDNA type 1 fragments
			Type2	Bam and bed files for ssDNA type 2 fragments
			dsDNA	Bam and bed files for dsDNA fragments
			Unclassified	Bam and bed files for unclassified fragments
			Flagstat	Mapping stats files
			Norm_factors	Normalization factors (library sizes) for each sample
bigwig	**kbrick_bigwig (optional)**	**deeptools/binsizeX**	Bigwig files (RPKM normalization) for ssDNA-derived fragments and dsDNA fragments	
			plot	Fingerprint and coverage plots computed by deeptools
			tab	Fingerprint and coverage stat tables computed by deeptools
		FRbigwig	Forward only/Reverse only bigwig files for ssDNA-derived fragments and dsDNA fragments	
	T1/T12	Bigwig and Bedgraph files for ssDNA type 1 fragments and optionally for type 1 fragments, normalization by total library size		
peaks	**with[out]-input**	**bed_shuffle**	Shuffled type 1 bed files before peak-calling	
		macs2	Raw peaks called by macs2 (bed, xls, and narrowPeak format)	
		saturation_curve (optional)	Saturation curve peak files and plots	
		Normalized	Normalized and recentered peaks (bedgraph files) using Replicate1 signal, and Replicate2 signal, and Replicate1 + Replicate2 concatenation if two replicates are provided	
		Finalpeaks	Copy of final set of peaks (filtered by IDR analysis or after replicate merging if IDR is not selected)	

(continued)

Table 5
(continued)

Result subfolder names in 02_results		Description		
idr (optional)	**narrowPeak_macs2pv*_macs2qv*1_idr_setup-***	**with[out]-input**	**Bfilt**	Blacklist filtered bed files
			macs2	Relaxed peak-calling peaks (macs2 narrowpeaks files)
			peaks	IDR final peak set (bed files)
			plots	Contains IDR plot files (png)
			pseudo_replicates	bed files for pseudo replicates samples
			unthresholded-peaks	unthresholded bed files
qc	**design**	**pipeline_info**	Copies of input design files	
	fastqscreen	Plots for fastqscreen screening		
	raw_fastqc	fastqc reports for raw reads		
	samstats	Mapping statistics tabs (samstats)		
	trimmed_fastqc	fastqc reports for trimmed reads		
	fingerprint	Fingerprint plots		
	flagstat	Mapping statistics files (samtools flagstat)		
	frip	FRIP statistics files		
	multiqc	**Ssds**	Tabs and plots about SSDS parsing statistics	
		***.multiQC.quality-control.report_data**	multiqc analysis report files	
		***.multiQC.quality-control.report_plots**	multiqc analysis report files	
		*.multiQC.quality-control.report.html	Pipeline analysis results HTML report	

Among interesting files that are generated by hotSSDS report, ssDNA-derived fragments composition and FRiP scores are provided. These files are located in 02_results/qc/multiqc/ssds (*see* Fig. 3). Also computed are the bigwig files allowing to inspect peaks distribution along the genome using a genome browser (such as IGB or IGV) (*see* Fig. 3f). An analysis report (html format) is automatically generated after each run, compiling main statistics about the data. It can be found in 02_results/qc/multiqc under the name *.multiQC.quality-control.report.html.

To go further in the exploration of results, we developed a second pipeline called hotSSDS-extra, which produces various analysis plots such as heatmaps, PCA, intersection plots (*see* Fig. 1). This second pipeline will take as inputs some of the outputs from hotSSDS pipeline:

- Peaks (hotspots): 02_results/peaks/with[out]_idr/finalpeaks
- ssDNA type1 bam files: 02_results/bwa/filterbam/flag_XX/parseitr/type1/bam
- ssDNA type1 fragments file: 02_results/bwa/filterbam/flag_XX/parseitr/type1/bed
- Bigwig files: 02_results/bigwig/kbrick_bigwig/deeptools/binsizeXX/*ssDNA_type1.deeptools.RPKM.bigwig

3.2 hotSSDS-extra Pipeline

The hotSSDS-extra pipeline is composed of 11 main processes to produce SSDS post-process analysis plots (all steps are optional). A more detailed description of each process can be found in Table 6.

3.2.1 Download the Pipeline

Using git (recommended):

```
git clone https://github.com/jajclement/hotSSDS-extra.git
cd hotSSDS-extra
```

Or download zip file using wget, then unzip file:

```
wget
https://github.com/jajclement/hotSSDS-extra/archive/refs/heads/master.zip
unzip hotSSDS-extra-master.zip
mv hotSSDS-extra-master hotSSDS-extra
cd hotSSDS-extra
```

3.2.2 Download Singularity Images

The procedure is very similar to the hotSSDS pipeline (*see* Subheading 3.1.2). Prior to run the pipeline using Singularity execution profile, it is necessary to download the images from Zenodo

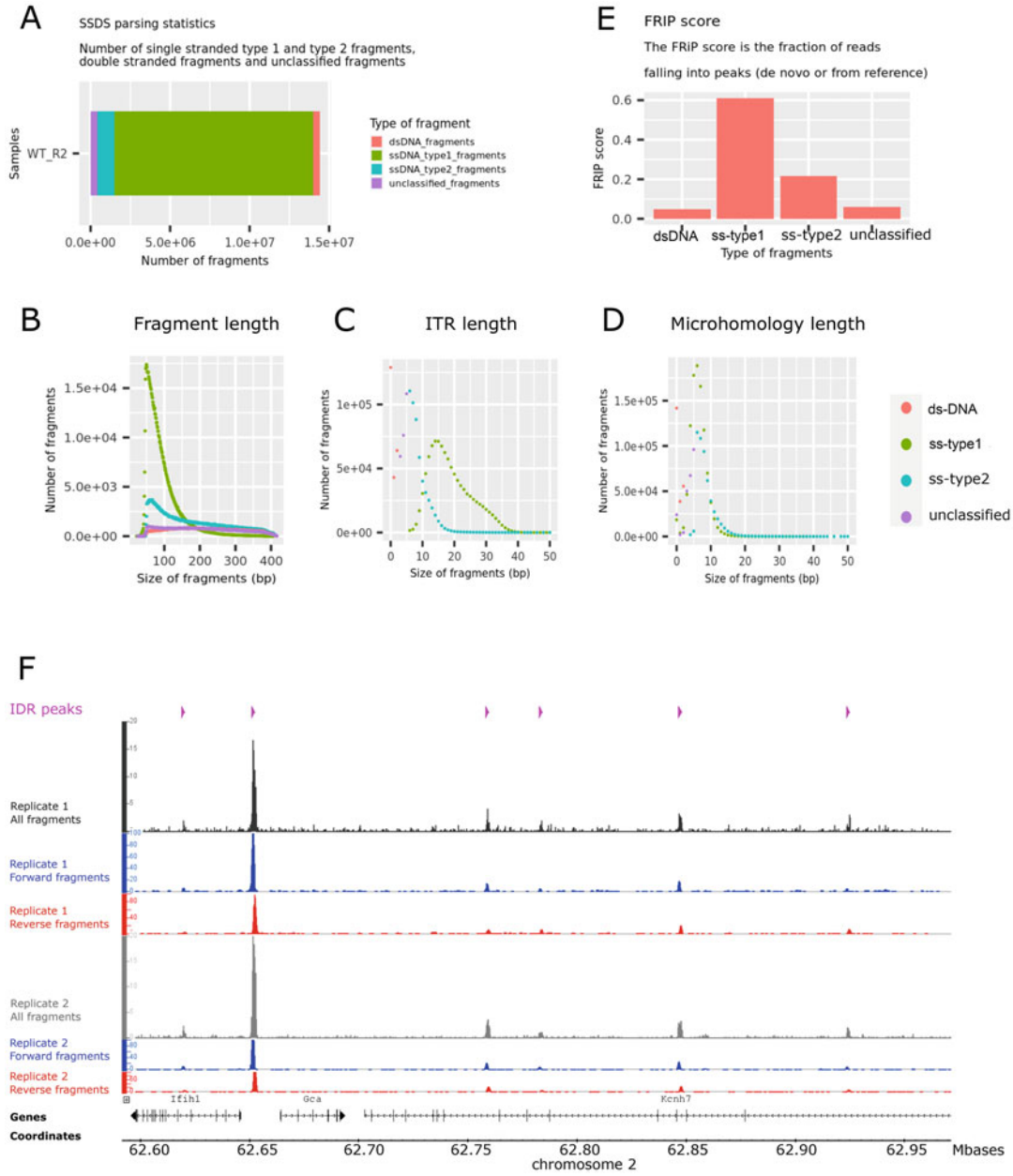

Fig. 3 The hotSSDS pipeline provides different types of output. (**a**–**e**) describe the raw data and allow one to check quality of the SSDS experiment and are part of the HTML report summarizing the analysis. The number of type 1-ssDNA, type 2-ssDNA, dsDNA, and unclassified fragments is provided for each sample (A), as well as the length of the fragment of the length (**b**), ITR (**c**), and the microhomology length (**d**) for each category of fragments. The fraction of reads inside IDR peaks for each fragment category (**e**) shows a strong bias for type 1-ssDNA for a good quality SSDS experiment. (**f**) Peaks and the SSDS signal can be examined along the genome by visualizing them in a genome browser tool (here IGB: https:/www.bioviz.org/). A 450 kb-portion of mouse chromosome 2 is shown, with SSDS total type 1-ssDNA fragment distribution (bigwig files) for both Replicate 1 (black) and Replicate 2 (gray) of test data. Having both fragments from the forward strand (blue) to the left or from the reverse strand (red) to the right is an additional guarantee of SSDS experiment quality. Peaks recovered after IDR are shown in pink

Table 6
Summary and description of the hotSSDS-extra pipeline steps

	Step	Description	Output file(s)
Clustering	Compute Matrix of read coverage at hotspots	Computes the read coverages of filtered type1 mapped bam files at hotspots (Deeptools multiBamSummary)	Compressed matrix of values as a numpy array (.npz file)
	Plot clustering and PCA	Plots an heatmap and a PCA (principal component analysis) of samples correlation based on read coverage matrix (Deeptools plotCorrelation and plotPCA)	Heatmap and PCA of samples (.pdf files)
F/R signal	Get Forward and Reverse separated signals	Gets coverage originated from the forward/reverse strand (Bedtools genomeCoverageBed and bedGraphToBigWig)	Forward/reverse separated coverage (.bigwig)
Heatmaps	Compute matrix of scores per hotspots	Calculates scores per genome regions for reverse+forward signal and/or only reverse and only forward signal (Deeptools computeMatrix)	Compressed matrix of values (.gzip file)
	Plot hotspot heatmap	Creates a heatmap for scores associated with hotspots for reverse+forward signal and/or only reverse and only forward signal (Deeptools plotHeatmap)	Heatmaps (.pdf files)
Annotation	Hotspot annotation	Annotates hotspots (Homer annotatePeaks)	Histograms and violin plots (.pdf files)
Overlaps	Plot peak sets overlaps	Computes overlap of bed sets using bedtools and intervene	Upset plots of intersects (.pdf file)

"hotSSDS and hotSSDS-extra Pipelines Singularity Images" open repository (https://zenodo.org/record/7783473). To do this, the two same options are available.

– **Run the pipeline using the option --get_sif**

The option --get_sif allows to launch a "dry run" that will check the existence of Singularity images in the pipeline directory. If not present, the pipeline will download them. Once download is completed, the pipeline stops. It can then be run again without the option --get_sif to perform SSDS analyses (*see* Subheading 3.2.5).

```
nextflow run main.nf -c conf/cluster.config
-params-file conf/test.json - profile <singular-
ity|mamba|conda|docker> --get_sif >& get_sif_-
main_log.txt 2>&1
```

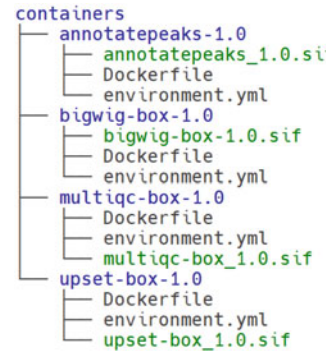

Fig. 4 Final containers repository file structure for the hotSSDS-extra pipeline

– **Download all singularity images independently**

Download all the four .sif files from Zenodo open repository at https://zenodo.org/record/7783473 and place them in hotSSDS-extra/containers folder so that the containers repository structure is consistent with the one shown in Fig. 4.

3.2.3 Configure Computing Parameter Files

Edit hotSSDS-extra/conf/cluster.config file to adjust the parameters to a computing cluster. *See* Subheading 3.1.3 instructions.

3.2.4 Edit/Create Parameter File

As for the hotSSDS pipeline, parameters can be set in two different ways: via parameter file, or directly in the Nextflow command-line. Parameters passed through command-line will overwrite those in the parameter file.

For *Mus musculus*-based analysis, parameter file hotSSDS/conf/mm10.json contains default parameters that can be overwritten and adapted, for example for another species. You can list all parameters using the following command:

```
nextflow run main.nf --help
```

A detailed description of available parameters can be found in Table 7.

3.2.5 Launch the Pipeline

Once computing config file have been set (*see* Subheading 3.2.3) and parameter file customized (*see* Subheading 3.2.4), the pipeline can be launched using the following command-line:

```
nextflow run main.nf -c conf/cluster.config
-params-file conf/mm10.json -profile <singularity|
mamba|conda|docker> --name "My_workflow_name" >&
main_log.txt 2>&1
```

Table 7
List of available parameters in the hotSSDS-extra pipeline

	Parameter name	Type	Default value	Description
Input data	params-file	FILE	hotSSDS-extra/conf/mm10.json	Path to parameters json file
	sample_name	STRING	DMC1-ChIP	Sample or group name used as reference
	finalpeaksbed	FILE	None	Filtered peaks in bed format (coming from hotSSDS pipeline, located in outdir/02_results/peaks/finalpeaks)
	peakreference	FILE	None	Reference peaks file in bed format, e.g., known B6 strain hotspots for *Mus musculus*
	Bamfolder	DIRECTORY	None	Path to bam folder containing filtered type 1 bam files from hotSSDS pipeline, located in outdir/02_results/bwa/filterbam/flag_*/parse_itr/type1/bam
	Bampattern	GLOB PATTERN	"*.bam"	Pattern for matching bam files in bamfolder
	bamreference	DIRECTORY	None	Path to reference bam folder containing filtered type 1 bam files for reference analysis (e.g., B6 strain for *Mus musculus*)
	bigwigfolder	DIRECTORY	None	Path to bigwig folder containing bigwig files from hotSSDS pipeline, located in outdir/02_results/bigwig/kbrick_bigwig/deeptools/binsize*
	bigwigpattern	GLOB PATTERN	"*ssDNA_type1.deeptools.RPKM.bigwig"	Pattern for matching bigwig files in bigwigfolder
	Bedfolder	DIRECTORY	None	Path to bed folder containing bed files to compute their intersect
	Bedpattern	GLOB PATTERN	"*.bed"	Pattern for matching bed files in bed folder
	Fragfolder	DIRECTORY	None	Path to the folder containing bed file with fragments after ITR parsing (ssDNA derived fragments identification) coming from hotSSDS pipeline, located in outdir/02_results/bwa/filterbam/flag_*/parse_itr/type1/bed
	Fragpattern	GLOB PATTERN	"*.bed"	Pattern for matching fragment bed files in fragfolder
	genome_gtf	FILE	hotSSDS-extra/data/mm10/mm10.refGene.gtf	Path to GTF (gene transfer format) genome annotation file (chromosome sizes file for reference genome) (Default file is for mm10 reference genome, downloaded from http://hgdownload.soe.ucsc.edu/goldenPath/mm10/bigZips/genes/mm10.refGene.gtf.gz 2023-04-20)

(continued)

Table 7
(continued)

	Parameter name	Type	Default value	Description
	genome_size	FILE	hotSSDS-extra/data/mm10/mm10.chrom.sizes	Path to genome size file: a two-column text file containing chromosome name in the first column and chromosome size in the second column (chromosome sizes file for reference genome) (Default file is for mm10 reference genome, downloaded from https://hgdownload.soe.ucsc.edu/goldenPath/mm10/bigZips/mm10.chrom.sizes 2021-01-11)
	genomebase	DIRECTORY	NULL	Path to genomes base folder
	Genomedir	DIRECTORY	NULL	Path to reference genome directory
	genome_name	STRING	mm10	Reference genome name
	genome_fasta	FILE	NULL	Path to genome fasta file (bwa index files must exist in the same folder)
	Fai	FILE	NULL	Path to genome .fai index file
General output	Name	STRING	hotSSDS-extra_pipeline	Analysis run name
	Outdir	DIRECTORY	hotSSDS-extra/name.outdir/02_results	Path to output directory
	publishdir_mode	STRING	"copy"	Mode for exporting each process output files to output directory (must be "symlink," "relink," "link," "copy," "copynofollow," "move," *see* https://www.nextflow.io/docs/latest/process.html)
Pipeline optional steps	with_clustering	BOOLEAN	True	If false then clustering step is skipped
	with_heatmap	BOOLEAN	True	If false then heatmap step is skipped
	with_FR_heatmap	BOOLEAN	True	If false then heatmap for reverse and forward step is skipped
	with_FR_bigwig	BOOLEAN	True	If false then bigwigs for reverse and forward step is skipped
	with_peak_annot	BOOLEAN	True	If false then peak annotation step is skipped
	with_plot_intersect	BOOLEAN	True	If false then bed intersection step is skipped
	with_report	BOOLEAN	True	If false then general report with multiqc step is skipped

Deeptools/bedtools/Homer configuration	corMethod	STRING	"spearman"	Correlation method for deeptools plotcorrelation process (valid options are spearman and pearson)
	corPlot	STRING	"heatmap"	Deeptools whattoplot option for plotcorrelation process (valid options are heatmap and scatterplot)
	heatmap_width	INTEGER	20	Deeptools heatmapwidth option for plotheatmap process
	matrix_downstream	INTEGER	2500	Deeptools downstream option for computematrix process
	matrix_upstream	INTEGER	2500	Deeptools upstream option for computematrix process
	gtf_id	STRING	"-gid"	By default homer processes the GTF file by transcript_id : let this parameter blank (i.e. "") if transcript_id is the relevant feature type in the provided GTF file, or set this parameter to "-gid" to process the GTF file by gene_id.
	figType	STRING	"pdf"	File format for plot files (valid options are pdf, svg, ps, tiff, png)
	intersect_options	STRING	"-f 1E-9"	intervene –bedtools-option (any of the arguments available for bedtool's intersect command)
	intersect_threshold	INTEGER	1	intervene --intersect_thresh option (minimum threshold to save the overlapping regions)
Singularity parameters	sif_url	URL	https://zenodo.org/record/7783473/files	URL to Zenodo open repository hosting pipeline singularity images to download
	get_sif	BOOLEAN	False	If true, check the existence of singularity images in containers/ directory and download them if needed (requires internet access)
Pipeline dependencies	Src	DIRECTORY	hotSSDS-extra/bin	Path to hotSSDS-extra pipeline source and binaries directory
	multiqc_configfile	FILE	hotSSDS-extra /conf/multiqc_config.yaml	Path to multiqc custom config file
Nextflow tower	with-tower	BOOLEAN	False	If true, enable job monitoring with Nextflow tower (https://tower.nf/); if true, you must generate an access token on tower website then export tower_access_token environment variable in your computing environment

As for the hotSSDS pipeline, it is highly recommended to launch the command in a batch job on the computing cluster, as its execution will take time and computing resources. It is also recommended to redirect the output of this main Nextflow command-line to an identified log file, which will be useful to monitor the pipeline execution.

3.2.6 Monitor the Pipeline

hotSSDS-extra pipeline monitoring is identical to hotSSDS pipeline (*see* Subheading 3.1.8).

3.2.7 Inspect Results

Similarly to the hotSSDS pipeline, three main folders are generated in the main output directory (specified with outdir parameter in command-line):

- **00_reports**: contains report files created by Nextflow each time the pipeline is run (*see* Table 4);
- **01_logs**: contains log files for all processes;
- **02_results**: contains result files, including:
  (a) **clustering** with PCA and heatmaps of sample clustering;
  (b) **heatmap** with heatmaps of signal distribution around peaks or regions (forward+reverse signal, only forward or only reverse signal);
  (c) **FRbigwig** with Reverse and Forward Type 1 fragments bigwig files;
  (d) **intersect** with upset plots of peak sets intersections;
  (e) **multiqc** with quality controls files and reports;
  (f) **annotation** with Peaks annotation plots (histograms and violin plot);
- **work**: is the working directory for Nextflow. The different plots produced by hotSSDS-extra pipeline are summarized in Fig. 5. As all steps are optional in the hotSSDS-extra pipeline, all output subfolders listed above under 02_results directory are optional too.

More details about output files can be found in Table 8.

An analysis report (html format) is automatically generated after each run, compiling main statistics about the data. It can be found in 02_results/qc/multiqc under the name *.multiqc.report.html.

4 Notes

1. All contributions are welcome, open an issue on the Github repository (https://github.com/jajclement/hotSSDS/issues or https://github.com/jajclement/hotSSDS-extra/issues) or submit pull requests.

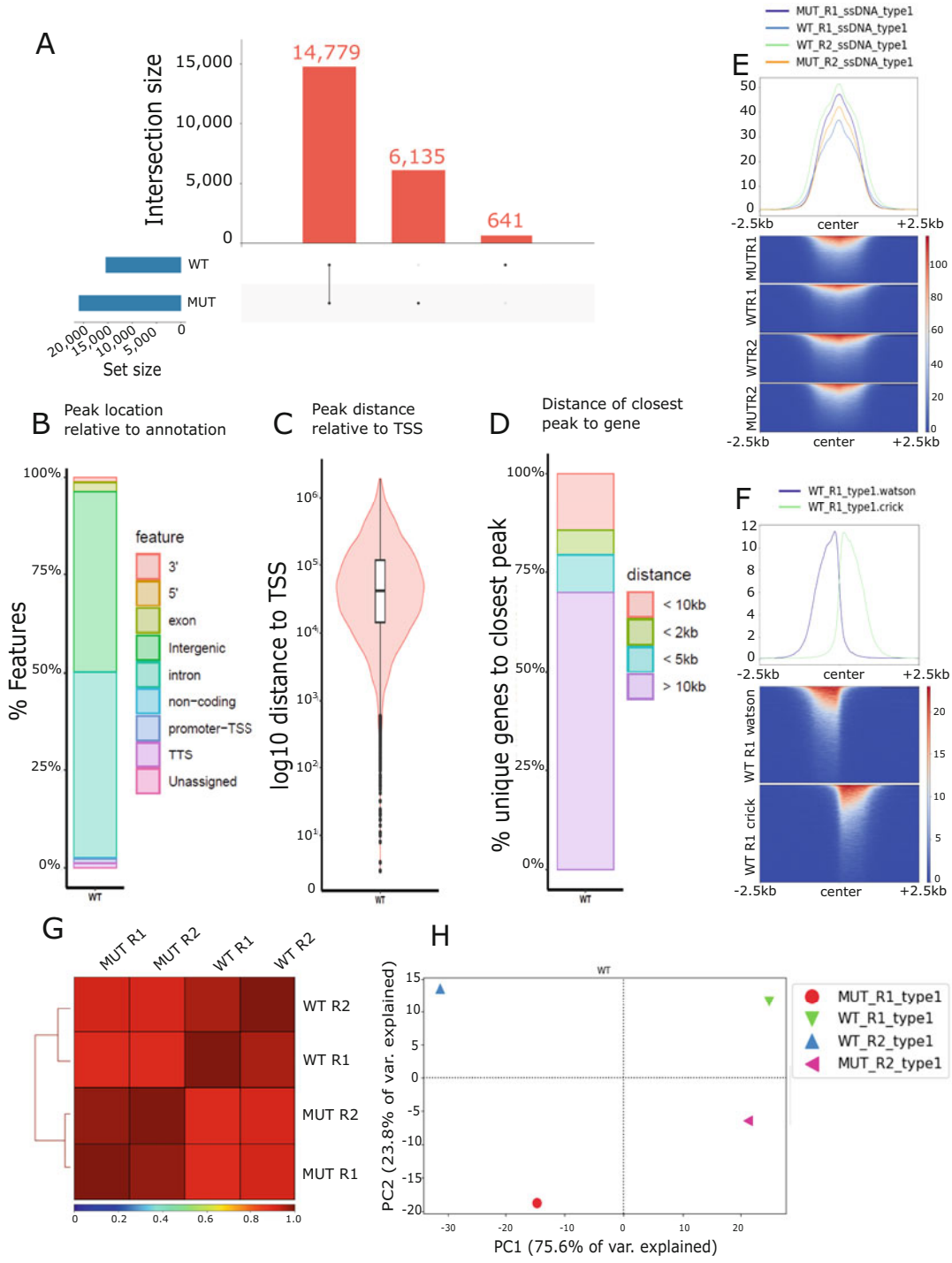

Fig. 5 The hotSSDS-extra pipeline provides different types of analysis output. (**a**) Upset plot showing the intersect between the different peaksets obtained with the hotSSDS pipeline. (**b**–**d**) and (**f**) are shown for one sample (mutant genotype) only. (B) Peak annotation. The proportion of peaks overlapping each feature is shown. (**c**) Distribution density of the peak distance to the closest transcription start site (TSS). (**d**) Distance of peaks to the closest gene. The proportion of peaks are shown for four distance categories. (**e**) Distribution of

2. Future enhancements include migration to Nextflow DSL2, which provides a syntax extension that allows the definition of module libraries and simplifies the writing of such complex data analysis pipelines (https://www.nextflow.io/docs/latest/dsl2.html). We are also working on migration to the analysis report from MultiQC to Rmarkdown to provide more flexibility in the layout and more readability of the results. Any additional functionality will be provided and documented on the same repository addresses.
3. hotSSDS pipeline specific parameters can also be passed through command-line using the double dash (`--with_trimgalore` for example). Nextflow native options are provided using single dash (`-resume` to resume a previously stalled workflow, see Nextflow documentation [16]).

Table 8
List of output files generated by the hotSSDS-extra pipeline

Result subfolder names in 02_results	Description	
annotation	Homer working and configuration files	
	plots	Peaks annotation plots (histograms and violin plot) in .pdf format
heatmap	**matrices**	Matrix binary files
	plots	Heatmaps (.png files) of signal (forward+reverse signal, only forward or only reverse signal)
FRbigwig	Reverse and Forward Type 1 fragments bigwig files	
intersect		
clustering	**matrices**	Matrix binary files
	plots	PCA and heatmap of sample clustering (.png files)
multiqc	***.multiqc. report_data**	multiqc analysis report files
	***.multiqc. report _plots**	multiqc analysis report files
	***. multiqc. report**.html	Pipeline analysis results HTML report

Fig. 5 (continued) signals around identified peaks. Upper plot is the average density plot. The bottom heatmaps represent the signal distribution across each peak for each sample (one box per sample); (**f**) Forward (blue) and reverse (green) signal distribution around peak for one sample. Upper plot is the average density plot. The bottom plots are heatmaps for each signal. (**g**) Spearman correlation heatmap of the SSDS signal for the four samples. (**h**) Principal component analysis of the SSDS signal calculated over the peaks for the four analyzed samples

4. Remember to use bash command `ls -a` to also display files whose names begin with a dot.
5. In addition to the points Subheadings 3.1.8 and 3.2.6, monitoring the pipeline can also be done by using Nextflow tower (https://cloud.tower.nf/). Sign in using your email address. Follow instructions received by email to connect and create a Token (upper-right scrolling menu > Your tokens > Add Token). Then, export tower_access_token environment variable in the computing environment before running the pipeline and use this Personal Access Token when running the pipeline by adding "`-with-tower <your_personal_access_token>`" to the command provided in Subheadings 3.1.6 and 3.2.6.
6. Although this methodology allows the estimation of hotspot intensity, interpreting or comparing the quantifications between two (or more) conditions should be done carefully. It was proposed to normalize the SSDS signal so that the sum of hotspot heats is identical in each sample (and equal to a control or reference genotype) [25]. This is a good option when the dynamics of DSB formation is comparable between conditions and has been checked (e.g., cytologically) beforehand. In some instances, this normalization is not relevant, e.g., due to altered DSB dynamics between tested genotypes/conditions, a difference in the cell population composition, or even a modified resection size. Quantitative comparisons should then be semi-quantitative: absolute values cannot be compared, but relative values can be. Whatever the situation, we recommend using DESeq2 to perform differential analysis between two (or more) conditions (*see* our previous work [24, 26]) and with appropriate peaksets (e.g., reference hotspots).
7. For the analysis of SSDS data from a newly investigated species, we identified some key points to pay attention to. First, since the methodology is based on the presence of microhomologies along the genome, it is important to check the microhomology distribution in the genome used as reference. This can be done using the code developed by the collaborator Makoto Kashima and available at https://github.com/Makoto-Kashima/microhomology.

   The default parameters provided in the pipeline are well adapted for mouse or human, but it could be necessary to adjust them for other species. For example, the IDR threshold depends on the genome size and total peak numbers (*see* recommendations https://hbctraining.github.io/Intro-to-ChIPseq/lessons/07_handling-replicates-idr.html). Peak-calling parameters could also require adjustments if the peak width or relative intensity to noise is different. In any case

including this particular one, we advise to always examine the signal distribution and to observe peak shape and distribution and their enrichment relative to background (for that, visualize the bigwig files in a genome browser such as IGB or IGV).

If the sample is originating from a non-inbred line, we warmly recommend using an input sample to control for any variations to the reference genome. We are currently testing the impact of single nucleotide polymorphism on ssDNA recovery. If we identify parameters to adjust regarding this specific point, information will be provided on the github repository, in our README document.

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18. Conda, https://docs.conda.io/en/latest/
19. Mamba, https://github.com/mamba-org/mamba
20. Docker, https://www.docker.com/
21. Singularity, https://sylabs.io/
22. git, https://git-scm.com/
23. wget, https://www.gnu.org/software/wget/
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Chapter 17

"MeiQuant": An Integrated Tool for Analyzing Meiotic Prophase I Spread Images

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Abstract

Immunocytochemical analysis of meiotic proteins on mouse chromosome spreads is one method of choice to study prophase I chromosome organization and homologous recombination. In recent decades, the development of microscopic approaches led to the production of a large number of images that monitor fluorescent proteins, defined as fluorescent objects, and a major challenge facing the community is the deep analysis of these fluorescent objects (measurement of object length, intensity, distance between objects, as well as foci identification, counting, and colocalization). We propose a set of tools designed from the macro language of the widely used image analysis software ImageJ (Schindelin et al., Nat Methods 9: 676–682, 2012), embedded in the "MeiQuant" macro, which are specifically designed for analyzing objects in the field of meiosis. Our aim is to propose a unified evolutive common tool for image analysis, with a specific focus on mouse prophase I meiotic events.

Key words Meiosis, Homologous recombination, Chromosome structure, Protein localization, Image analysis, Foci count, Intensity measurement, ImageJ, MeiQuant

1 Introduction

Sexually reproducing organisms rely on the specialized cell division of meiosis to generate haploid cells that differentiate into gametes. This halving of genomic content is mediated by one round of replication, followed by two successive divisions (meiosis I and II). The homologous chromosomes (homologs) segregate at meiosis I, hence referred to as the reductional division [2]. To properly segregate at meiosis I, homologs need to pair and establish physical connections. In various organisms including mice, which is the original focus of the method described in this chapter, this connection results from the repair of DNA double-strand breaks (DSBs) by homologous recombination (HR) during the prophase of the first meiotic division (prophase I) [3].

Marco Barchi and Massimo De Felici (eds.), *Germ Cell Development: Methods and Protocols*, Methods in Molecular Biology, vol. 2770, https://doi.org/10.1007/978-1-0716-3698-5_17,

Meiotic HR occurs in the context of a meiosis-specific chromosome structural reorganization. This process is highly dynamic and the structural changes observed by cytological analysis at the chromosome scale were used to define the different substages of prophase I: leptotene, zygotene, pachytene, and diplotene. Prophase I initiates at leptotene with the formation of a proteinaceous axis, on which chromatin loops of the pairs of sister chromatids are anchored. The meiotic chromosome axes are composed of various proteins (e.g., SYCP3, HORMAD1, which are commonly used to identify the axes and mentioned in this chapter) and DNA. At leptotene, HR initiates by the programmed formation of DSBs. Then, the pairing of the homologous chromosomes starts and culminates with the formation of the synaptonemal complex (SC). The SC is a tripartite structure, formed by two lateral elements linked by a central structure. The lateral elements are the axes of both homologs that are connected by the central element, which is a proteinaceous structure (e.g., SYCP1 protein is one of the components of the central element used to detect its formation) that establishes a connection that propagates along the axes through a "zipperlike" process. The cytological detection of the first SC fragments defines the beginning of the zygotene substage. The assembly of the SC along the full length of the homologous chromosomes defines the pachytene stage. At the end of this stage, the recombination intermediates are resolved. Eventually, the SC disassembles during the diplotene stage and homologous chromosomes remain connected by the chiasmata, which are the cytological correlates of crossing-over (CO).

During prophase I, the meiotic HR and the structural reorganization of the chromosomes are two interdependent mechanisms that are essential for completing meiosis I and involve numerous protein activities. Since decades, the spatiotemporal pattern of recruitment of the proteins involved in these processes has been studied intensively, principally to (1) decipher the sequence of events that leads to proper homolog segregation at meiosis I, (2) get insights regarding the molecular functions of the different proteins involved, and (3) assess the impact of mutations that alter the molecular processes of HR or chromosome structure. To achieve this goal, monitoring of prophase I proteins by immunocytofluorescence analyses were widely developed, in particular on chromosome spreads obtained from mouse meiocytes. By combining antibodies raised in different organisms against various proteins that participate either in shaping the structure of meiotic chromosomes or in the molecular process and regulation of HR, the localization of these proteins detected as fluorescent objects on chromosome spread wide-field images can be analyzed on a huge number of wide-field microscopy images. One main challenge facing the community is then the thorough characterization of these fluorescent objects, in particular their shape, size, number, and the

distance between them. In order to analyze quantitatively these parameters on large numbers of cells, automated image analysis tools are particularly needed.

Our approach consisted in making use of the macro language that is integrated within the widely employed image analysis software, ImageJ software1. This software is commonly used to examine microscope images. We chose to use the macro language because it enables users to conveniently customize or adjust the tools that are provided. Our primary goal is to propose a standardized and adaptable tool for nuclear spread image analysis that can be used by the broader community, with a particular emphasis on mouse prophase I meiotic events. Despite some limitations of the macro language, it remains straightforward for anyone familiar with ImageJ to modify its code, thereby expanding its possible applications.

2 Materials

2.1 2D Chromosome Spreads

The "MeiQuant" macro (also referred as "Meiosis bar") was initially designed to analyze images obtained from immunostained prophase I mouse chromosome spreads, prepared using the dry down technique [4]. Immunostaining protocol used in the example images is described elsewhere [5–8].

2.2 Images

It is recommended to acquire high resolution widefield fluorescence microscopy 2D images. A high numerical aperture (NA) 63× or 100× magnification immersion objective is recommended (e.g., NA > 1.3). The refractive index of the sample mounting medium should match that of the immersion medium of the objective to avoid deformation along the axis or unwanted spherical aberrations. Regarding excitation, a stable LED excitation light source and selective band-pass fluorescence filter sets should be used. To ease the process of acquisition of hundreds of nuclei images, a high sensitivity (high quantum efficiency, low noise) camera with a wide detector (such as sCMOS cameras) allows acquisition of large field of views, while a motorized XY stage can be used for mosaic acquisition. For accurate measurements, especially for colocalization analyses, it is highly recommended to have a pixel size that fulfills Shannon-Nyquist's sampling density criterion [9–11]. Importantly, other techniques can be used to produce images (*see* **Note 1**).

2.3 Images Preprocessing

To get better quality images, we suggest using deconvolution when using widefield images. This process can improve the resolution, contrast, and signal-to-noise ratio of the image, making identification of axes and foci easier [12, 13].

1. First, remove the background locally. We suggest using Huygens Professional (SVI, Hilversum) background estimation algorithm (radius of 0.7 μm). This step is important because the macro's tools used to analyze the image do not use a local threshold algorithm.
2. Estimate and correct the images for photon shot noise. The signal-to-noise ratio (SNR) of widefield images using the full dynamic range of the camera have a typical SNR of 100.
3. Perform deconvolution with a maximum likelihood estimation (CMLE in Huygens Professional) algorithm. If this option is available in the deconvolution suite used, consider using computed theoretical point spread function (PSF) or real, acquired PSF images. We use theoretical PSFs as compared to measured PSF acquired with our 100× alpha Plan-Apochromat 1.46 oil DIC objective and 160 nm fluorescent beads. Indeed, we found that measured PSF does not give better results.

2.4 Input Images File Format

The MeiQuant bar tools will open any Bio-Formats compatible image's file formats (http://www.openmicroscopy.org/bio-formats/). Users are advised to refer to their version of the ImageJ bioformats plugin.

2.5 Versions and Plugins Used

1. The MeiQuant macro (Meiosis bar) is an ImageJ/Fiji's macro that can be downloaded here: https://github.com/MontpellierRessourcesImagerie/meiosis_bar. A comprehensive manual can be downloaded as well, which fully describes some tools that are not included in this manuscript.
2. The macro uses the Actionbar plugin. This can be downloaded using the built-in updater of Fiji (help > Update > Manage update sites), through selection of the IBMP-CNRS update site. For the purpose of axes length measurements, the tool uses the skeletonize 2D/3D plugins (visit https://github.com/fiji/Skeletonize3D for installation if not using Fiji). The macro was tested using ImageJ 1.53t, Java 1.8.0_172, and a 64-bits windows OS.

3 Methods

The "MeiQuant" macro (General menu window "Meiosis bar," displayed in Fig. 1a) provides a button enabling the cropping and classification of meiosis stages for single nucleus images during prophase I. Along with this, the macro offers four primary tools consisting of modular blocks (referred to as modules in Fig. 1b–e) to analyze the generated single nucleus images. The initial tool, "Measure axis length" (Fig. 1b), is employed to identify chromosome axes and determine their overall length. The second tool

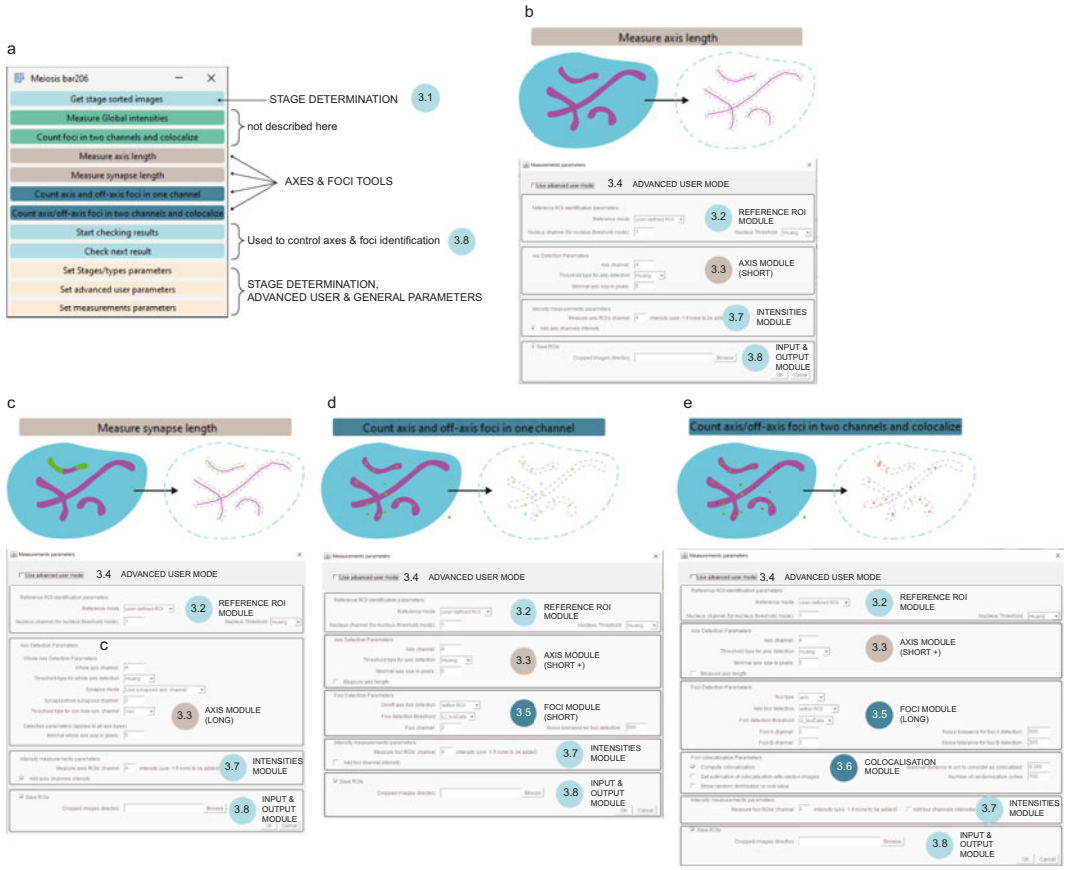

Fig. 1 The main meiosis bar (**a**) with its associated tools, modules, and functions. The Measure axis length (**b**), Measure synapse length (**c**), Count axis and off-axis foci in one channel (**d**), and Count axis/off-axis foci in two channels and colocalize (**e**) tools are presented with their respective modular architectures

("Measure synapse length") builds upon the functionality of the first, by incorporating the detection and measurement of the synapsed sections of the axes, based on one additional staining (Fig. 1c). The third tool ("Count axis and off-axis foci in one channel") enables the counting of foci in relation to the axes (Fig. 1d). Finally, the fourth tool ("Count axis/off-axis foci in two channels and colocalize") identifies axes and identifies the foci of two different stainings, whether on or excluded from the axes (Fig. 1e). All tools combine different modules, which are detailed below using the numbering indicated in Fig. 1b–e. The module descriptions and the associated step-by-step figures are numbered correspondingly. For instance, **step 1** of the reference region of interest (ROI) module, which involves setting the reference ROI method, is explained in Subheading 3.2, **step 1** and is indicated by the Subheading 3.2, **step 1** sticker in Fig. 2d). The macro also includes buttons that facilitate the control of the correct

segmentation of relevant features ("Start checking results" and "Check next result"), such as axes or foci, or allow for settings of parameters that are shared across the different tools. Additionally, the macro contains extra tools that are not directly relevant to meiosis prophase I and are therefore not covered in this chapter, such as the "Measure global intensities" tool and the "Count foci in two channels and colocalize" tool, which enables the detection and analysis of foci of two stainings nucleus-wide.

3.1 Stage Determination

This formatting step is intended to produce images of single nuclei and tag them with a meiotic prophase I substage prefix.

1. Before starting the analysis, first click on the "Get stage sorted images" button (Fig. 1a). In the "File Parameters" window that appears (Fig. 2a), provide the file extension that corresponds to the image files you wish to analyze (e.g., .ics or .tif), which must be compatible with Bio-Formats to be processed.
2. There are various modes available for displaying images, each with its own specific features. In "composite" mode, the stack is shown as a composite image. In grayscale mode, each channel is presented in grayscale, with the selected channel (either nucleus or axis) displayed by default. This choice has no impact on subsequent steps. However, if you opt for "grayscale nucleus" or "grayscale axis," the channel corresponding either to the nucleus (e.g., DAPI) or to the axis (e.g., SYCP3) must be specified. In composite mode, these options have no effect.
3. Choose a drawing tool (either freehand or magic wand) to draw the outline of the nucleus.
4. Enter the appropriate number of nucleus stages or types to distinguish, up to seven. This information is used to construct the Crop stage bar (an example is displayed in Fig. 2c). For stage determination, (*see* **Note 2**).
5. Select the folder containing the images to process by browsing in the Raw Image directory field and click "OK."
6. In the window that opens (Fig. 2b), type the prefixes that will specify the nucleus stage of the cropped images (*see* **Note 3**). This step generates a Crop stage bar with buttons (Fig. 2c). For subsequent analyses, the prefixes of the images must match with the current selection of stage prefixes. If needed (e.g., for analyzing a previous series of cropped images with a different list of prefixes), the list of prefixes can be adjusted with the option "Set stages/types parameters" from the main menu (Fig. 1a) (see comprehensive manual).
7. Once the raw image is opened and the crop menu is displayed, draw the outlines of the nucleus of interest with the drawing tool selected at **step 3**. Add the selection to the ROI Manager

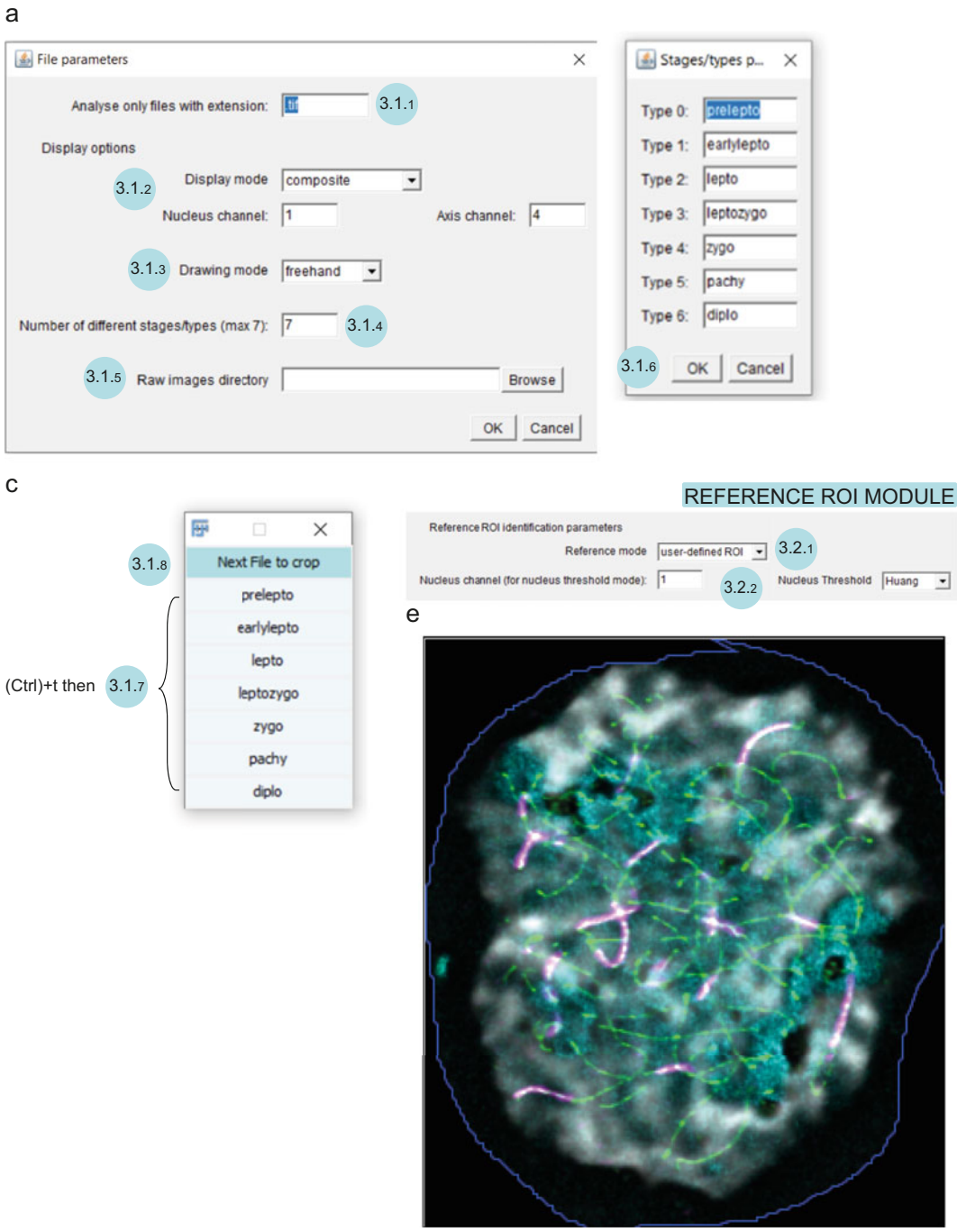

Fig. 2 Illustration of the process of formatting images and setting the reference ROI. Panels a–c demonstrate the various steps of the stage determination module, where parameters are entered to display and set the images appropriately (**a**) and create a customized bar for stage determination (**a**, **b**), which is displayed in panel c. (**d**) Step-by-step description of the reference ROI module. (**e**) An example of a user-defined ROI obtained with the reference ROI module

list using the shortcut displayed with "Edit > Selection > Add to Manager" (Ctrl+t, or just t). Repeat drawing and adding selections for all nuclei of the same stage present on the image, then select the appropriate meiosis stage with its prefix button (zygo for instance, Fig. 2e). Each cropped nucleus is saved as a new image file, with pixels outside the selection being set to black. The file is saved in a subfolder called "processedData" as a .tif file, with its stage prefix and a specific identification suffix (_0 for the first one, then _1, and so on). The associated user-defined ROI is saved in the same folder as a "companion" .roi file in the same "processedData" folder. Repeat these steps for each stage if the raw image contains nuclei of more than one stage.

8. Press the "Next file to crop" button to process the next image.

3.2 Reference ROI Module for Reference ROI Identification

The analysis tools provided by this macro only examine a specified ROI within the image referred to as "reference ROI" (e.g., the surface covered by a nucleus, defined as described below). The reference ROI module is used to determine the reference ROI.

1. When opening any analysis tool from the macro, you are prompted to choose the reference mode that will determine the reference region of interest (ROI) used for that specific analysis (as shown in Fig. 2d). This can be done by selecting either the "User-defined ROI" option, which uses the ROI previously saved in the "companion" .roi file (generated in Sect. 3.1), or the "Nucleus threshold" option, which automatically applies a threshold to the selected nucleus channel (e.g., DAPI).
2. In the case of the "Nucleus threshold" option, set the nucleus channel and select one of the built-in automatic threshold algorithms in ImageJ (by default, Huang, Triangle, or Moments). The list of default methods can be expanded to the full list of available thresholds by referring to the comprehensive manual (option in "Measurement parameters" menu, Subheading 3.7, **step 2**). Figure 2e shows an example of a reference ROI that can be obtained using user-defined ROI.

3.3 Axis Module for Axis Segmentation and Length Measurements

This module enables the segmentation of axes, creating Axis ROIs that are then used for skeletonization and length measurement. The module comes in two versions: the "short" version (Fig. 3a, top panel) is used for identifying whole axes, while the long version (Fig. 3a, bottom panel) is designed to identify both whole axes and their synapsed sections that define the synaptonemal complex (SC) using two staining (e.g., SYCP3 for axis and SYCP1 for SC identification, respectively [14, 15]). This "long" version is used in the "Measure synapse length" tool.

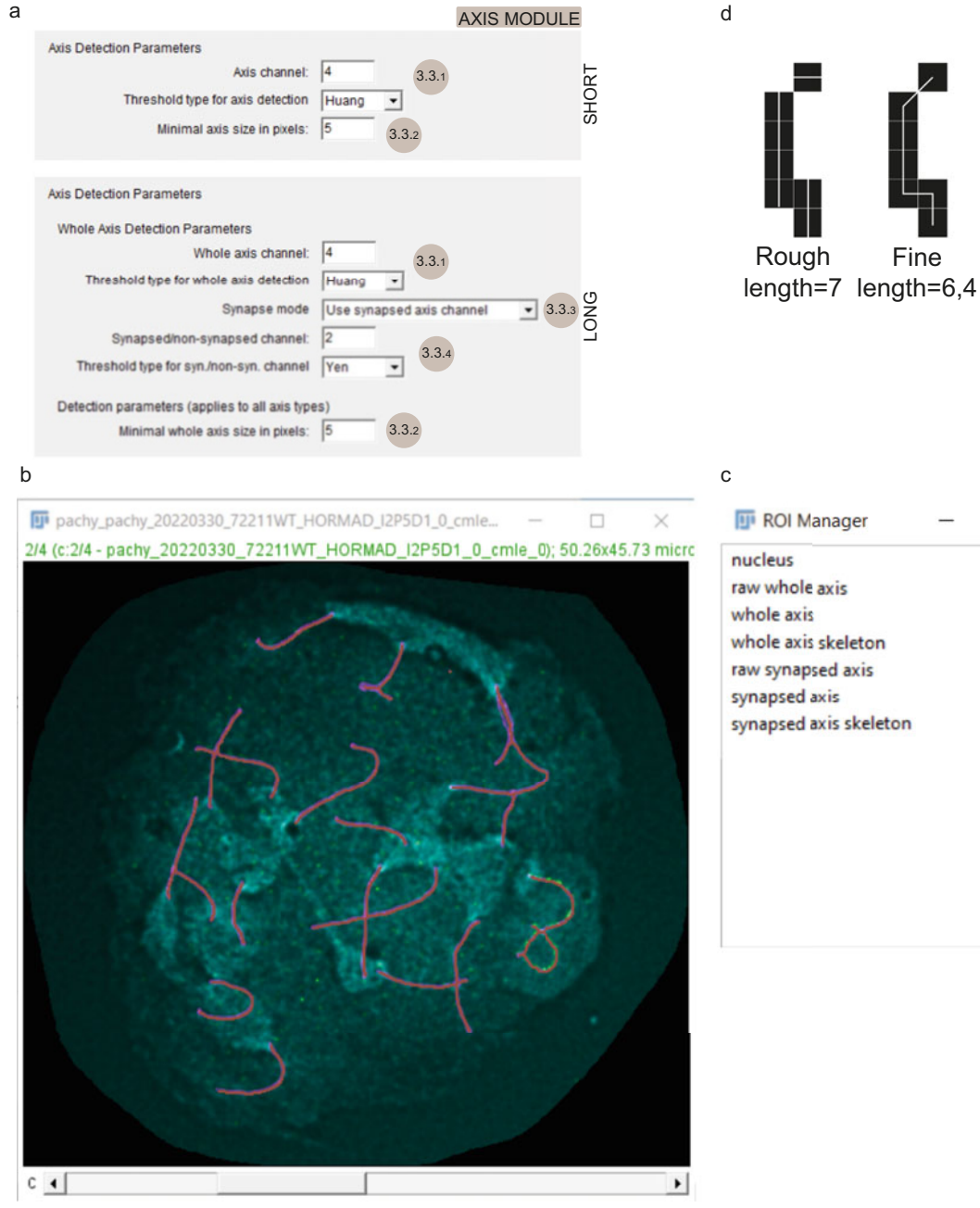

Fig. 3 Synaptonemal complexes module. (**a**) Step-by-step description of its short (whole axis only, top panel) and long (whole axis and synapsed/non-synapsed axis, bottom panel) versions. (**b**, **c**) An example of a segmented image and the corresponding ROIs, as generated using the long version of the module. (**d**) Actual lengths measured with the rough and fine methods (white lines). Measured lengths of the white lines are indicated

1. First, select the whole axis staining channel and choose the appropriate automatic threshold method to segment it (Fig. 3a).
2. Next, the module uses ImageJ's Analyze Particle command to identify the axis elements within the reference selection. You can set a minimum pixel number size to exclude small elements, but be careful not to set it too high for early stages (leptotene) when only short axis stretches are present (Fig. 3a). Some additional exclusion criteria are available in advanced user mode (such as exclusion based on elements' shape or location near the image's edges). In brief, this module combines the elements that meet all selected criteria to create a raw axis selection (as shown in Fig. 3c). Algorithm details are detailed in the comprehensive manual.

   If using the long version of the module, which is used for "Measure synapse length," follow these additional steps:
3. Set the synapse mode to "Use synapsed axis channel" (**step 3**) if the staining used (referred to as synapsis staining below) corresponds to a synapsed section of the axis (e.g., SYCP1 staining). If using a non-synapsed marker (e.g., HORMAD1 staining [16]), select "Use non-synapsed axis channel." The purpose of this setting is solely to track which staining was used for synapsis and does not impact the analysis in any way.
4. Set the synapsis staining channel and the automatic threshold to use (**step 4**). The synapsed/unsynapsed sections are detected similarly to axis sections at **steps 1** and **2**, but the Analyze Particle command is run on the whole axis selection (defined in **steps 1** and **2**) instead of the reference ROI. Additionally, if small whole axis elements are excluded by using a minimum particle size (**step 2**), this exclusion criterion also applies to the synapsed/non-synapsed elements. Therefore, if such short elements are not detected, consider lowering the minimum whole axis size at **step 2**.

In conclusion, given the algorithm's design, for whole axes, the raw selection is restricted to the reference ROI; for specifying synapsed/non-synapsed axes in a second phase, the raw selection is now restricted to the raw whole axis ROI.

By default, the raw and (final) axis selections are the same, but advanced user parameters can be used to apply pruning to the axis raw selection (as displayed in Fig. 4a). Then, the raw selection is further processed into a final (whole axis or synapsed/unsynapsed) axis selection (*see* Subheading 3.4).

The (final) axis selection is then used as a binary mask for skeleton analysis. This step uses the skeleton 2D/3D plugin (preinstalled in Fiji releases). A binary skeleton is produced and used to draw a skeleton selection. An example is depicted in Fig. 3b. The ROI manager displays all generated ROIs (Fig. 3c). The skeleton

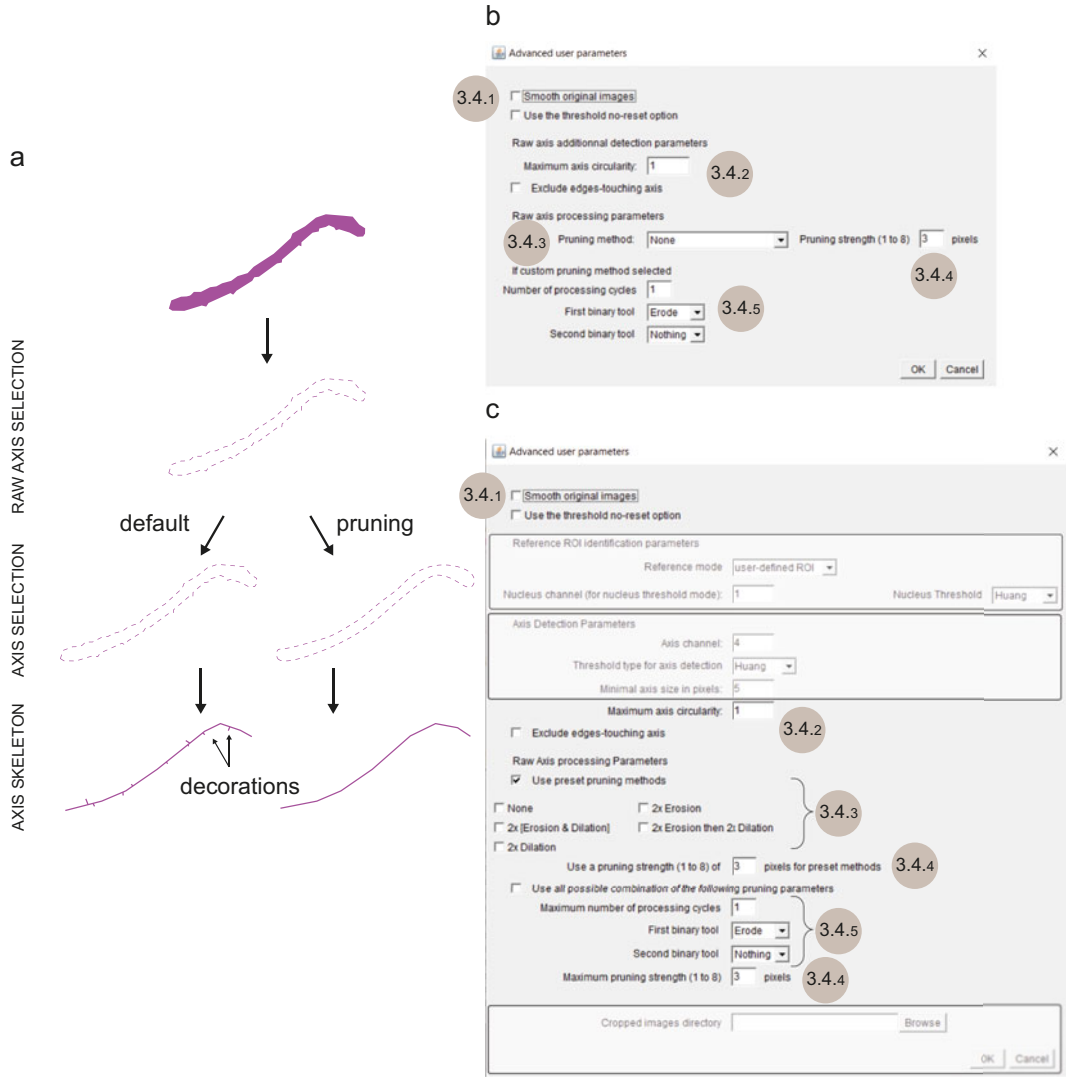

Fig. 4 Advanced user parameters. (**a**) Pruning process (right) for more accurate measurement of axis length by removing unwanted skeleton decoration caused by discontinuities in the raw axis selection (left). (**b**, **c**) Step-by-step description of the advanced user parameters window, displayed when the option is selected in the main tools (**b**), or when the "Set Advanced user parameters" button of the main macro is used (**c**)

length is measured using either a "rough" or "fine" method (Fig. 3d, white lines on the left and right panels, respectively). Default method is the more precise (fine) one, but it can be changed if needed (through "Set measurements parameters," Fig. 1a). Note that if using the "short +" version of the module (e.g., with foci tools), skeletonization and length measurement can be skipped. An example of whole axis and synapsed axis identification is shown in Fig. 3b.

3.4 Advanced User Parameters for Axis Identification

Advanced user parameters offer improved feature detection capabilities, and the two primary options available are smoothing and pruning. The comprehensive manual also describes a third parameter, known as the "Use the threshold no-reset option," which is of more specific interest.

While additional processing of the raw axis may not be necessary for high magnification widefield images, the main smoothing and pruning options are especially effective when examining super-resolution images under conditions where there is substantial photon shot noise or low signal intensity. In such cases, processing the raw axis ROI is crucial for obtaining accurate length measurements, as failing to do so can lead to unwanted skeleton decorations being included in the overall length calculation. There are two ways to use the advanced user mode. The first involves ticking the "Use advanced user mode option" box from each tool's window (*see* Fig. 1b–e), which triggers the Fig. 4b menu. The second method is to preset the advanced user parameters using the corresponding button on the main meiosis bar (Fig. 1a), which then triggers the Fig. 4c menu.

1. It is recommended to use a smoothing convolution filter. Tick the "Smooth original images" option (**step 1**).
2. For a better raw axis segmentation, additional exclusion criteria can be used. For instance, the "Maximum axis circularity" parameter can be adjusted to set an upper circularity threshold and select only elongated elements. Another criterion is to "exclude edge-touching axis" by removing axis elements that touch the border of the image (**step 2**). It is important to refer to the comprehensive manual for a detailed description of these options, as they can have side effects.
3. Binary mask methods can be used to remove (prune) unwanted decorations from the axis's skeleton. If you use the "Use advanced user parameters" option of one of the four tools described (Fig. 4b), you can select a preset pruning method or choose "custom" to use another combination.
4. Set a pruning strength to determine the harshness of the pruning rules. This value ranges from 1 to 8 (**step 4**—see comprehensive manual for details).
5. If using the "custom method," users can set the first binary tool to be used and consider using a second one. They can also set the number of cycles each binary tool will run. The second binary tool cycle starts after the end of the first tool cycle (**step 5**).

The "Set Advanced User parameters" button of the main meiosis bar can be used to fine-tune the best pruning parameters. It can be used as a stand-alone tool and utilizes the Reference ROI

Module (Subheading 3.2) and the axis module (Subheading 3.3). Users can try all preset methods with a common strength value by selecting them, or they can set the binary tools to be tested and the maximum number of cycles and strength. The algorithm will try all custom methods corresponding to all possible combinations of cycles and strength values. All corresponding (final) axis and skeleton ROIs are generated. To visualize these ROIs, users should refer to Subheading 3.8.

3.5 Foci Module for Foci Identification and Counting

The module enables the detection of foci based on their on-axis or off-axis position. It uses ImageJ's "Find Maxima" command (make sure to use ImageJ version 1.52 or later). To detect foci, follow these steps:

1. Select a method for identifying the type of foci to detect (either on- or off-axis) with the rolling menu "(On/off) Axis foci detection" (Fig. 5a), with two modes available: "within ROI" and "within mask" (*see* **Note 4**). In "within ROI" mode (Fig. 5b), foci are first detected within the entire reference ROI, then they are classified as "axis" or "off-axis" foci depending on whether the maximum is located within or outside the axis ROI. In "within mask" mode (Fig. 5c), intensities are cleared outside (top panel) or inside the axis ROI (bottom panel) for detecting axis or off-axis foci, respectively, then "Find Maxima" is run. Thus, in "within mask" mode, when a focus particle overlaps the edge of the axis, two maxima may be detected (axis and off-axis, as shown in top and bottom panels of Fig. 5c, respectively), resulting in the double counting of certain foci.
2. To identify local maxima using ImageJ "Find Maxima" command, first set the foci channel and the noise tolerance value, which determines the prominence value (Fig. 5a). The command compares the intensity of each pixel to its neighbors and ignores pixels that do not stand out from the surrounding area by more than the set prominence value. The long version of the module, shown in the lower panel of Fig. 5a, enables detecting foci from a second channel corresponding to a second staining. Set a foci detection threshold method (Fig. 5a). Foci are primarily detected by locating their respective maxima. When using an automatic foci detection threshold to identify the maxima, the search is restricted to thresholded areas.
3. Foci are characterized by both their maxima and outlines, also called "particles" (Fig. 5b). The same foci detection threshold is used to generate the particle outlines. There are two methods available for segmenting particles (*see* **Note 5**). In the first method, with no threshold applied (Foci detection threshold "None"), all pixels with an intensity larger than the intensity of

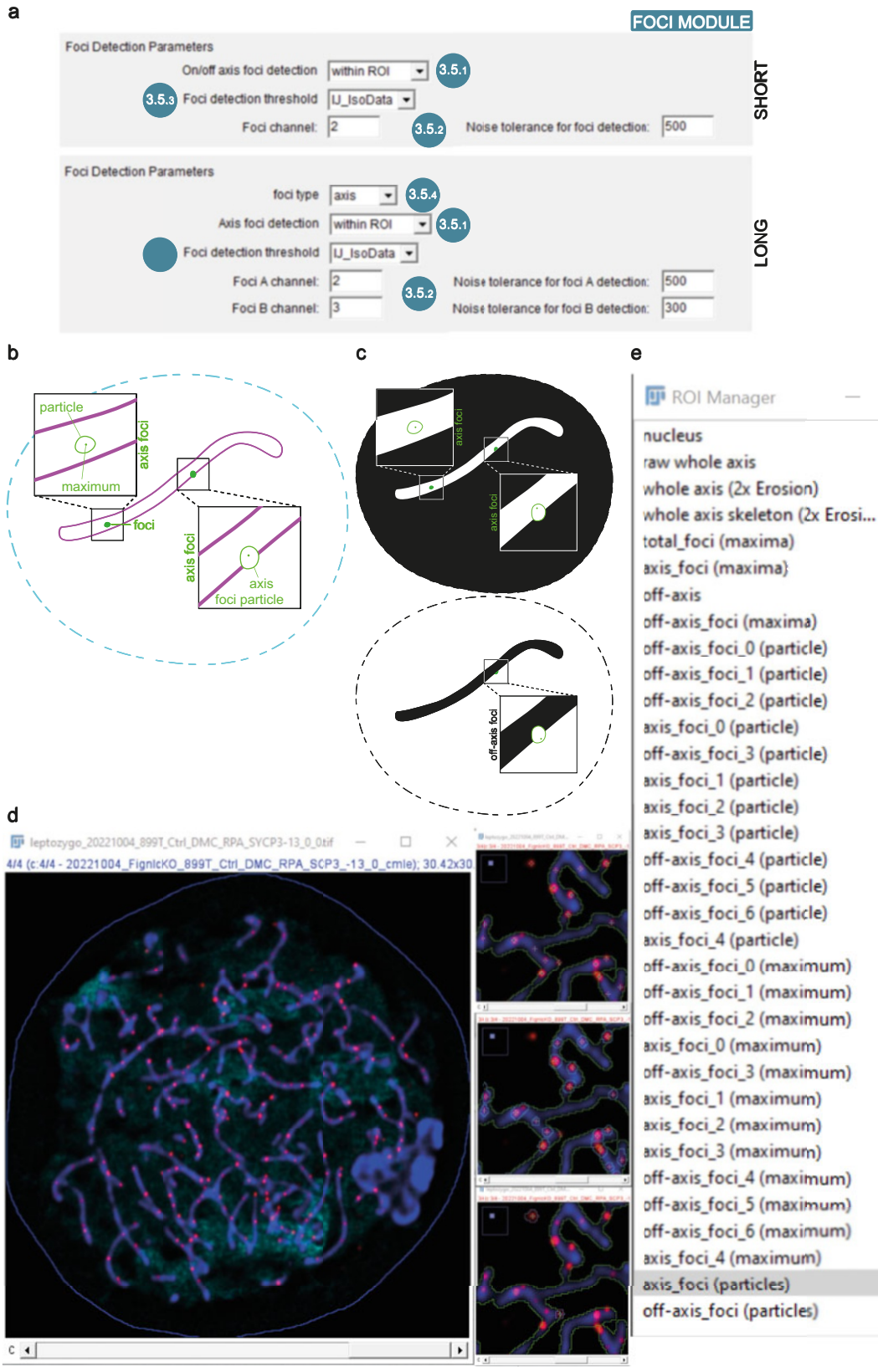

Fig. 5 Foci module. (**a**) Step-by-step description of the short (1 color, top) or long (2 colors, bottom) versions of the module. (**b**) Foci detection using the within ROI method. Foci are identified through maximum and particle

their neighboring local maximum minus the selected prominence are considered as part of the focus particle. The second method uses an automatic threshold to identify particles, independent of each maximum's intensity. Additional automatic thresholds can be applied if needed, as specified in the manual.

The algorithm generates a temporary "total particles" ROI that is further split into individual particles to compare them with the axis or off-axis maxima selections. Each individual particle ROI that matches with an axis (or off-axis if analyzed) maximum gets a new ID, and a matching maximum selection is also created. Both particle and associated maximum share the same ID.

Importantly, the maximum of, for example, an axis focus is necessarily within the axis ROI, but the associated focus particle may extend outside of the axis ROI (examples are shown in the insets on the right-hand side of all panels of Fig. 5b, c). This is a key feature of the foci module. Identifying particles is time-consuming and may be skipped (by unchecking "Show individual foci's values" of the "Set measurements parameters" menu in the main meiosis bar).

4. With the extended version of the module, such as in the "Count axis/off-axis foci in 2 channels and colocalize" tool (Fig. 1e), the analysis is limited to one specific type of foci (either axis or off-axis) that is selected with the "Foci type" button (Fig. 5a). To analyze both types of foci in two channels on the same series of images, run the analysis separately for each type of foci.

3.6 Colocalization Module

The colocalization module (Fig. 6b) offers several options to analyze the colocalization of foci within two channels (termed fociA and fociB channels) and assess its significance. This analysis locates every focus from each channel at the position of its maximum and measures pairwise distances between focus maxima in fociA and fociB channels (Fig. 6a). Two foci are considered as colocalized when their maxima are closer to each other than a defined "threshold distance." It is recommended to set the minimal resolution

Fig. 5 (continued) selections (top inset). The location of the foci is defined by its maximum, while the particle is associated with its maximum and can spread outside/inside the axis (bottom inset). (**c**) Foci detection using the "using mask" method. (**d**, **e**) Example image and associated ROIs of the foci module, showing single particle selections, along with their corresponding single maximum selections (**e**). (**d**) Main panel: whole image with DAPI (cyan), SYCP3 (blue), RPA (red), and user reference ROI (blue line). Top inset: axis ROI (green line), axis focus maxima (crosses with green center), off-axis focus maxima (crosses with red center); middle inset: axis ROI (green line), axis focus maxima (crosses), axis focus particles (blue lines); bottom inset: axis ROI (green line), off-axis focus maxima (crosses), off-axis focus particles (purple lines)

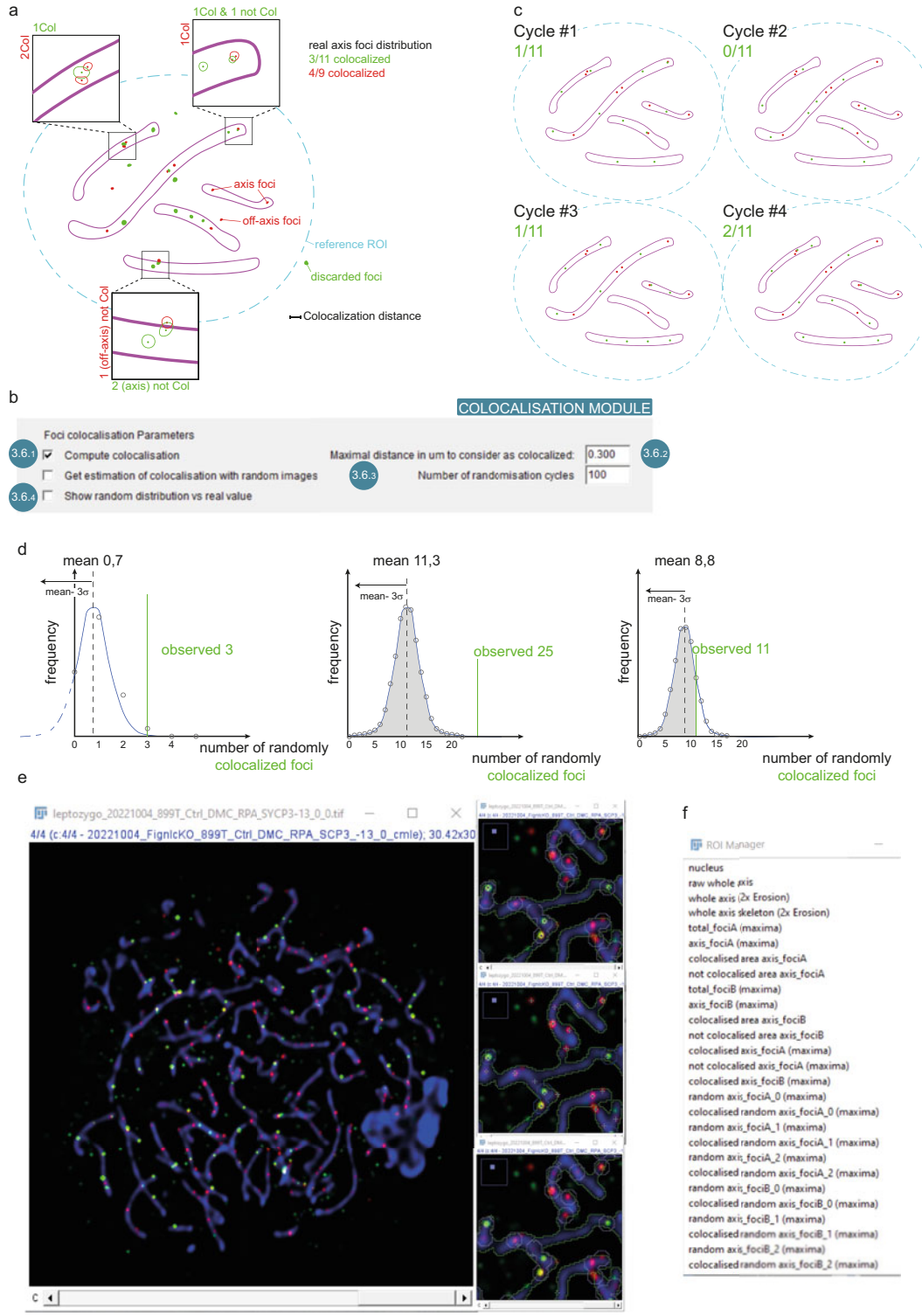

Fig. 6 Colocalization module. (**a**) The number of colocalized foci can be different for the two channels (top insets). No colocalization is detected when an axis focus of one channel colocalizes with an off-axis focus of the other channel (bottom inset). Colocalization is analyzed using maxima (upper-right inset). (**b**) Step-by-step

distance of the microscope setup as colocalization threshold distance, typically 230–250 nm for a 1.4 NA lens and a widefield setup. The reason behind this is that two foci from the same channel, closer than the minimal resolution distance, cannot be distinguished as different [17]. The minimal resolution distances can be either theoretical resolution distances [18] or measured actual values [19].

1. To analyze foci colocalization, enable the "Compute colocalization" checkbox (shown in Fig. 6b). Because it is combined with the long version of the foci module (bottom panel of Fig. 5a), the colocalization module only compares two sets of foci of the same type (either axis foci or off-axis foci), as selected at Subheading 3.5, **step 4**. Even if a green (fociA channel) axis focus is actually within the colocalization distance of a red (fociB channel) off-axis focus, they will still not be considered as colocalized (Fig. 6a, lower inset). The tool "Count foci in two channels and colocalize" allows for assessing the colocalization status of all foci within a nucleus (precisely, within a reference ROI, *see* Subheading 3.2), with the shortcoming of ignoring the axis/off-axis status of the foci.
2. Specify a threshold distance (in μm) below which two foci will be considered colocalized (Fig. 6c). To measure the distance to the nearest maximum of the other channel, a Euclidean distance map is calculated, as explained in the comprehensive manual. A "colocalized area" selection is then produced in the ROI manager (Fig. 6f). Maxima of fociA channel foci that fall within the fociB channel "colocalized area" selection are classified as fociA colocalized foci, numbered and added to the fociA colocalized maxima selection, and vice versa to build a selection of fociB foci colocalized with fociA foci. An instance of colocalization analysis is shown in Fig. 6e. The numbers of colocalized foci in two channels may differ when two or more foci are within colocalization distance of the same focus from the other channel (insets in Fig. 6a) (*see* **Note 6**).

Fig. 6 (continued) description of the colocalization module. (**c**) Example of four cycles of randomization of the green foci within the axis ROI, with the number of randomly colocalized green foci indicated in the upper-left corner of each image. (**d**) Example of random foci colocalization count distributions (gray circles), with a blue line representing a Gaussian fit. Mean randomly colocalized values are indicated. Left: "clipped" distribution, middle and right: normal distributions. Middle: The *p*-value shows that the observed, real value cannot be obtained randomly. (**e**, **f**) Example of a two-colors foci colocalization image and generated ROIs. (**e**) Main panel: whole image of same nucleus as in Fig. 5d, with SYCP3 (blue), DMC1 (green, fociA channel), and RPA (red, fociB channel). Top inset: axis ROI (green line), FociA total focus maxima (crosses with pink center), FociB axis focus colocalization area ROI (purple circles); middle inset: axis ROI (green line), FociB total focus maxima (crosses with green center), FociA axis focus colocalization area ROI (blue circles); bottom inset: as in top inset, but with randomly generated axis fociA focus maxima (crosses with green center)

3. Random colocalization. A certain level of observed colocalization, expressed as the ratio of, for example, the number of axis fociA that are colocalized with axis fociB to the total number of axis fociA, might occur by chance. To estimate in each nucleus the level of colocalization expected to occur by chance and the significance of the observed colocalization level, the colocalization module contains an option to generate multiple random focus distributions in each channel (Fig. 6b). To activate it, tick the "Get estimation of colocalization with random images" checkbox (Fig. 6b). Then, foci of a given channel (e.g., fociA) are shuffled within the right region of interest (i.e., either the whole axis ROI when selected foci type at Subheading 3.5, **step 4** is "axis," or the complementary off-axis ROI if foci type is set to "off-axis"). Finally, the location of each randomly localized fociA focus (as shown in Fig. 6c for four randomization cycles) is compared with the experimental localizations of fociB foci. The process is repeated for a custom number of randomization cycles (Fig. 6b). It is recommended to run at least 30 randomization cycles, to increase the chance for the number of colocalized randomly localized foci to fit a normal distribution (*see* next Step). By default, the tool displays in the ROI manager three random focus distributions for both channels, but this can be changed in the "Measurements parameters" menu (*see* Subheading 3.7, **step 2** and the comprehensive manual). The mean number of randomly colocalized foci of a given channel is then calculated (Fig. 6d). This process is run for every nucleus of the selected series.
4. Calculation of the *p*-value. To assess the significance of the observed colocalization proportion in every nucleus, select the "Show random distribution vs real value" option (Fig. 6b). If the mean number of randomly colocalized foci is greater than zero, and assuming a normal distribution, a frequency plot is drawn. If the plot is not clipped (left panel in Fig. 6d), a Gaussian fit is applied (blue line in every panel of Fig. 6d) and a *p*-value calculated (gray area below the blue line). Refer to the comprehensive manual for the calculation details. A *p*-value equal to 1 (panel in Fig. 6d) means that the observed distribution cannot be obtained by chance. In other cases (left and right panels in Fig. 6d), more advanced statistical tests are needed. The frequency plot can be saved as a .tif file (in the "controlData" folder) and reopened with ImageJ to obtain the plot's coordinates (by clicking the "List" button) for a more thorough statistical examination.

3.7 Intensities Module

The previous modules are focused on measuring the axis/SC length or the number of foci, with the option of analyzing focus colocalization. In addition, intensities of axes/SC or foci can be

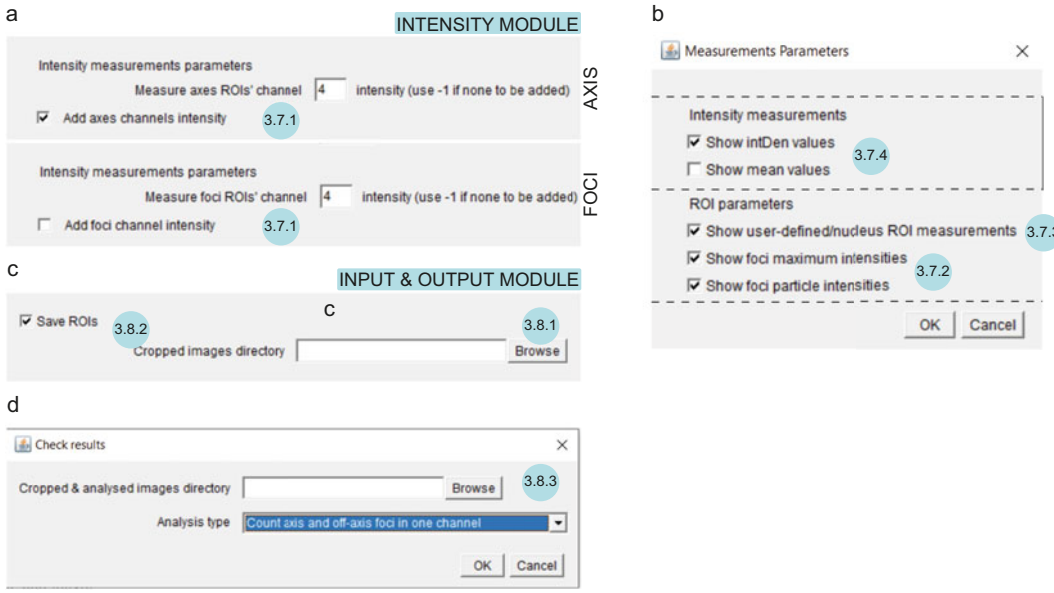

Fig. 7 Intensity and "input & output" modules. (**a**, **c**) Step-by-step description of the intensity (**a**) and "input & output" (**c**) modules. (**b**) Truncated General parameters window showing the additional measurements and ROI parameters used for intensity measurements, with other checkboxes hidden (dashed lines). (**d**) The Check Results window is triggered by clicking the "Start checking results" button on the main meiosis bar, allowing users to review the accurate detection of features

measured with the intensity module. Users need to specify the channels, selections, and measurement methods for intensity measurement.

1. *Channels.* To measure intensities in a given channel, use either the "Axis" or the "Foci" version of the intensity module (top and bottom panels of Fig. 7a, respectively). Users can include the channels used for segmenting the axes (whole axis channel, synapsed/non-synapsed axis channel if any) or foci (one or two channels) by selecting the checkbox "Add axes channels intensity" or "Add foci channels intensity," respectively.
2. *Selections.* The feature ROI is automatically included in the selection list whenever a channel measurement is requested in previous step. For example, with axis tools, the intensities of the axis channel within the axis ROI (and within the synapsed/unsynapsed axis ROI when relevant) are measured. When using foci tools, focus intensities are measured, with the choice of measuring intensities of focus maxima or focus particles. To do so, before running any focus tool, click the "Set measurements parameters" button in the General menu window (Fig. 1a), which opens the measurement parameters window. A truncated version of this window is shown in Fig. 7b. Specific

checkboxes in ROI parameters section permit to add/remove a foci selection type (Fig. 7b).

3. *Other selections.* By default, intensity measurements are taken within the reference selection obtained by the reference ROI module (Subheading 3.2). However, users can skip through the ROI parameters checkbox "show user-defined/nucleus ROI measurements" (Fig. 7).
4. In the Measurements Parameters window, users can choose between several measurement methods (Fig. 7b). Integrated densities (intDen, the sum of uncalibrated raw intensity values for all pixels within the selection) are used by default, but mean values measurement (mean of the uncalibrated raw intensity values for all pixels within the selection) can be selected with the appropriate checkbox in the "Intensity measurement" section. The intensity value of a maximum is always measured as integrated density, as it is a point (single pixel) selection. When using a multipoint maxima selection, the "intDen" value is the sum of the selected pixel integrated densities.

3.8 Input & Output Module

All analysis tools in the meiosis macro share a common "input & output" module. Users can also choose to save the ROIs if they want to check the accuracy of feature identification. All tools share the same module.

1. The first step consists in specifying the input folder that contains the images to analyze ("cropped images directory" field, Fig. 7c) that should have already gone through the determination process outlined in Subheading 3.1, as the .roi file may be necessary for the reference ROI module (Subheading 3.2).
2. Users can choose to save the ROIs to check the accuracy of feature identification (Fig. 7c).
3. If the ROIs were saved, use the "Start checking results" button on the General menu window (Fig. 1a) to verify visually the accuracy of the results. The Check Results window displays the correct settings (i.e., the folder containing the series of analyzed image files in "Cropped and analyzed images directory" and the performed analysis in "analysis type") if the tools were used recently. The first analyzed image is displayed, along with the ROIs, and users can scroll through the different generated selections in the ROI manager to ensure that their detection is accurate. To move on to next image, click the "Check next result" button (Fig. 1a).

4 Notes

1. Alternative techniques than widefield microscopy can be used to generate images, such as Grid Projection Microscopy (aka apotome), or confocal microscopy can be used. Super-resolution images are also an option. Keep in mind that the meiosis bar macro was originally designed for widefield images. If a different technique is used, parameters may need adjustment. Confocal and STED microscopy can produce high photon shot noise, so additional processing may be needed for accurate results (as included in the meiosis bar macro). Moreover, as synaptonemal complex lateral elements (i.e., axes) get separated using STED or SIM microscopy, length measurements parameters should be adapted.
2. For routine experiments, staging criteria were as follows using the staining of SYCP3 axial protein, γH2AX DSB marker, and SYCP1 central element protein. Pre-leptotene nuclei had weak SYCP3 nuclear signal and no or very weak γH2AX signal and no SYCP1 signal; early leptotene nuclei were γH2AX-positive and with only short SYCP3 fragments and no SYCP1 signal; mid leptotene nuclei were γH2AX-positive and with short and long SYCP3 fragments and no SYCP1 signal; late leptotene were γH2AX-positive and with only long SYCP3 fragments; zygotene nuclei had long SYCP3 fragments, partially synapsed homologs with SYCP1 signal and were γH2AX-positive; and pachytene cells had all 19 autosomes fully synapsed with long SYCP3 signal that overlap with SYCP1 and γH2AX restricted to the sex chromosomes. Diplotene stage have desynapsed chromosomes with long SYCP3, which overlap in the remaining synapsed portion with SYCP1 and γH2AX-positive restricted in the sex body.
3. Folder or file name from the root of the folder hierarchy to image files must not contain any space, otherwise the images are not considered. For this reason, in Subheading 3.1**, step 6**, the stage prefixes must contain no space (use underscores instead).
4. In Subheading 3.5, **step 1**, if "within mask" mode is chosen, the intensity of all pixels outside the selected ROI (axis or off-axis) is set to 0. In some image acquisition settings, the default baseline intensity may be set to a value significantly different from 0 (high photon shot noise). For instance, with our STED acquisition setting (Abberior Instruments), the baseline is set to the value of 32,768, and signal intensities do not reach values higher than a few hundred units above that level. In that case, "within mask" mode does not allow

detecting axes or foci reliably, and "within ROI" mode must be chosen.

5. For counting or colocalizing foci, the focus maxima is sufficient. If measurements of focus areas or integrated intensities are involved, focus particles should be defined. In this case, it is essential to use an automatic threshold in order to define the focus particle boundaries independently of the focus maximum intensity. Otherwise, the particle boundaries are determined only from the set "noise tolerance," i.e., the absolute intensity difference with the value at the maximum, which results often in stronger foci being associated to a smaller area particle. Select a "Foci detection threshold" different from "None" in the foci module (Subheading 3.5, **step 3**). Particle areas are not showed in the output, but can be determined easily as the ratio of integrated intensity over mean intensity.
6. An important aspect of the colocalization algorithm (Subheading 3.6, **step 2**) is that colocalization is determined only by the location of the focus maxima. During the process of identifying individual focus particles (as described in Subheading 3.5, **step 3**), any particle that matches a colocalized maximum will be considered colocalized. As a result, the tool does not perform any "particle" colocalization analysis, such as the overlap-based calculation found elsewhere [17]. Even if the particle of one fociA channel focus does not overlap with the maximum of one foci B channel focus, if their maximum-to-maximum distance is below the distance threshold, the particle will be deemed colocalized (for instance, the green particle of the upper left inset of Fig. 6a is considered colocalized, even though the red maximum does not match the green particle, because the red-to-green maxima distance is below the "colocalization distance").

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