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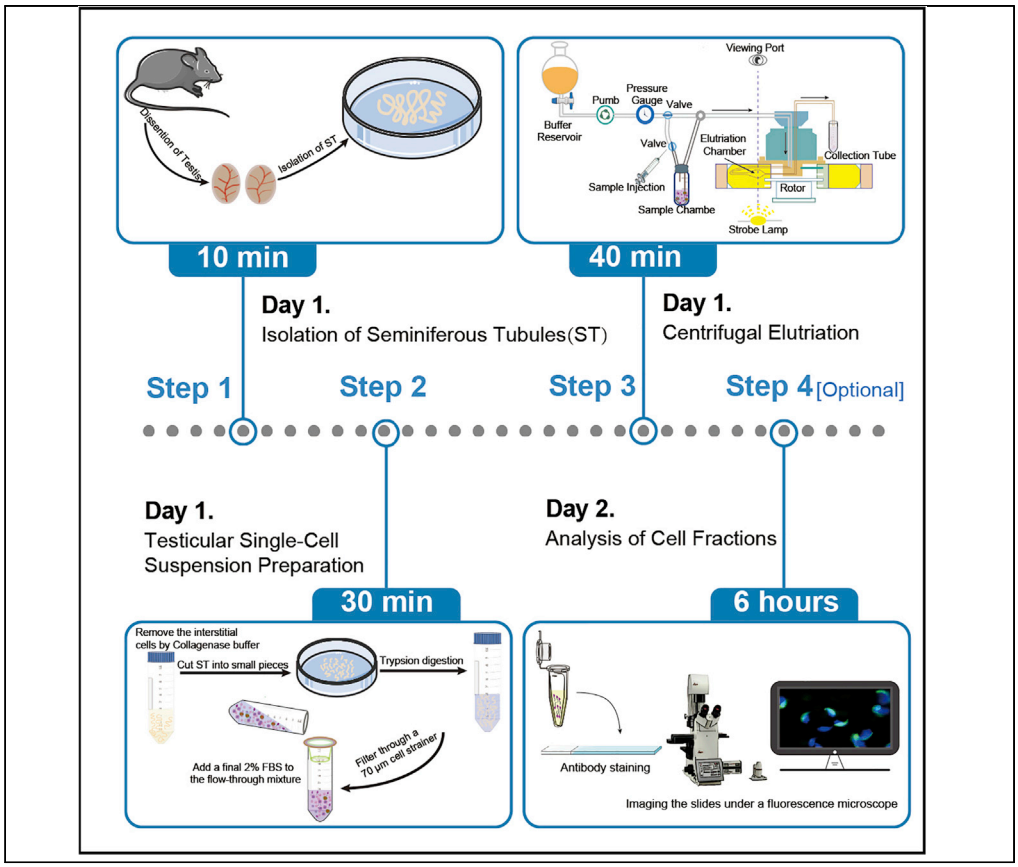


Germline development is challenging to study due to the diversity of cell types in mammalian testis. Here, we present an optimized protocol, namely centrifugal elutriation, that allows the simultaneous isolation of mouse germ cells at different stages with high purity within ~2 h. This approach exploits the JE-5.0 centrifugal elutriation system that fractionates cells based on differential sedimentation gravity. We herein provide the optimized parameters and procedures for isolation of elongating spermatids, round spermatids, and pachytene spermatocytes from adult mouse testes.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Highlights

Optimized protocol for efficient preparation of testicular single-cell suspension

Rapid purification of three germ cell populations with high viability from mouse testes

Easily scaled up and down depending on downstream applications

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# Optimized protocol for isolation of germ cells from mouse testis by centrifugal elutriation

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## SUMMARY

**Germline development is challenging to study due to the diversity of cell types in mammalian testis. Here, we present an optimized protocol, namely centrifugal elutriation, that allows the simultaneous isolation of mouse germ cells at different stages with high purity within ~2 h. This approach exploits the JE-5.0 centrifugal elutriation system that fractionates cells based on differential sedimentation gravity. We herein provide the optimized parameters and procedures for isolation of elongating spermatids, round spermatids, and pachytene spermatocytes from adult mouse testes.**

**For complete details on the use and execution of this protocol, please refer to Bao et al. (2018).**

## BEFORE YOU BEGIN

In mammals, the germ cell development undergoes three successive processes - mitotic proliferation (spermatogonia), meiotic division (primary and secondary spermatocytes), and haploid development (round and elongating/elongated spermatids). Over the past decades, several approaches have been developed to purify one population or several specific developmental stages of germ cells from the murine testis. For example, the STA-PUT, which is based on the differential sedimentation velocity, is most commonly adopted since it is easy to set up and is less costly; However, this approach usually takes 3–6 h and is vulnerable to external disturbance (Bryant et al., 2013; Kim et al., 2021). Flow cytometry usually produces a highly purified population of germ cells, but the yield is low and is technically demanding (Valles and Huynh, 2020). In this protocol, we present an optimized, highly reproducible protocol, to simultaneously isolate different stages of germ cells from adult mouse testis within a ~2-h time frame. For specific applications that require highly purified populations of sub-stage meiotic cells from juvenile mouse testes, the parameters are also appended in this protocol.

### Preparation of solutions

Required solutions used in this protocol can either be prepared in advance and stored as indicated, or made fresh on the day of the experiment. Please refer to the materials and equipment for a complete list of solution recipes.

### Institutional permissions

All mouse experiments were reviewed and approved by the Animal Care and Use Committee of the University of Science and Technology in China (USTC). The operations in mice complied with institutional rules.

### Preparation for equipment

Timing: 10 min

During the intervals of single-cell preparation, one can set up the elutriation machine concurrently to shorten the waiting time before single-cell loading.

1. Machine Set-up.
   a. Set an incubator to 37°C.
   b. Set up and verify the tubing system following the manufacturer's instructions (Beckman Avanti J-26S XP elutriator equipped with JE 5.0 elutriation systems and a standard elutriation chamber).
   c. Rinse the elutriator system with at least 500 mL sterile water (1000 rpm, 20 mL/min). Then replenish with elutriation buffer (>200 mL). Turn on/off the centrifugation repeatedly for several times and push out any visible bubbles.

   *Note:* To fully eliminate potential bubbles inside the tubing system, start the centrifuge for running at 1000 rpm for a while (e.g., 5 min), and then turn it off while the pump continues running. Repeat this process for several times until no visible bubbles are present.

2. Reagent Set-up.
   a. 1 L of sterile water.
   b. Prepare 100 mL of 6% Hydrogen peroxide.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Lectin PNA From Arachis hypogaea (peanut), Alexa Fluor™ 488 Conjugated; Working dilution 1:1000 | Invitrogen | MEF#L21409; RRID: AB_2315178 |
| DDX4, VASA Polyclonal antibody; Working dilution 1:200 | Proteintech | Cat#51042-1-AP; RRID: AB_2092998 |
| SYCP3 Polyclonal antibody; Working dilution 1:400 | Proteintech | Cat#23024-1-AP; RRID: AB_11232426 |
| Anti-phospho-Histone H2A.X (Ser139) antibody; Working dilution 1:800 | Millipore | Cat#05-636-I; RRID: AB_2755003 |
| CoraLite 488-conjugated Goat Anti-Mouse IgG(H+L); Working dilution 1:500 | Proteintech | Cat# SA000013-1; RRID: AB_2810983 |
| Rhodamine(TRITC)-conjugate Goat Anti-Rabbit IgG(H+L); Working dilution 1:500 | Proteintech | Cat#SA00007-2; RRID: AB_2889939 |
| Chemicals, peptides, and recombinant proteins | | |
| Collagenase Ⅳ | Sigma-Aldrich | Cat#C5138 |
| Trypsin | Sigma-Aldrich | Cat#T9201 |
| DNase I | Sigma-Aldrich | Cat#DN25 |
| Fetal Bovine Serum | Gibco | Cat#10099-141 |
| Bovine Serum Albumin | Sigma-Aldrich | Cat#A2153 |
| HBSS- with $Ca^{2+}$/$Mg^{2+}$ | Servicebio | Cat# G4204 |
| HBSS-without $Ca^{2+}$/$Mg^{2+}$ | Servicebio | Cat# G4203 |
| PBS | Servicebio | Cat# G4202 |
| 0.5 M EDTA | Sangon Biotech | Cat# B540625 |
| Sodium chloride | Sigma-Aldrich | Cat#S9625 |
| Sodium bicarbonate | Sigma-Aldrich | Cat#S8875 |
| Potassium chloride | Sigma-Aldrich | Cat#P4504 |
| D(+)-Glucose monohydrate | Sigma-Aldrich | Cat#49159 |
| Magnesium sulfate heptahydrate | Sigma-Aldrich | Cat#M1880 |
| Calcium chloride dihydrate | Sigma-Aldrich | Cat#C3881 |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| Potassium phosphate monobasic | Sigma-Aldrich | Cat#P5379 |
| 60 mm cell culture and tissue dishes | Jetbiofil | Cat#TCD010060 |
| ProLong™ Diamond Antifade Mountant with DAPI | Invitrogen | Cat# P36971 |
| 4% PFA in PBS | Aladdin | Cat#P395744 |
| 0.4% trypan blue | Solarbio | Cat#C0040 |
| IHC PAP pen | Abcam | Cat#BC005 |
| Triton X-100 | Sigma-Aldrich | Cat#9036-19-5 |
| Hydrogen peroxide 30% aqueous solution | Sinopharm | Cat#10011218 |
| Tween-20 | Beyotime | Cat#ST825 |
| Experimental models: Organisms/strains | | |
| Mouse: C57BL/6 male mice | Beijing Vital River Laboratory | n/a |
| Software and algorithms | | |
| GraphPad Prism 8 software | GraphPad | https://www.graphpad.com |
| ImageJ software | National Institutes of Health | https://imagej.net/software/fiji/ |
| Other | | |
| 70 μm Cell Strainer | Biosharp | Cat# BS-70-CS |
| Plastic tubes (50 mL) | Corning | Cat#352063 |
| e-HC Electronic Pipette Filler | Bio-DL | Cat#73631451 |
| Forceps | Accuspire | Cat#55-424 |
| Large Capacity Benchtop Centrifuge Refrigerated | Herexi | Cat#TGL20MW |
| High-speed Refrigerated Micro centrifuge | DLAB | Cat#D3024R |
| Benchtop Shaking Incubator | ZHICHENG | Cat#ZWYR-240 |
| Olympus BX53 upright microscope | Olympus Life Sciences | n/a |
| THUNDER Imager Live Cell | Leica | n/a |
| Peristaltic pump | Masterflex | Model#77200-60 |
| Silicone tubing | Masterflex | Cat#96410-25 |
| Elutriator | Beckman | Avanti J-26S XP |
| Elutriation systems | Beckman | JE-5.0 |
| Elutriation Chamber | Beckman | 356943 |

## MATERIALS AND EQUIPMENT

| **10×KREBS stock: Store at 4°C** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| NaCl | 1.19 M | 139.5 g |
| KCl | 47.75 mM | 7.12 g |
| D(+)-Glucose monohydrate | 100.92 mM | 40 g |
| $MgSO_4 \cdot 7H_2O$ | 11.95 mM | 5.89 g |
| $CaCl_2 \cdot 2H_2O$ | 12.86 mM | 3.78 g |
| $KH_2PO_4$ | 11.98 mM | 3.26 g |
| $ddH_2O$ | n/a | 2000 mL |
| **Total** | **n/a** | **2000 mL** |
| Filter through 0.22 μm and autoclave, and store at 4°C for several months, pH 4.2. | | |

| **1×KREBS: Store at 4°C** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| 10×KREBS stock | n/a | 200 mL |
| $NaHCO_3$ | 25.24 mM | 4.24 g |
| $ddH_2O$ | n/a | 1800 mL |
| **Total** | **n/a** | **2000 mL** |
| 1×KREBS is prepared freshly, pH 7.6. | | |

| Elutriation buffer: Store at 25°C | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| 1×KREBS | n/a | 1796.4 mL |
| 0.5 M EDTA | 1 mM | 3.6 mL |
| BSA | 0.1%(w/v) | 1.8 g |
| **Total** | **n/a** | **1800 mL** |
| To be prepared fresh. | | |

*Note:* The supplement of a chelating agent, EDTA, serves to both inhibit enzymatic activity and reduce cell clumping by chelating $Ca^{2+}$/$Mg^{2+}$.

| DNase I stock: Store at −20°C | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| DNase I | 10 mg/mL | 10 mg |
| HBSS-with $Ca^{2+}$/$Mg^{2+}$ | n/a | 1 mL |
| **Total** | **n/a** | **1 mL** |
| DNase I stock should be stored at −20°C for long-term storage. | | |

| Collagenase stock: Store at −20°C | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Collagenase IV | 50 mg/mL | 50 mg |
| HBSS-with $Ca^{2+}$/$Mg^{2+}$ | 1 mL | 1 mL |
| **Total** | **n/a** | **1 mL** |
| Collagenase stock should be stored at −20°C for long-term storage. | | |

| Trypsin stock: Store at −20°C | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Trypsin | 100 mg/mL | 100 mg |
| HBSS-without $Ca^{2+}$/$Mg^{2+}$ | n/a | 1 mL |
| **Total** | **n/a** | **1 mL** |
| Trypsin stock should be stored at −20°C for long-term storage. | | |

| Collagenase buffer: Store at 4°C | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Collagenase stock | 0.25 mg/mL | 50 μL |
| DNase I | 5 μg/mL | 50 μL |
| BSA | 0.5 mg/mL | 5 mg |
| HBSS-with $Ca^{2+}$/$Mg^{2+}$ | n/a | 9.9 mL |
| **Total** | **n/a** | **10 mL** |

*Note:* To be prepared fresh.

| Trypsin buffer: Store at 4°C | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Trypsin stock | 1.0 mg/mL | 200 μL |
| DNase I | 5 μg/mL | 100 μL |
| HBSS-without $Ca^{2+}$/$Mg^{2+}$ | n/a | 19.7 mL |
| **Total** | **n/a** | **20 mL** |

*Note:* To be prepared fresh. This buffer does not contain $Ca^{2+}$/$Mg^{2+}$, which inhibit the enzymatic activity of trypsin.

| **Sodium citrate buffer** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Sodium Citrate | 0.01 M | 2.94 g |
| $H_2O$ | n/a | 1000 mL |
| **Total** | **n/a** | **1000 mL** |
| To be prepared fresh, pH 6.0. | | |

| **Blocking buffer: Store at −20°C** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| BSA | 5%(w/v) | 0.5 g |
| 1×PBS | n/a | 10 mL |
| **Total** | **n/a** | **10 mL** |

## STEP-BY-STEP METHOD DETAILS

### Preparation for testicular single-cell suspension

Timing: 1 h

1. Isolation of mouse testes.
   a. Sacrifice two adult mice by cervical dislocation or isoflurane inhalation in a closed chamber. Wet the lower abdomen by spraying with 70% Ethanol, and use a surgical scissor and forceps to open the abdominal cavity (Figure 1A).
   b. Pull out the perigonadal white adipose tissue (pgWAT) with a tweezer. Dissect the two whole testes off the epididymis, remove any leftover fat, and keep them in cold 1×KREBS in a 60 mm dish on ice.
   c. Under dissection microscopy, carefully remove the tunica albuginea with a pair of sharp forceps one by one for each testis, and pool them in a fresh drop of 500 μL 1×KREBS (Figure 1C).
2. Testicular Germ Cell Suspension Preparation.

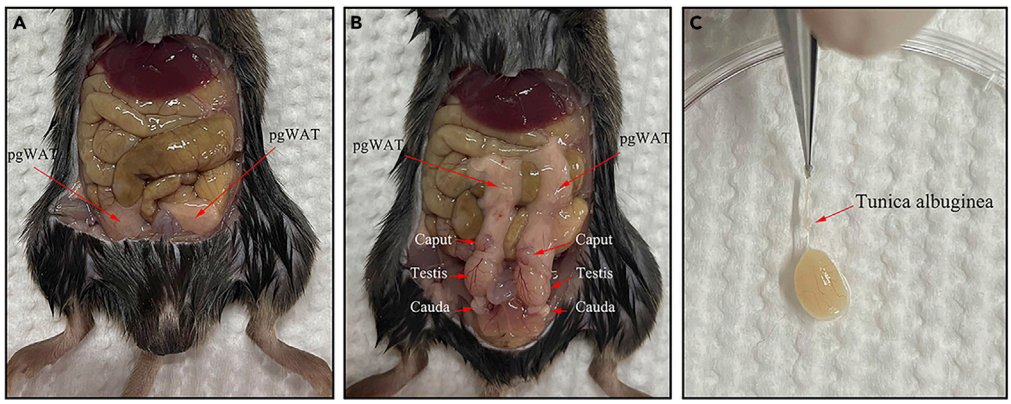


**Figure 1. Dissection of the mouse testis**

(A) Exposure of the perigonadal white adipose tissue (pgWAT) after opening the lower abdomen of an adult male mouse.

(B) Exposure of the testis and the cauda epididymis by pulling out the pgWAT with forceps.

(C) Morphology of the testis after peeling off the tunica albuginea.

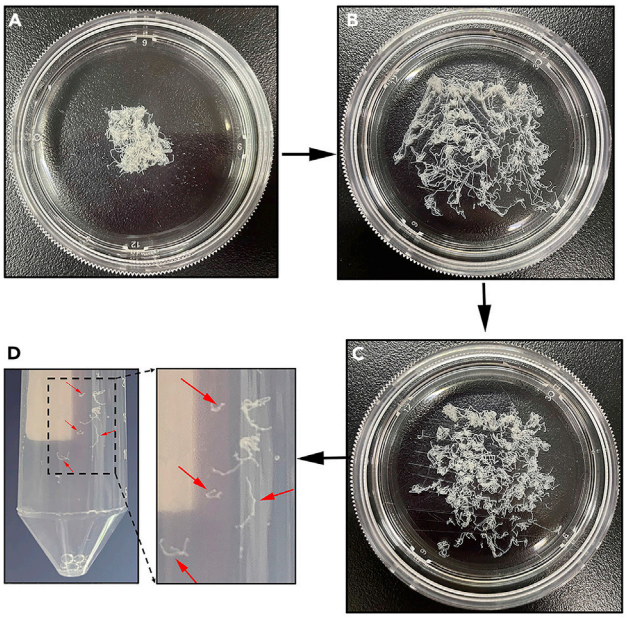


**Figure 2. Digestion of the seminiferous tubules into single-cell suspension**

(A) The morphology of the well-dispersed seminiferous tubules after removal of the tunica albuginea.

(B) The morphology of the intact seminiferous tubules following the removal of interstitial cells through digestion by Collagenase IV.

(C) Manual fragmentation of the seminiferous tubules using a sharp blade before trypsin digestion.

(D) The turbid solution owing to single-cell release and the residual fragmented seminiferous tubules after initial trypsin digestion. Red arrows point to small pieces of undigested seminiferous tubules.

a. Briefly pull apart the seminiferous tubules using a pair of forceps to expose the interstitial cells (e.g., Leydig cells). See Figure 2A for an example of slightly dispersed seminiferous tubules.
b. Transfer the spermatogenic tubules to a 50 mL centrifuge tube containing 10 mL of freshly prepared collagenase buffer.
c. Incubate the centrifuge tube in a 37°C incubator at 80 rpm for 5 min.

*Note:* This step is to roughly digest the interstitial cells out of the seminiferous tubules. Do not over-digest the tubules, which might cause over fragmentation resulting in a loss of germ cells. It is beneficial to gently disperse the tubules by pipetting up and down several times briefly with a wide-bore tip (by cutting out the end of a 1 mL tip).

d. Top up the collagenase buffer to a final 40 mL with fresh ice-cold 1×KREBS, and let the tubes stand straightly on ice to allow the tubules to settle down for ~1 min.

*Note:* Watch carefully, and once the tubules all settle down to the bottom of the tube, carefully discard the supernatant using a 10 mL pipetman.

e. Add fresh 40 mL ice-cold 1×KREBS solution and gently mix. Repeat this wash step about 2–3 times until the supernatant turns from turbid to clear.

*Note:* The initial supernatant consists of abundant interstitial cells, e.g., Leydig cells, and thus looks highly turbid. During a short time frame of settlement on ice, the big chunk of tubules settles down faster to the bottom while the interstitial cells remain in the supernatant. Therefore, repeated wash with fresh 1×KREBS will eliminate interstitial cell contamination. Do not let the tube stand on ice too long, which might cause concurrent sedimentation of somatic cells.

f. After the last wash, carefully discard the residual 1×KREBS, transfer the tubules-containing solution into a 60 mm dish, use forceps to disperse the seminiferous tubules gently, and cut them into small pieces with sterile surgical blades. See Figure 2 for the representative morphology of the dispersed tubules before (B) and after cutting (C).

*Note:* This step is optional. However, it will enhance the digestion efficiency for trypsin enzyme in the following steps, and thus increase the germ cell yield.

g. Add 10 mL of freshly prepared trypsin buffer (1 mg/mL of trypsin in $Ca^{2+}$/$Mg^{2+}$-free 1×HBSS, plus 5 μg/mL DNase I), mix gently and place it in a 37°C incubator and shake for 10 min (~80 rpm).

*Note:* At this step, the buffer has no $Ca^{2+}$/$Mg^{2+}$, which guarantees the optimal reaction milieu for trypsin digestion.

h. Add 10 mL of 1×HBSS (1×HBSS with $Ca^{2+}$/$Mg^{2+}$, and plus 50 μL DNase I stock), then gently disperse the digested tubules with a 1 mL wide-bore tip every five minutes, shake for 10–20 min.

⚠ CRITICAL: Check the condition of the tubules. Once the solution turns cloudy and only a tiny fraction of fragmented tubules is present (Figure 2D), transfer the tube on ice and let the tubules settle down.

*Note:* We found that the gentle pipetting greatly facilitates the trypsin digestion and thus boosts the cell yield. In addition, it is critical to use narrow-bore tips when tubules are fragmented into small pieces. Do not over-digest the cells, which likely causes cell death and DNA release resulting in cell clumping.

i. Carefully transfer the supernatant containing single cells to a new 50 mL tube and quench the trypsin digestion by adding a final 2% FBS.
j. To the residual cell pellet containing undigested tubules, add ~5 mL of freshly prepared trypsin buffer, mix gently and place it in a 37°C incubator and shake for 5–10 min (~80 rpm).
k. Combine both trypsin-digested single-cell suspensions into one 50 mL tube. Pellet the cells by centrifugation at 700 g for 5 min at 4°C. Gently discard the supernatant.

*Note:* Centrifugation with a swinging bucket rotor at low speed will preserve the viability of germ cells.

l. Resuspend the cell pellet in 10 mL elutriation buffer supplemented with DNase I (5 μg/mL), gently pipetting up and down.

*Note:* Use the elutriation buffer containing BSA. It helps reduce the loss of germ cells throughout the buffer flowing through the elutriation tubing system. In addition, the supplement of DNase I is helpful in preventing the cell clumping before and during centrifugal elutriation.

m. (Optional) Add 90 μL of the germ cell suspension to 10 μL trypan blue (0.04%), incubate for 3 min, then take 10 μL of the cell suspension and count the number of living and dead cells under a microscope.
n. Filter through a 70 μm cell strainer. Add a final 2% FBS to the flow-through mixture. Keep on ice before elutriation.

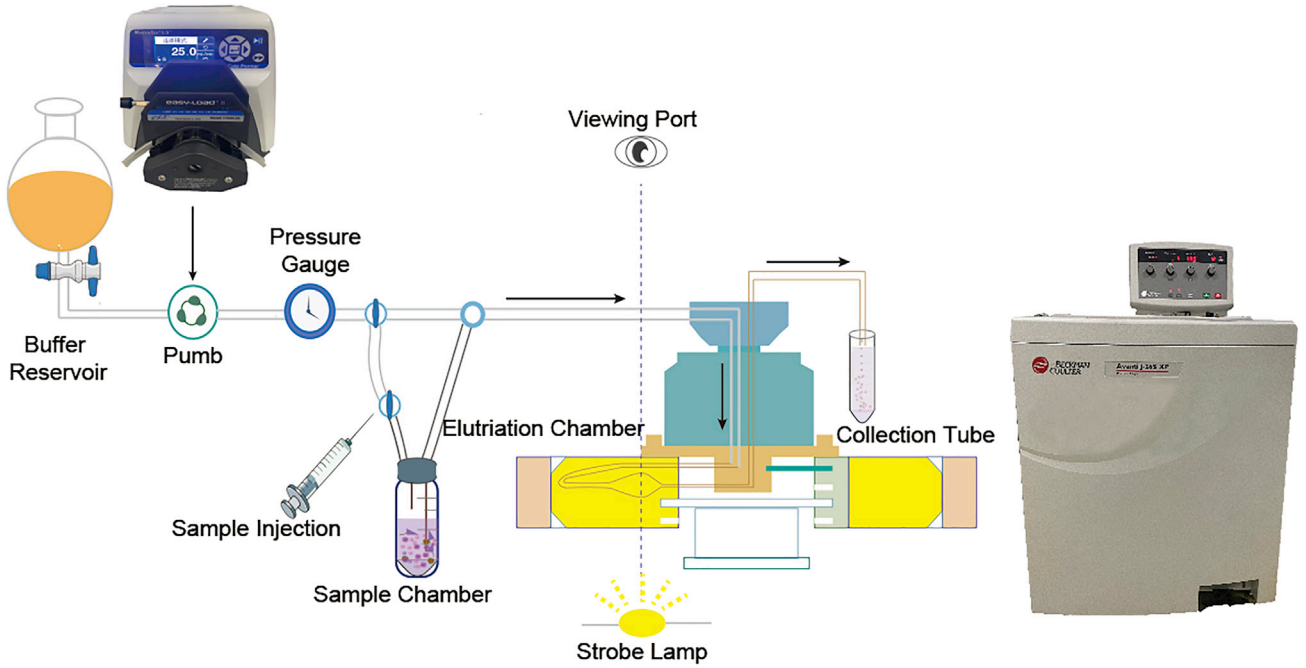


**Figure 3. Schematic diagram of the tubing pipeline connecting the buffer reservoir, sample chamber, and elutriator in the JE5.0 elutriator system (Beckman Coulter)**

Single cells are sequentially purified into different fractions based on their distinct sedimentation velocity as implemented by the centrifugal force and pumping.

*Note:* At this step, we recommend going straightly to the next step for elutriation without stop because long-term storage of single-cell suspension on ice will reduce cell viability and cause cell clumping.

## Centrifugal elutriation

Timing: 45 min

The setup of the elutriation system (Figure 3) can be accomplished among the single-cell digestion procedures. The readers are strongly recommended to refer to the operational manual of Beckman JE5.0 system prior to first-time use. The parameters provided below are specifically for purification of germ cells from 2–10 adult mouse testes (from 1–5 mice).

3. Elutriation system set up.
   a. Set up the Centrifugal elutriator parameters: Speed: 3000 rpm; Temp: 20°C; Timer: HOLD Pump speed: 5.0 mL/min.

   *Note:* It is important to the buffer temperature must be constant from reservoir to elutriation chamber.

   b. Fill the buffer reservoir tank with 1.5 L of elutriation buffer.
   c. Prior to testicular single-cell suspension loading to the sample chamber, switch the 3-way bypass valve to the position that allows single-cell suspension to flow into the chamber, but the air is restrained to enter the system.

**Table 1. Elutriator settings (Standard Chamber)**

| Fraction # | RPM (centrifuge) | Volume (mL) | Flow rate (mL/min) (pump) |
|---|---|---|---|
| 1 | 3000 | 200 | 13.4 |
| 2 | 3000 | 100 | 18.2 |
| 3 | 3000 | 100 | 23.6 |
| 4 | 2250 | 150 | 19.6 |
| 5 | 2250 | 100 | 21 |
| 6 | 2250 | 100 | 23.6 |
| 7 | 2250 | 150 | 34 |
| 8 | 2250 | 150 | 40.5 |
| 9 | 2250 | 100 | 45 |

Note: The parameters provided in this table are specifically optimized for a standard chamber in the JE5.0 machine. If using a Sanderson chamber in the JE5.0 machine, the flow rate needs to be re-calculated based on the formula: [flow rate (Standard Chamber) =flow rate (Sanderson Chamber) *1.35].

*Note:* Make sure that the bypass valve is closed.

d. Set the initial flow rate of the peristaltic pump at 5.0 mL/min.

*Note:* Determine an initial flow rate that will ensure all size ranges of particles to be fractionated are retained.

e. Put the collection tube into the buffer reservoir chamber so that the buffer will circulate inside the tubing system at 4°C. Start the elutriator and the pump.

4. Elutriation process.
   a. Right before cell loading, gently pipet up and down and filter the cell suspension through a 70 μm cell strainer into a new 50 mL tube on ice.

*Note:* High cell density (>$10^8$/ mL) can cause cell re-aggregation, and the maximum volume of cell suspension can be determined according to the injection device.

   b. Open the injector valve and inject the cell suspension into the sample chamber.
   c. Once the samples are loaded, open the 3-way valve to connect the buffer reservoir chamber to the elutriator.
      i. Let the buffer run out until all the cells flow into the sample reservoir.
      ii. Once the cell suspension has completely entered the elutriation chamber, close the injection valve.
      iii. Refer to the procedure in Table 1 for cell collection.

*Note:* Just before the suspension lowers down to the bottom of the syringe, turn the bypass valve back toward the syringe to stop the sample flow. This will prevent any air bubbles from entering the line.

   d. Start fraction collection (Table 1):
      i. Fraction 1 (pump: 13.4 mL/min; elutriator: 3000 rpm): Collect four 50 mL tubes (200 mL in total): Major cell population-elongating spermatids ;
      ii. Fraction 2 (pump: 18.2 mL/min; elutriator: 3000 rpm): Collect two 50 mL tubes (100 mL in total): Major cell population-elongating spermatids;
      iii. Fraction 3 (pump: 23.6 mL/min; elutriator: 3000 rpm): Collect two 50 mL tubes (100 mL in total): Major cell population- elongating spermatids and round spermatids;
      iv. Fraction 4 (pump: 19.6 mL/min; elutriator: 2250 rpm): Collect three 50 mL tubes (150 mL in total): Major cell population-round spermatids;

v. Fraction 5 (pump: 21 mL/min; elutriator: 2250 rpm): Collect two 50 mL tubes (100 mL in total): Major cell population-round spermatids;
vi. Fraction 6 (pump: 23.6 mL/min; elutriator: 2250 rpm): Collect two 50 mL tubes (100 mL in total): Major cell population-round spermatids;
vii. Fraction 7 (pump: 34 mL/min; elutriator: 2250 rpm): Collect three 50 mL tubes (150 mL in total): Major cell population- round spermatids and pachytene spermatocytes;
viii. Fraction 8 (pump: 40.5 mL/min; elutriator: 2250 rpm): Collect three 50 mL tubes (150 mL in total): Major cell population-pachytene spermatocytes.
ix. Fraction 9 (pump: 45 mL/min; elutriator: 2250 rpm): Collect two 50 mL tubes (100 mL in total): Major cell population-pachytene spermatocytes.

***Note:*** Cell fractionation is achieved by adjusting the pump speed and elutriator speed. Collect 50 mL suspension each time using a 50 mL tube.

e. Once all fractions are collected, turn off the centrifuge, and let all leftover elutriation buffer run out.
f. Perform the system cleaning: Turn off the elutriator, and replenish the buffer reservoir tank with 100 mL of freshly prepared 6% Hydrogen peroxide, followed by 500 mL of sterile water to rinse and sterilize the tubing system.

***Note:*** This cleaning step can be executed during the waiting periods of the centrifugation in the following steps.

g. Pellet the cells by centrifugation of each fraction at 700 g for 5 min at 4°C.

***Note:*** Centrifugation using a swimming bucket rotor at low speed will preserve the viability of germ cells.

h. Carefully discard the supernatant, add 1 mL of 1×KREBS to each tube and resuspend the cell pellet. Transfer the cell suspension into a 1.5 mL centrifuge tube and repeat centrifugation at 700 g for 5 min at 4°C.

***Note:*** Depending on downstream applications, washing by 1×KREBS will eliminate BSA contamination.

i. After the last wash, carefully remove all supernatant and freeze down the cell pellet at −80°C, or leave an appropriate volume of the supernatant as needed and resuspend the cells, proceed to downstream operations (RNA extraction, protein extraction, etc).

***Note:*** To achieve optimal results, it is necessary to thoroughly sterilize and clean the whole connecting tubing system in the elutriation machine at the end of each time. This will eliminate potential bacterial fermentation or other contaminations inside the chambers and the tubes.

5. Optional: Validation of cell purity in each fraction by DAPI staining.

***Note:*** This procedure is only required for the initial validation of cell purification. Once the parameters are optimized, it can be skipped owing to the high reproducibility by centrifugal elutriation.

a. Label an appropriate number of glass slides. Draw a ring of ~1 cm in diameter with a grease pen and let the grease dry.
b. Transfer 20 μL of 4% paraformaldehyde (PFA) into each ring on the slides.
c. Immediately add 2 μL of the purified cell suspension from each fraction. Repeat for each fraction.

*Note:* Always keep all cell suspensions on ice.

d. Let the slides stand at 20°C–25°C for ~1 h until samples become dry, or cover them in a chamber and leave 8–12 h.
e. Wash slides three times for 5 min each with 1×PBS at 20°C–25°C.
f. Remove the excess buffer by gently tapping one corner of the slide on the desk or using lint-free kimwipes.
g. Aspirate ~10 μL of DAPI-containing mounting medium to cover the circled samples (Antifade Mountant with DAPI, Invitrogen).
h. Image the samples under a fluorescence microscope to evaluate the cell type enriched in each fraction.

*Note:* Different cell types can be defined by their distinct nuclear morphology as visualized by DAPI staining.

6. Optional: Indirect fluorescence staining.
   a. Spread 2 μL of cell suspension with 20 μL of fixative solution (4% PFA and 0.5% Triton X-100 in PBS) inside a grease pen-circled ring. Dry the slides at 20°C–25°C for ~1 h.
   b. After the buffer is gone, wash three times with 1×PBST (1×PBS with 0.1% Tween-20) for 5 min each at 20°C–25°C.
   c. (Optional) Perform antigen retrieval by submerging the slides in boiled 0.01 M sodium citrate buffer (pH 6.0).

   *Note:* It is not advisable to boil the slides directly in the buffer, as it likely causes cell detaching off the slides. In contrast, microwave the buffer tank until it becomes boiling (>95°C). Then immerse the slides in the boiled buffer till it cools down. Repeat this process to ensure the slides are heated > 30 min in the citrate buffer.

   d. Wash three times with 1×PBST for 5 min each at 20°C–25°C.
   e. For nuclear antigens, permeabilize the samples with 0.5% Triton X-100 in 1×PBST, for 15 min at 20°C–25°C.
   f. Wash three times with 1×PBST for 5 min each at 20°C–25°C.
   g. Block the slides with 5% BSA (Blocking buffer) for 1 h at 20°C–25°C.
   h. Remove the excess blocking buffer, incubated with primary antibodies as follows:
      i. PNA (1:1000 dilution in blocking buffer) that stains the acrosome in round and elongating spermatids;
      ii. DDX4 antibody (1:200 dilution in blocking buffer) that labels chromatoid body (CB) in round spermatids;
      iii. γH2AX antibody (1:800 dilution in blocking buffer) that recognizes sex body (XY body) in meiotic spermatocytes;
      iv. SYCP3 antibody (1:400 dilution in blocking buffer) that recognizes the lateral element of the synaptonemal complex.
   i. Incubate the slides at 4°C for 12 h in a light-proof humidified chamber.
   j. The next day, recover the slides to 20°C–25°C for 30 min. Wash three times with 1×PBST for 5 min each at 20°C–25°C.
   k. Incubate slides with secondary antibodies diluted in 5% BSA (CoraLite 488-conjugated Goat Anti-Mouse, 1:500; Rhodamine (TRITC)-conjugated Goat Anti-Rabbit, 1:500), for 1 h at 20°C–25°C in a dark humidified chamber.
   l. Wash three times with 1×PBST for 5 min each at 20°C–25°C.
   m. Remove the excess buffer by tapping one corner of the slide on the desk gently and add ~10 μL of mounting medium (Antifade Mountant with DAPI, Invitrogen). Cover the samples with appropriate coverslips.

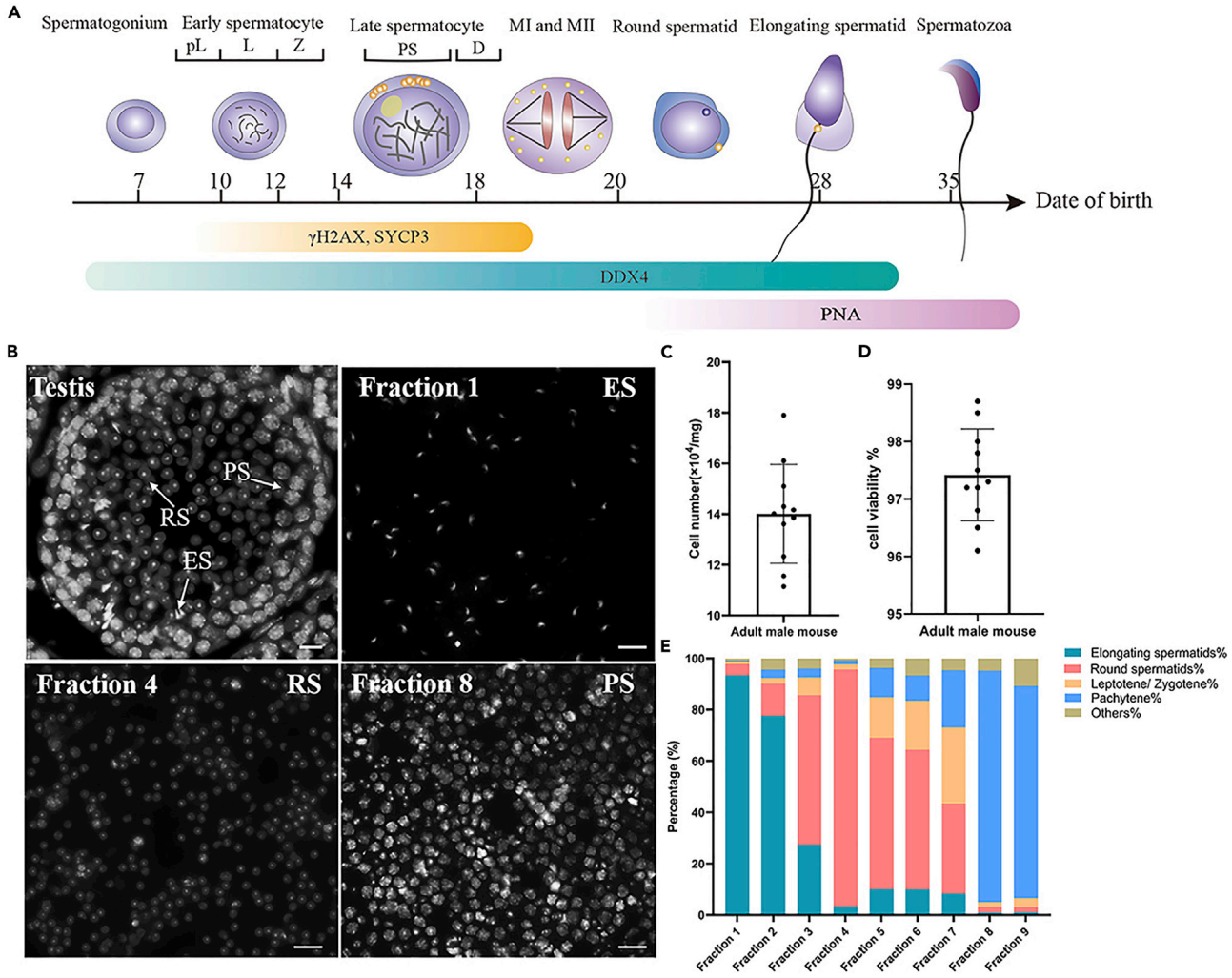


**Figure 4. Representative morphological images of the cell fractions and cell purity calculation**

(A) Schematic diagram showing the developmental sub-stages of germ cells after birth in mice. The markers delineated below were used to distinguish various sub-stages of germ cells. pL, pre-Leptotene; L, Leptotene; Z, Zygotene; PS, Pachytene Spermatocytes; D, Diplotene.

(B) Representative morphology of pseudo-colored images of DAPI-stained nuclei in testicular cross-sections and enriched fractions as labeled. ES, Elongating Spermatids; RS, Round Spermatids; PS, Pachytene Spermatocytes. Scale bars: 20 μm.

(C) The average number of germ cells purified from an adult male mouse (n=10).

(D) The average cell viability of germ cells purified from an adult male mouse (n=10). The values are defined as mean ± SD.

(E) Relative quantification of the enriched stage of germ cells in each fraction. Individual cells belonging to a specific stage as defined by γH2AX, SYCP3 and DAPI staining, including elongating spermatids, round spermatids, leptotene/zygotene, pachytene and other cells, were manually counted using the ImageJ tool (n=10).

n. Image the samples with a wide-field Leica THUNDER Imager microscopy. Images can be processed using the Image J software.

## EXPECTED OUTCOMES

We herein provide the detailed parameters and procedures for simultaneous isolation of multiple stages of germ cells from 1–5 adult male mice at one time. Single-cell preparation by collagenase IV and trypsin digestion will yield approximately 1.5 × $10^5$ cells per mg of testis (Figure 4), and the enriched percentages of round spermatids and spermatocytes were higher than 90%, respectively (Figure 4E). Owing to the highly condensed nuclei, the elongating and the elongated spermatids come out first from the elutriator chamber and are thus enriched in the initial fractions #1–2. Due

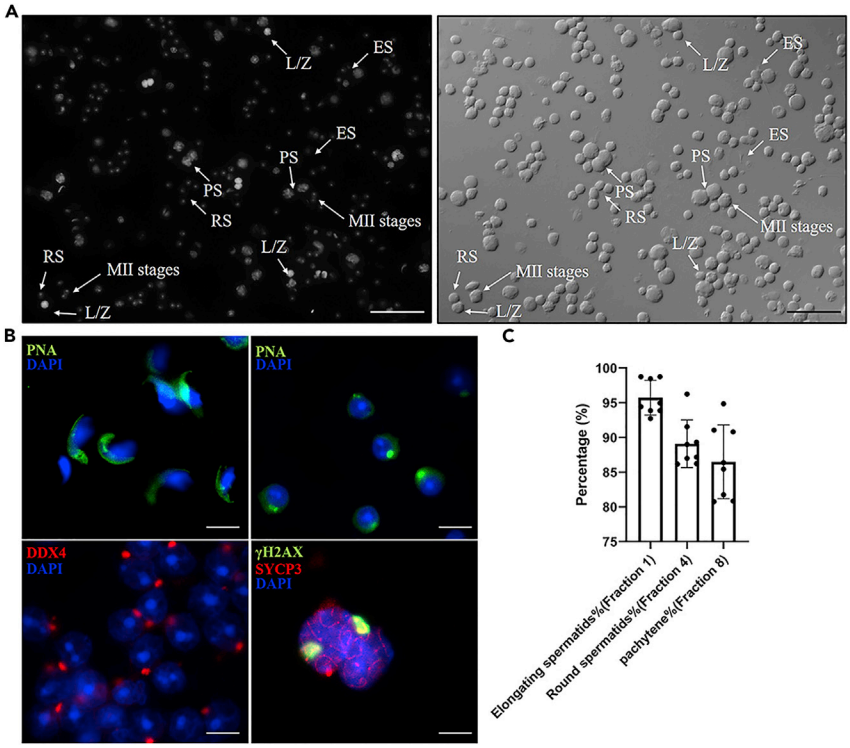


**Figure 5. Verification of the germ cell enrichment by immunofluorescence staining**

(A) DAPI-stained nuclei (left) and phase-contrast images (right) of diverse stages of germ cells prior to centrifugal elutriation. Scale bar: 50 μm.

(B) Validation of cell enrichment by immunofluorescence staining with PNA (Fraction 1 and 4), DDX4 (fraction 4), γH2AX and SYCP3 (fraction 8). Scale bars: 10 μm.

(C) Relative quantification of the enriched germ cell stages as indicated in Fraction 1, 4 and 8. Cells were manually counted using the ImageJ software. The values are defined as mean ± SD.

to the smaller size of round spermatids, they flow out faster than spermatocytes and are hence enriched in subsequent fractions #3–7. Given that round spermatids are the predominant cell type within the seminiferous epithelium in adult mouse testis, we found the percentage of round spermatids in fraction #4 enriched higher than 95% (Figures 4 and 5). Furthermore, the spermatocytes can also be readily enriched, and its purity is as high as 85% in fraction #8. Depending on the downstream applications and requirement, enriched cells in a single fraction can be directly utilized, or pooled together for some experiments that require an adequate number of cells at the cost of cell purity. Thanks to the fast elutriation process (<1 h), we have found the average cell viability is higher than 90% in all fractions.

Nonetheless, we have difficulty in purifying the leptotene/zygotene cells from adult mouse testes because they are most often intermixed with round spermatids as seen in fractions #5–7 (Figure 4E). Alternatively, in cases wherein a high purity of leptotene/zygotene cells is required, the readers can utilize juvenile male mice (Table 2). The testicular epithelium from a male mouse younger than 20 days of age exclusively comprises meiotic germ cells but is absent in round spermatids. In this scenario, we showed that the enriched percentage of leptotene/zygotene cells reached higher than 80% in mice between 14–17 days (Figure 6). Interestingly, previous studies have shown that the heterogeneous cell populations in varied cell cycle stages can be separated from each other through centrifugal elutriation (Banfalvi, 2008, 2017; Delgado et al., 2017; Liu et al., 2021). We thus envision that a further optimization will refine the purification of more sub-stages of meiotic cells from mice at different ages in the future.

**Table 2. Elutriator settings for purification of germ cells from juvenile mice (14–17 days)**

| Fraction# | RPM(Centrifuge) | VOLUME(mL) | Flow rate (mL/min) (pump) |
|---|---|---|---|
| 1 | 2250 | 100 | 19.6 |
| 2 | 2250 | 100 | 21 |
| 3 | 2250 | 200 | 23.6 |
| 4 | 2250 | 150 | 34 |
| 5 | 2250 | 150 | 40.5 |
| 6 | 2250 | 100 | 45 |

## LIMITATIONS

The commonly adopted STA-PUT method relies on natural gravity balance through the continuous BSA gradient, and is thus time-consuming and extremely vulnerable to external disturbance. The flow cytometry usually necessitates dye-labelling and thus produces highly purified cells, but with low yield and low viability. In comparison, the centrifugal elutriation method described herein is highly reproducible and time-saving with high yield and high viability. Owing to the differential sedimentation velocity, a plethora of populations of germ cells at varied developmental stages can be sequentially sorted out, which is well-controlled following the force balance between centrifugation and pumping.

Therefore, once the parameters are optimized, the researchers can go directly to set up the parameters without further calibration and do not need to visually check cell purity in each fraction

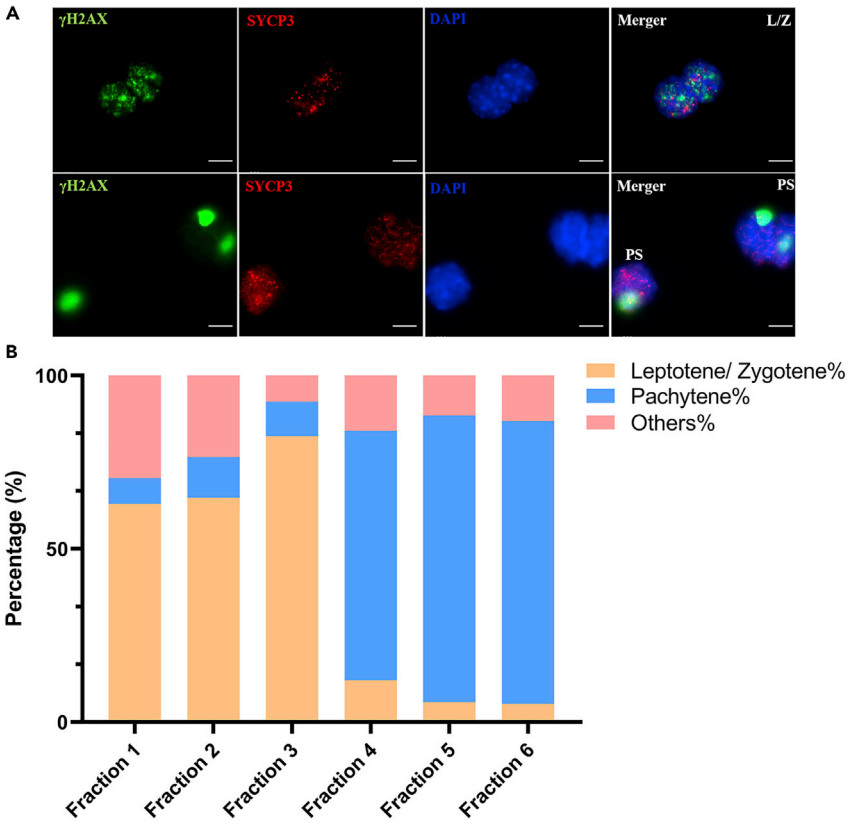


**Figure 6. Validation and counting of enriched germ cells in collected cell fractions from juvenile mouse testis (14∼17-day old)**

(A) Representative images of enriched meiotic germ cells as defined by γH2AX and SYCP3 staining. Scale bars: 10 μm.

(B) Relative quantification of the enriched percentages for the indicated types of germ cells in each fraction (14–17-day mice). Enriched cells were classified into leptotene/zygotene, pachytene and other cells.

repeatedly every time. However, there are some intrinsic limitations for application of centrifugal elutriation. For instance, the sedimentation velocity resembles each other between leptotene/zygotene and round spermatids, hence it is difficult to get a highly purified population of leptotene/zygotene meiotic cells since they only account for a small percentage in adult mouse testis. Nonetheless, we can utilize the juvenile mouse testes, which only possess meiotic cells without haploid spermatids, to get highly purified sub-meiotic spermatocytes. For purification of spermatogonia, it is advisable to use testes from ~P7 mice, when only spermatogonia and Sertoli cells are found within seminiferous tubules. There is no doubt that further optimization of the parameters would facilitate the purification of more sub-stages of germ cells in the future.

## TROUBLESHOOTING

### Problem 1

Insufficient digestion for preparation of single-cell testicular suspension.

### Potential solution

The enzymatic activity of trypsin is inhibited in the presence of $Ca^{2+}$/$Mg^{2+}$, we thus strongly recommend to use $Ca^{2+}$/$Mg^{2+}$-free buffer for digestion of seminiferous tubules into single cells. In addition, we found that the second-step trypsin digestion of the undigested tubules after initial trypsin digestion (two-step trypsin digestion presented in this protocol) will improve the yield. Inadequate digestion will yield less number of germ cells.

### Problem 2

Cell clumping or cell re-aggregation.

### Potential solution

Cell clumping or re-aggregation is commonly observed for single-cell preparation from any tissues due to the release of the sticky genomic DNA molecules from the dead cells. Therefore, always avoid the over-digestion of fragmented seminiferous tubules by trypsin (<20 min). In addition, always gently pipetting up and down when wash or transfer germ cells. The inclusion of 0.1–0.5% BSA and $Ca^{2+}$/$Mg^{2+}$-chelating EDTA, as well as the supplement of DNase I are helpful to reduce the aggregation among single cells.

### Problem 3

Cell aggregation within tubing system during loading.

### Potential solution

We noticed that single cells re-aggregate during flowing through the sample reservoir chamber to the elutriation chamber for germ cells derived from genome-edited mouse models. If that happens, we recommend to increase the pump speed to 10 mL/min or higher during loading.

### Problem 4

Low cell yield and low cell viability.

### Potential solution

During digestion of intact seminiferous tubules into single cells, we recommend to boost the enzymatic digestion efficiency by gently pipetting up and down the cell suspension using wide-bore tips. This treatment will shorten the digestion time and lower the enzyme concentration, so as to increase the cell yield and cell viability.

### Problem 5

Loss of cell pellet during cell collection.

### Potential solution

At the last steps of cell collection, we recommend centrifugation using swinging-bucket rotors that will better preserve the intact cell pellet and cell viability compared with fixed-angle rotors.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jianqiang Bao (jqbao@ustc.edu.cn).

### Materials availability

No new materials were generated.

### Data and code availability

This study did not generate/analyze new datasets or code.

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## AUTHOR CONTRIBUTIONS

J.B. and Y.C. designed and optimized protocols. Y.C. and L.M. performed the experiments and analyzed the data. J.B. and Y.C. wrote the manuscript. All authors contributed to writing and editing the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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