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TITLE:

Enrichment of Pachytene Spermatocytes and Spermatids from Mouse Testes using Standard Laboratory Equipment

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KEYWORDS:

testis, spermatogenesis, round spermatid, pachytene spermatocyte, male germ cells, cell fractionation, discontinuous density gradient

SUMMARY:

Presented here is a protocol for enriching pachytene spermatocytes, round spermatids, and elongating spermatids from adult mouse testes using a discontinuous bovine serum albumin density gradient with standard laboratory equipment.

ABSTRACT:

To characterize each step of spermatogenesis, researchers must separate different subpopulations of germ cells from testes. However, isolating discrete populations is challenging, because the adult testis contains a complex mix of germ cells from all steps of spermatogenesis along with certain populations of somatic cells. Over the past few decades, different techniques such as centrifugal elutriation, fluorescence-activated cell sorting (FACS), and STA-PUT have been successfully applied to the isolation of germ cells. A drawback is that they all require dedicated devices and specialized training. Following principles underlying the STA-PUT method, a simple protocol has been developed for the isolation of pachytene spermatocytes, round spermatids, and elongating spermatids from mouse testes. After preparing a single cell suspension of testicular cells, specific cell populations are enriched by gravity sedimentation through a discontinuous bovine serum albumin (BSA) density gradient. The cell fractions are then manually collected and microscopically analysed. This modified density gradient for round spermatids (MDR) sedimentation protocol can be widely applied, because it requires only standard laboratory equipment. Furthermore, the protocol requires minimal starting materials, reducing

its cost and use of laboratory animals.

INTRODUCTION:

Much is still unknown about the molecular and biological events taking place during mammalian spermatogenesis, a complex process in which spermatogonial stem cells transform into highly specialized spermatozoa^{1,2}. Spermatogenesis takes place inside the seminiferous tubules of the testis. The tubules contain a mixture of germ cells from each step of differentiation, including spermatogonial stem cells, mitotically dividing spermatogonia, meiotic spermatocytes, and postmeiotic spermatids (which undergo haploid differentiation from round spermatids to elongating spermatids, and finally to mature spermatozoa). Somatic cells of the testis include Sertoli cells that are intermingled with germ cells inside the seminiferous tubules, peritubular myoid cells that form walls of the tubules, and testosterone-producing Leydig cells in the interstitial space between tubules.

Studying molecular and biochemical processes during spermatogenesis often requires separation of distinct germ cell populations from a complex mixture of testicular cells. Many different strategies have been developed for cell enrichment. The most successful methods are STA-PUT velocity sedimentation by unit gravity³⁻⁶, centrifugal elutriation based on counterflow centrifugation^{7,8}, and fluorescence-activated cell sorting (FACS) that separates cells according to DNA content and/or specific markers. These methods are commonly used among spermatogenesis researchers and allow for the efficient enrichment of specific germ cell types. However, a limitation of these techniques is that they require specialized, expensive hardware that require expertise.

Presented here is a simple and inexpensive protocol to isolate enriched populations of the three most abundant cell populations of mouse testes: round spermatids, pachytene spermatocytes, and elongating spermatids. This protocol is referred to as the modified density gradient for round spermatids (MDR), because it works particularly well for enriching round spermatids. The MDR method is based on the same principles as the STA-PUT velocity sedimentation, yet it requires only standard laboratory equipment. Living cells are allowed to sediment through a manually prepared discontinuous bovine serum albumin (BSA) density gradient inside a standard 50 mL tube under the earth's gravitational field. Larger cells move faster through the gradient, which separates different populations of germ cells. After sedimentation, enriched fractions of the three cell types are manually collected. The purity of these enriched cell populations is comparable to those obtained by STA-PUT and centrifugal elutriation.

In addition to covering the construction and use of the BSA gradient for velocity sedimentation, the protocol also describes a digestion method to release testicular cells from seminiferous tubules. The protocol was modified from that developed by Romrell et al.⁹ and includes sequential digestions with collagenase IV and trypsin. Sequential digestions combined with use of a bicarbonate buffer (i.e., the Krebs solution) have been shown to greatly enhance the separation and viability of the germ cells⁹.

During MDR enrichment, cells spend around 4 h together outside the environment of the

seminiferous tubules and are not subjected to stressful mechanical forces, which allows for the collection of highly viable cellular fractions for downstream analysis. In addition, similar to the centrifugal elutriation and STA-PUT, the MDR protocol does not require any chemical treatment or labelling of cells, which also helps to maintain their viability. Importantly, as little as two adult mouse testes are sufficient for the MDR isolation and therefore, one adult mouse provides enough enriched cells for both RNA and protein analysis. Standard STA-PUT protocol recommends use of as many as 12 adult mice for cell isolation⁶; although, based on prior experience, it is known that successful isolation can be done from three to four adult mice. The lowest amount of starting material reported to be sufficient for centrifugal elutriation is six mouse testes (three mice)⁸. Therefore, besides eliminating the need for expensive specialized equipment, the MDR protocol reduces the number of laboratory animals required.

PROTOCOL:

The maintenance of laboratory mice and all experiments were performed in accordance with the relevant guidelines and regulations for the care and use of laboratory animals.

1. Equipment and reagent set-up

1.1. Set the water bath to 37 °C.

1.2. Set the cell culture incubator to 34 °C, 5% CO₂, 95% humidity. Put the tube rotator inside the incubator.

NOTE: Incubators require a long time to change internal temperature. If an incubator constantly set at 34 °C is not available, set one up 1 day before the experiment.

1.3. Prepare and label the appropriate amount of microscopy glass slides. Draw a ring of ~1 cm in diameter with a grease pen and let the grease dry.

1.4. Prepare 1x Krebs buffer, pH 7.8 (**Table 1**). Put two conical tubes with 50 mL of 1x Krebs to 34 °C to pre-warm for steps 2.5–2.8. Store the rest of the 1x Krebs at 4 °C or on ice.

1.5. Prepare BSA solutions. First prepare 25 mL of 10% (w/v) BSA solution in Krebs by dissolving 2.5 g of BSA in 1x Krebs buffer to a final volume of 25 mL. Dilute the 10% BSA solution with 1x Krebs buffer to obtain different BSA concentrations (**Table 2**). Keep all BSA solutions at 4 °C.

NOTE: Prepare solutions the same day of the procedure and store at 4 °C until use.

1.6. Prepare the digestion enzymes by weighing the correct amount of trypsin and collagenase to 50 mL conical tubes (**Table 3**).

2. Animal dissection and preparation of germ cell suspension

NOTE: This takes approximately 1 h to complete.

2.1. Sacrifice an adult male mouse (aged 7 weeks or older, testis weighing 80–120 mg depending on the strain and age) via cervical dislocation or CO₂ asphyxiation.

2.2. Spray the ventral abdomen of the mouse with 70% ethanol. Open the abdominopelvic cavity using scissors, making a V-shaped opening.

2.3. Pulling on the epididymal fat pad with forceps, locate the testes and remove them with scissors. Avoid disturbing the tunica albuginea. Place the testes on a 6 cm Petri dish containing 1x Krebs.

2.4. Decapsulate the testes and discard the tunica albuginea. Slightly disperse the seminiferous tubules by gently teasing them apart with forceps.

2.5. Use forceps to transfer the seminiferous tubules into a 50 mL conical tube containing 2 mL of freshly prepared collagenase solution (**Table 3**).

2.6. Incubate the tubules in the 37 °C water bath for 3 min. Agitate gently by rocking the tube.

NOTE: Freely floating tubules should occur within 3 min due to the removal of interstitial cells. The physiological temperature for testicular cells is 34 °C; therefore, long digestions are usually done at this temperature. However, the short 3 min digestion can be conducted at 37 °C (recommended temperature by the manufacturer). Note that if 34 °C is used, the digestion time should be re-optimized.

2.7. Add at least 40 mL of warm 1x Krebs and allow the tubules to sediment (~1 min) at room temperature (RT). Remove the supernatant and repeat 1x.

2.8. Add 25 mL of freshly prepared trypsin solution (**Table 3**), place the tube on the tube rotator inside the 34 °C incubator, and incubate for 15–20 min (~15 rpm). Sporadically check the status of the tubules. Once the solution becomes cloudy and only small pieces of tubules remain, place the tube on ice and immediately proceed to the next step.

NOTE: To avoid overdigestion and cell lysis, move swiftly to the following washing steps. Some protocols include the deactivation of trypsin by fetal bovine serum (FBS). In this protocol, FBS treatment is omitted, and instead, trypsin is removed by immediate centrifugation and subsequent washes with cold 1x Krebs.

2.9. Filter the solution through a 40 µm cell strainer into a new 50 mL conical tube on ice.

2.10. Centrifuge 600 x g for 5 min at 4 °C to pellet the cells.

NOTE: Centrifugation with too strong of a force may harm the cells.

2.11. Remove supernatant by carefully pouring it out.

2.12. Tap the cell pellet to resuspend the cells in the remainder of 1x Krebs.

2.13. Add at least 40 mL of cold 1x Krebs to the resuspended cells.

2.14. Repeat steps 2.10 and 2.11.

2.15. Tap the tube with the cell pellet to resuspend the cells. Add 1 mL of 0.5% BSA in 1x Krebs and with a cut pipette tip resuspend the cells by pipetting the solution up and down. Avoid making bubbles.

2.16. Finally, add 1–3 mL of 0.5% BSA in 1x Krebs so that the final volume is ~3 mL. Filtrate the germ cell suspension through a 40 µm cell strainer and proceed immediately to loading the cells on the BSA gradient.

3. Separation of germ cells through the discontinuous BSA gradient

NOTE: This section takes approximately 2 h to complete. Start preparing the discontinuous BSA gradient during the wash steps (steps 2.10–2.14) to load the cells as soon as the pretreatment is ready.

3.1. Accommodate a 50 mL tube vertically on ice so that it is possible to see one side of the tube. Alternatively, run the protocol in a cold room at 4 °C, in which case no ice is needed.

3.2. Cut the tip of a 10 mL serological pipette about 5–10 mm from the tip to obtain a larger aperture (**Figure 1A**), and mount it on the pipette controller (**Figure 1C**).

NOTE: This will decrease the velocity during pipetting, which facilitates stacking of the different BSA solutions. Alternatively, use a 1 mL mechanical pipette with a smooth piston and cut the pipette tips with an aperture of about 3 mm in diameter (**Figure 1B**).

3.3. Start by pipetting 5 mL of 5% BSA solution to the bottom of the 50 mL tube.

3.4. Slowly pipet 5 mL of 4% BSA solution on top of the 5% solution. Start by gently touching the surface of the 5% solution with the cut tip of the pipette and layer the two solutions while carefully maintaining contact with the surface, ensuring to not allow the pipet tip to become immersed.

NOTE: A clear line should be visible between the two layers at the end of this step.

3.5. Repeat step 3.4 with the other BSA solutions obtain a discontinuous gradient from 5% up to 1% (**Figure 1D**).

3.6. Carefully load the single-cell suspension on top of the gradient without disturbing. Let the cells sediment through the gradient for 1.5 h at 4 °C or on ice.

4. Collection of enriched germ cell fractions

NOTE: This section takes approximately 30 min to complete.

4.1. Mount a 1 mL pipette tip onto a 1 mL mechanical pipette and cut the tip so that the aperture is ~3 mm in diameter (**Figure 1B**).

4.2. Carefully collect ~1 mL fractions in separate 1.5 mL tubes, starting from the top of the BSA gradient, and store them on ice. Number the tubes in the same order as the fractions will be collected.

NOTE: At this point, there should be a series of ~28 tubes containing cell suspensions. Use a 1 mL mechanical pipette with a smooth piston and cut the pipette tips to minimize the risk of disturbing the BSA gradient.

4.3. Centrifuge the germ cell fractions at 600 x *g* for 10 min at 4 °C.

4.4. Careful not to disturb the pellet, discard most of the supernatant and resuspend the cell pellet by flicking the tube. Add 1 mL of ice-cold 1x Krebs buffer to each tube and repeat centrifugation.

NOTE: Repeat the washing step if BSA interferes with downstream analysis.

4.5. Discard most of the supernatant but leave ~100 µL after the final wash. Resuspend the cell pellet carefully.

NOTE: Resuspending the cells carefully ensures that the sample taken from this solution represents all cells of the given fraction. Keep the cell suspensions on ice while preparing the slides.

5. Analysis of cell fractions

NOTE: Analysis takes 2 h to complete.

5.1. One at a time, pipet 20 µL of 4% paraformaldehyde (PFA) inside each grease pen ring on a numbered microscopy slide.

5.2. Immediately add 2 µL of the resuspended cell suspension from the corresponding fraction. Repeat for each fraction.

NOTE: While preparing slides, keep all cell suspensions on ice.

5.3. Dry the slides at RT for 1 h to overnight (O/N).

NOTE: During this step, after taking a sample from each fraction, the cells can be processed for downstream analyses or storage (section 6).

5.4. Rinse the slides once with 1x PBS and mount with mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Table of Materials).

5.5. Analyze the slides under a fluorescence microscope to estimate which particular germ cell type is enriched in each fraction.

NOTE: Different cell types can be recognized by their characteristic nuclear morphology visualized by DAPI staining. **Figure 2A** shows representative fractions enriched in elongating spermatids, round spermatids and pachytene spermatocytes. The round spermatid nucleus is small (6–7 μm in diameter) and round (**Figure 2A**). Mouse round spermatid nuclei are also characterized by a single, round heterochromatin structure called the chromocenter. Nuclei of elongating spermatids have a typical elongated shape and reduced nuclear size due to chromatin condensation (**Figure 2A**). Pachytene spermatocyte nuclei are much larger (diameter more than 10 μm) and more irregular in shape, with areas of densely packed chromatin distributed throughout (**Figure 2A**).

5.6. If necessary, perform immunostaining in addition to the DAPI staining to support the recognition of the different cell types.

5.6.1. In this case, spread 2 μL of cell suspension with 20 μL of a fixing solution (4% PFA and 0.1% nonionic surfactant in PBS) inside a grease pen ring. After drying the slides, postfix the cells with 4% PFA for 10 min at RT.

5.6.2. Rinse with PBS, then permeabilize with 0.1% nonionic surfactant in PBS for 5 min. Rinse with PBS and deactivate any remaining PFA by incubating slides in 100 mM NH_4Cl for 5 min.

5.6.3. Rinse with PBS and proceed to a standard immunofluorescence protocol consisting of blocking and incubations with specific primary antibodies and fluorochrome-labeled secondary antibodies. Mount the slides with antifade mountant with DAPI (Table of Materials) and allow them to set for 24 h.

NOTE: Examples of useful markers for primary antibody incubations include peanut agglutinin (PNA; 1:2,000 in blocking solution) that stains the acrosome in round and elongating spermatids, anti-DDX4 antibody (1:200 in blocking solution) that labels a single cytoplasmic granule in round spermatids, and anti- γH2AX antibody (1:100 in blocking solution) that recognizes sex bodies in pachytene spermatocytes. See the representative results and **Figure 2C,D** for examples of immunofluorescence analysis of the fractions.

6. Processing the remaining samples for storage

NOTE: Section 6 takes approximately 20 min to complete.

6.1. Once a sample from each fraction has been taken for a microscopy slide, add 1 mL of ice-cold 1x Krebs to each fraction and centrifuge the cells down at 600–13,000 $\times g$ for 10 min at 4 °C.

NOTE: A low-speed centrifugation results in a loose pellet, which makes it difficult to completely remove 1x Krebs, while a high-speed centrifugation may harm the cells. Choose the centrifugation speed according to specific requirements of the downstream protocol.

6.2. Remove and discard the supernatant and continue with preferred downstream analysis.

NOTE: At this point, the cells can be snap-frozen in liquid nitrogen and stored at -70 °C. Once familiar with this technique, it is possible to continue with the downstream protocol of preference directly, but it is always advisable to take a sample and ensure the purity of each fraction. Total RNA can be extracted, quantified, and analysed by methods such as reverse transcription polymerase chain reaction (PCR) and RNA sequencing. Total protein extracts can also be prepared from which immunoprecipitation or western blotting analysis can be performed (Figure 3).

REPRESENTATIVE RESULTS:

Sufficient enrichment of a germ cell type is usually considered to be above 80%⁶. The MDR protocol works particularly well for enriching round spermatids. A high number of >90% pure round spermatids can routinely be obtained using this technique. Optimal fractions of pachytene spermatocytes and elongating spermatids are enriched to ~75% and ~80%, respectively. Elongating spermatids tend to stay on top of the gradient and are collected with the first fraction. In the example shown, fraction 1 contained ~80% of elongating spermatids (Figure 2B). Most elongating spermatids obtained with this technique have condensed nuclei, while non-condensed early elongating spermatids are scarce (Figure 2A). The following fractions contain enriched round spermatids. In the example, the enrichment of round spermatids was more than 90% in fractions 2, 3, and 4, and enrichment above 80% was seen altogether in seven fractions (2–8) (Figure 2B). Due to their large size, pachytene spermatocytes sediment faster and are collected last. In the purification example, enrichment was around 75% in fractions 14 and 15 (Figure 2B).

While nuclear morphology, visualized by DAPI staining, usually suffices for recognition of the cells, immunofluorescence analysis can be performed to support the analysis. PNA stains the developing acrosomes of round and elongating spermatids, and the acrosomal appearance can be exploited to further categorize round spermatids into steps 1–8 of differentiation¹⁰. Distinguishing PNA-stained elongating and round spermatids relies on the differences in their nuclear shape (Figure 2C, left panel). Anti-DDX4 antibody is a useful marker for the round spermatid fractions since it visualizes one single DDX4-positive perinuclear granule, the

chromatoid body (CB), in the cytoplasm of each round spermatid. This CB-specific staining is easy to distinguish from more widely distributed cytoplasmic staining in spermatocytes (**Figure 2C**, middle panel). Pachytene spermatocytes can be recognized by anti- γ H2AX antibody that labels the nuclear sex body specifically appearing at the pachytene phase of the first meiotic division (**Figure 2C**, right panel). In this purification, PNA staining revealed that MDR enriched round spermatid fractions contained a mixture of round spermatids at various steps of differentiation, all containing their signature structure, the DDX4-positive CB (**Figure 2D**, RS fraction). Anti- γ H2AX further validated the enrichment pachytene spermatocytes in fraction 16 (**Figure 2D**, PSpc fraction).

The cell counting revealed that the number of cells in the round spermatid and pachytene spermatocyte fractions is adequate for various downstream analyses. Round spermatid fractions (5–8) each contained around 2.5×10^6 cells. Therefore, by pooling these fractions, it is possible to obtain more than 10 million cells. Pachytene spermatocyte fractions (14 and 15) usually contained somewhat fewer cells. In this isolation, around $1.5\text{--}2.0 \times 10^6$ cells per fraction were counted. The first fraction contained 0.75×10^6 elongating spermatids.

As shown in **Figure 3A**, the total RNA amount obtained from the majority of fractions ranged from 0.5–2.5 μ g, which is sufficient for downstream RNA analyses such as reverse transcription PCR, RNA sequencing, or visualizing RNA on a gel. The amount of protein obtained from each fraction typically ranges from 20–140 μ g (**Figure 3B**), which is sufficient for several western blots. Whole cell lysates were prepared from collected fractions, and western blot analysis was performed using antibodies detecting DDX4, PIWIL1, and PIWIL2, which are all highly expressed in pachytene spermatocytes and round spermatids as well as the ubiquitously expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

In this protocol, 10% of the protein lysates derived from single fractions was sufficient to clearly detect all these proteins on a standard western blot setting (**Figure 3C**). The amount of protein in one fraction was also shown to be sufficient for immunoprecipitation using an antibody against PIWIL1, as well as for the detection of co-immunoprecipitated PIWIL2 (**Figure 3D**). Furthermore, this protocol has been successfully used to obtain enriched fractions of pachytene spermatocytes and round spermatids from control and genetically modified mice for downstream applications such as quantitative reverse transcription PCR¹¹ and high throughput RNA sequencing¹².

FIGURE AND TABLE LEGENDS:

Figure 1: Preparation of a discontinuous BSA gradient. (A) A 5 mL serological pipette tip for preparation of the gradient. (B) A 1 mL pipette tip for preparation of the gradient and collection of the germ cell fractions after sedimentation. (C) The equipment needed for preparation of the gradient. (D) Lateral view of the discontinuous BSA density gradient from the 5% (bottom) to the 1% (top) BSA solution; the 2% and 4% BSA solutions are blue in color for better visualization.

Figure 2: Representative images of the collected cell fractions and the enrichment analysis. (A) DAPI-stained testicular cells. The upper row shows testicular cells in the intact seminiferous

epithelium of a PFA-fixed, paraffin-embedded testis section (left) or in suspension (right). The lower row shows fractions of enriched elongating spermatids (ES), round spermatids (RS), or pachytene spermatocytes (PSpc). Scale bars = 20 μ m for the upper row and 10 μ m for insets of the lower row. **(B)** Relative quantification of the different germ cell types in each fraction. Cells were manually counted using the ImageJ software and classified into RS, ES, PSpc, Sertoli cells, and other cells. The fraction numbers and respective percentages of BSA in the gradient are indicated on the x-axis. **(C)** Immunofluorescence analysis of testicular cells. Left panel: rhodamine-labelled PNA stains the acrosome in both ES (yellow arrow) and RS (white arrow). Middle panel: DDX4 antibody stains a single perinuclear granule in RS, which is easy to distinguish from the more diffuse cytoplasmic signal in spermatocytes (blue arrow). No DDX4 signal is detected in ES (orange arrow). Right panel: γ H2AX antibody recognizes the characteristic nuclear sex body present only in pachytene spermatocytes (γ H2AX-negative cells marked by a white asterisk). Scale bars = 10 μ m. **(D)** MDR enriched cell fractions were labelled with PNA (RS fraction), anti-DDX4 (RS fraction), and anti- γ H2AX (PSpc fraction) to further validate the cell enrichment in each fraction. Scale bars = 10 μ m.

Figure 3: Downstream analyses after MDR cell enrichment. **(A)** RNA was extracted from each fraction after a representative MDR cell enrichment and quantified. The fraction numbers and respective percentages of BSA in the gradient are indicated on the x-axis. **(B)** Proteins were extracted from each fraction by lysing the cell pellet in radioimmunoprecipitation assay (RIPA) buffer then quantified. **(C)** Whole cell protein extracts were prepared and analysed by western blotting using antibodies against DDX4, PIWIL1, PIWIL2, and GAPDH. 10% of the lysate was loaded from each indicated fraction. **(D)** Immunoprecipitation was performed from indicated fractions using anti-PIWIL1 followed by western blotting with anti-PIWIL1 and anti-PIWIL2 antibodies. The input sample includes a mixture of protein lysates from different fractions, and the control immunoprecipitation (IP) using rabbit IgG was also performed from a mixed lysate.

Figure 4: Schematic representation of MDR protocol and time required for completing each step. Starting material is composed of two testes from an adult mouse. The average number of cells and the amount of RNA and protein obtained from one fraction are indicated.

Table 1: Preparation of Krebs buffer.

Table 2: Preparation of BSA solutions.

Table 3: Preparation of the digestion enzymes.

DISCUSSION:

Presented here is a simple and inexpensive protocol to isolate enriched populations of round spermatids, pachytene spermatocytes, and elongating spermatids using standard laboratory equipment (overview of the protocol shown in **Figure 4**). Though no expertise or expensive machinery is required, there are some critical steps that must be considered during tissue digestion, construction of the gradient, and loading of the cell suspension onto the gradient.

Germ cells are released from seminiferous tubules by two consecutive enzymatic digestions. The first digestion with collagenase IV separates seminiferous tubules by removing interstitial cells. Prolonged digestion time may damage the tubules and lead to loss of spermatids, as (if released from the tubules during this step) they will be discarded in the following steps. The second digestion step with trypsin releases germ cells from seminiferous tubules. There may be occasional cell lysis and typically some clumps form due to released genomic DNA. Exceeding the suggested duration or temperature of the digestion is not advised, as this can lead to poorer viability, increased cell lysis, and clumping. If mild clumping does occur, the clumps can be ignored. However, in cases of significant clumping and loss of cells, trypsin digestion time or concentration should be reduced. It should also be noted that the enzymatic activity of trypsin may vary between batches and during prolonged times of storage. The amount of DNase I during trypsin digestion can also be increased to remove excess clumps, but this should be considered a secondary solution. It is important to obtain a homogenous single cell suspension at the end of pre-treatment, since clumped cells will sediment faster, contaminating the fractions and disrupting the gradient.

Building the gradient may require some practice. If there is discomfort using a 5 mL pipette tip with a pipette controller, it is recommended to use a normal 1 mL mechanical pipette with a smooth piston then cut the pipette tips to an aperture of ~3 mm in diameter (**Figure 1B**). A wider aperture and smooth loading of the BSA solutions will decrease the risk of mixing the gradient. When properly prepared, it is possible to see the boundaries between adjacent BSA solutions due to their different refractive indices. The gradient should be produced directly prior to use. It should also be noted that any small shaking or vibration may disturb the gradient, so the gradient should be set in an environment where it will not be disturbed.

Loading of the cell suspension onto the gradient must be done very carefully. After loading, the cell suspension should stay on top of the gradient, from which the cells will slowly start to sediment through the first layer. If large groups of cells are seen moving fast through the gradient, the cells were likely not resuspended carefully or there is excess clumping. If the cells do not stay on the top of the discontinuous BSA gradient upon loading but immediately sink between the 1% and 2% BSA layers (step 3.6), the cell suspension is likely too dense. This protocol has been optimized using two testes of an adult mouse (80–120 mg/testis) as starting material; although, successful isolations using reduced amounts of starting material has been performed. To upscale the protocol and obtain more enriched germ cells from higher numbers of testes, more 50 mL tubes with the gradient should be introduced.

The protocol was initially developed and optimized to enrich haploid round spermatids from adult mouse testes, and the purity of round spermatid fractions is expected to be more than 90%. In addition to highly pure round spermatid fractions, satisfactory results for the enrichment of pachytene spermatocytes and elongating spermatids were obtained. It should be noted that erythrocytes may contaminate the elongating spermatid fractions, and further steps to eliminate them should be taken if their presence is expected to interfere with downstream analyses. We have not been able to enrich other cell types such as premeiotic or early meiotic cells (prior to pachytene phase) from adult mice using the MDR protocol.

485
486 Additionally, STA-PUT sedimentation has been successfully used to obtain enriched fractions of
487 spermatogonia or preleptotene, leptotene, and zygotene spermatocytes using juvenile testes
488 collected at given timepoints after birth¹³. This approach takes advantage of the appearance of
489 these cell types during the first wave of spermatogenesis. The same approach can likely be
490 applied to MDR enrichment, but it has not yet been tested in practice. Another method that is a
491 good option for the purification of premeiotic and meiotic cells at specific stages of
492 differentiation is FACS, which has the important advantage of allowing the isolation of specific
493 cells types based on the presence specific markers^{14–17}.

494
495 Overall, the MDR velocity sedimentation serves as a useful method for germ cell enrichment.
496 While this method is not superior to other well-established methods in terms of the purity or
497 quantity of enriched cells, its clear advantages are its simplicity and low set-up costs. This,
498 together with the low amount of required starting materials, render this protocol a great option
499 for researchers in the spermatogenesis field and those in other fields who may not wish to invest
500 in specialized hardware or large groups of animals.

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507 **DISCLOSURES:**

508 The authors have nothing to disclose.

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Figure 1

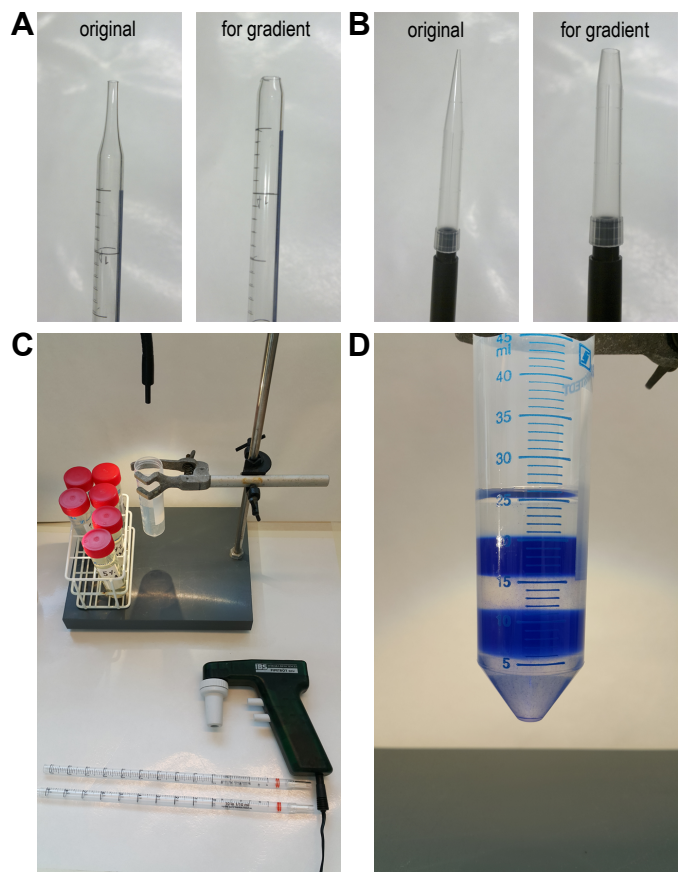


Figure 2

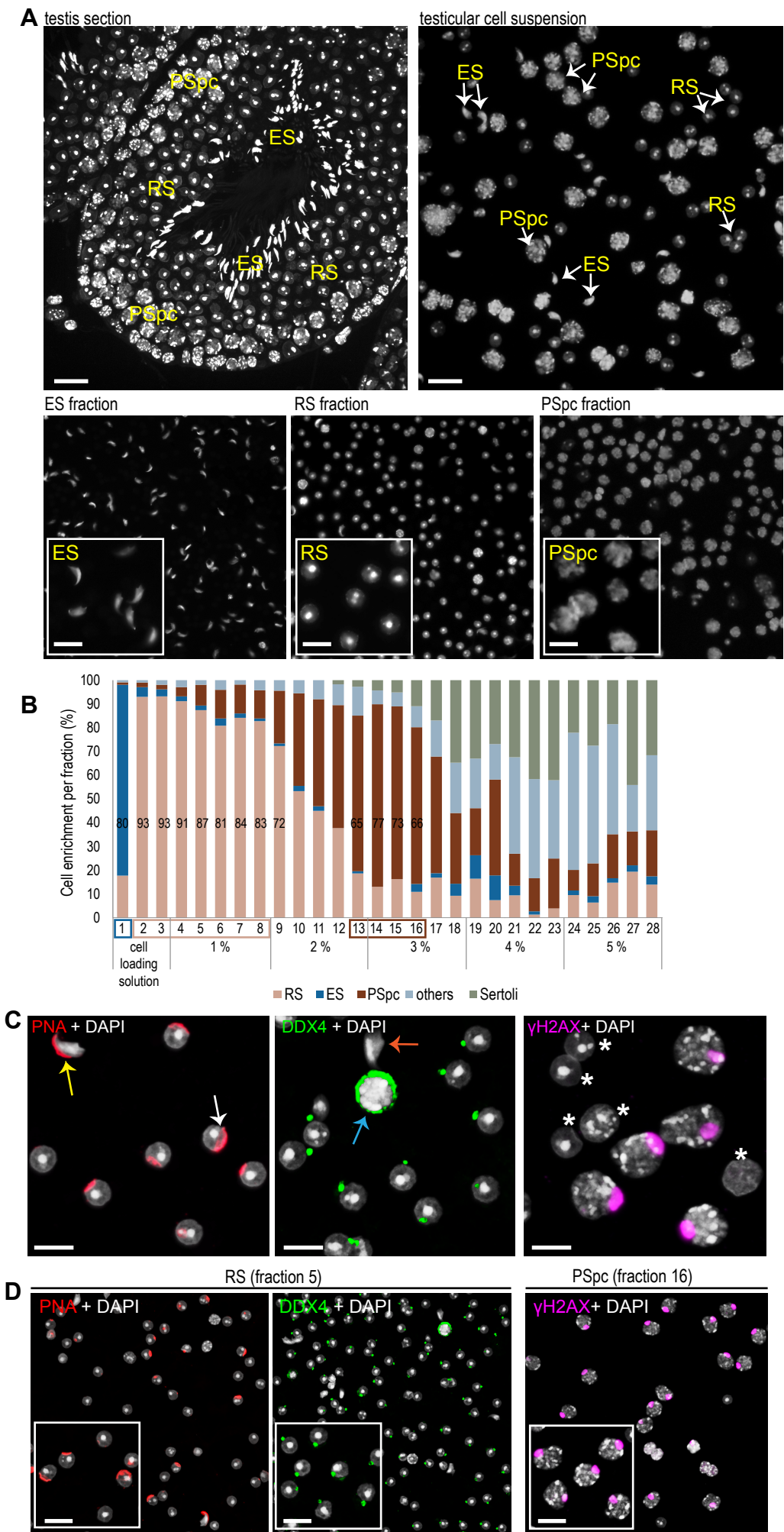


Figure 3

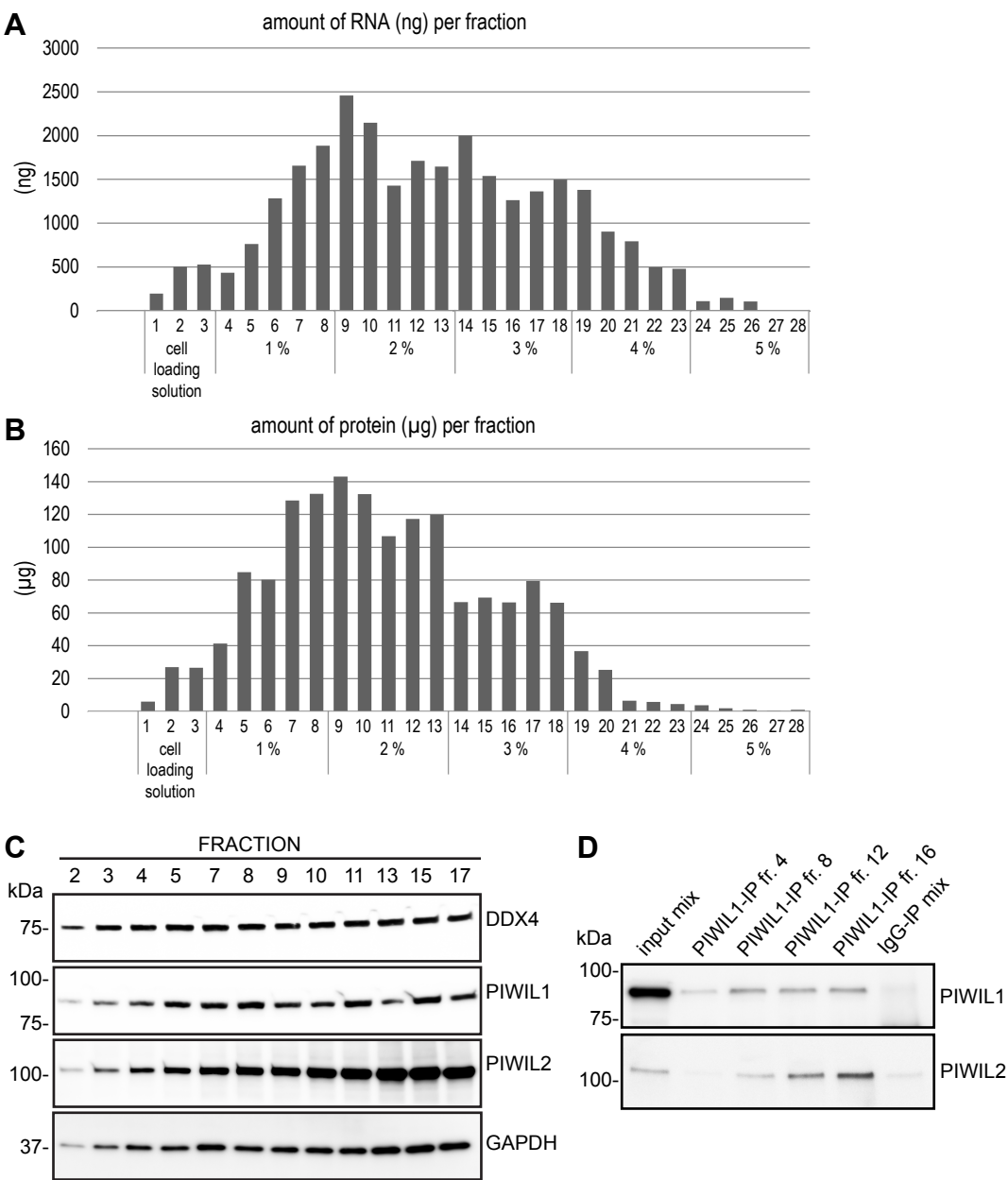
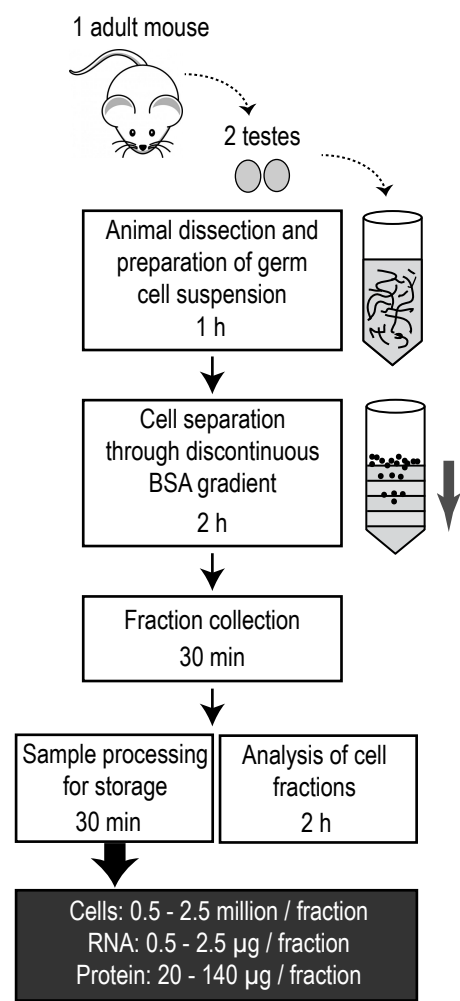


Figure 4



Buffer	Reagents	Preparation	Storage
Krebs buffer (10x)	3.26 g KH_2PO_4 139.5 g NaCl 5.89 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 50 g dextrose 3.78 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 7.12 g KCl	Bring to 2 L with H_2O , filter 0.22 μm and autoclave.	Can be stored at 4 °C for several months
Krebs buffer (1x)	4.24 g NaHCO_3 200 mL 10x Krebs buffer	Dissolve NaHCO_3 to 100 mL of H_2O , add 200 mL of 10x Krebs buffer, and bring to 2 L with H_2O	To be prepared fresh

BSA concentration (w/v)	10% BSA solution	1x Krebs buffer
0.50%	0.5 mL	9.5 mL
1%	1 mL	9 mL
2%	2 mL	8 mL
3%	3 mL	7 mL
4%	4 mL	6 mL
5%	5 mL	5 mL

Digestion solution	Working concentration	Reagents	Preparation
Collagenase	1 mg/mL	Collagenase IV	Weigh 2 mg of collagenase IV to a 50 mL conical tube, and add 2 mL of warm 1x Krebs buffer right before the digestion (step 2.3).
Trypsin	0.6 mg/mL >3.2 ku/mL	Trypsin DNase I	Weigh 15 mg of trypsin to a 50 mL conical tube, and add 25 mL of warm 1X KREBS buffer and 40 µL of DNase I right before the digestion (step 2.6).



Name of Material	Company	Catalog #	Comments
4% Paraformaldehyde	Preference of researcher		
AlexaFluor488 donkey anti-rabbit IgG	Thermo Fisher Scientific	A-21206	
AlexaFluor647 donkey anti-mouse IgG	Thermo Fisher Scientific	A-31571	
Bovine Serum Albumin (BSA)	Sigma	A9647	
CaCl ₂ ·2H ₂ O	Preference of researcher		
Collagenase IV	Sigma	C5138	
Complete protease inhibitor cocktail	Roche	11836145001	
DDX4 antibody	Abcam	ab13840	
Dextrose	Preference of researcher		
DNase I	Worthington	LS006355	
GAPDH	HyTest	5G4	
HRP-linked anti-mouse IgG	GE Healthcare Life Science	NA931	
HRP-linked anti-rabbit IgG	GE Healthcare Life Science	NA934	
KCl	Preference of researcher		
KH ₂ PO ₄	Preference of researcher		
MgSO ₄ ·7H ₂ O	Preference of researcher		
NaCl	Preference of researcher		
NaHCO ₃	Preference of researcher		
NH ₄ Cl	Preference of researcher		
Pierce BCA protein assay kit	Life Technologies	23227	
PIWIL1	Cell Signaling Technology	G82	
PIWIL2, clone 13E-3	Millipore	MABE363	
Prolong Diamond Antidafe Mountant with DAPI	Thermo Fisher Scientific	P36962	for Alexa Fluor immunostainings
rabbit IgG	Neomarkers	NC-100-P	
Rhodamine-labelled Peanut agglutinin (PNA)	Vector Laboratories	RL-1072	
RIPA buffer			50 mM Tris-HCl, pH 7.5, 1% NP-40, 0.5% w/v sodium deoxycholate, 0.05% w/v SDS, 1 mM EDTA, 150 mM NaCl, 1x protease inhibitor cocktail, 0.2 mM PMSF and 1 mM DTT
TRIsure	Bioline	BIO-38033	
Triton X-100	Preference of researcher		nonionic surfactant
Trypsin	Worthington	LS003703	
VECTASHIELD Antifade Mounting Medium with DAPI	Vector Laboratories	H-1200	for standard DAPI analysis of cell fractions
γH2AX antibody	Millipore	05-636	

Name of Equipment	Company	Catalog #	Comments
0.22 μm filter	Sartorius	Sartolab BT 180C6	or equivalent
1 ml mechanical pipette	Preference of researcher		
1.5 mL or 2 ml tubes	Preference of researcher		
40 μm cell sieves for 50 mL tubes	Greiner Bio-One	542040	or equivalent cell strainer
5 mL serological pipettes	Sarstedt	86.1254.001	or equivalent
50 mL conical tubes	Preference of researcher		
6 cm Petri dishes	Preference of researcher		
cell culture incubator	Preference of researcher		
centrifuge for 50 mL tubes	Preference of researcher		
grease pen for microscopy glass slides	Preference of researcher		
HulaMixer Sample Mixer	Thermo Fisher Scientific	15920D	or equivalent cell rotator
microdissection forceps	Preference of researcher		
microdissection scissors	Preference of researcher		
microscopy glass slides and coverslips	Preference of researcher		
Nanodrop 1000	Thermo Scientific		
Pipetboy Acu 2	Integra	155 000	or equivalent pipette controller
refrigerated centrifuge for 1.5 mL tubes	Preference of researcher		
tips for 1 ml mechanical pipette	Preference of researcher		
water bath	Preference of researcher		



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Author(s):	Matteo Da Ros, Tiina Lehtiniemi, Opeyemi Olotu, Oliver Meikar, Noora Kotaja

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RESPONSE LETTER

Matteo Da Ros , Tiina Lehtiniemi, Opeyemi Olotu, Oliver Meikar, Noora Kotaja:

Simple protocol to enrich pachytene spermatocytes and spermatids from mouse testes using standard laboratory equipment

We greatly appreciate all relevant editorial and reviewers' comments. We have now addressed them and revised the manuscript accordingly (modified text shown in red in the revised manuscript). You can find point by point responses below. We have included new cell counting data in the text, as well as new figures (Figure 2C and D, Figure 3C and D), and modified the text accordingly. We feel that the revision helped us to improve the manuscript, and we hope that it is now suitable for publication.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript and edited the language.

2. Summary: Please use complete sentences.

We have modified the summary to use complete sentences.

3. Abstract: Please expand to include an overview of the method.

The abstract has been modified to include a description of the method.

4. Please remove commercial language in your manuscript: HulaMixer, milliQ, etc.

Modified.

5. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

Modified.

6. 2.1: Please specify the age and type of the mouse.

Point 2.1., page 4: we included this information in the protocol.

7. Tables: Please use the period symbol (.) for the decimal separator.

Tables 2 and 3 were revised.

8. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol.

We have ensured that the Table of Materials contains all relevant information.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript is well written and clearly describes step-by-step how purified fractions of male germ cells can be prepared, starting from a single mouse. The interesting aspect of this work is the possibility of obtaining enriched populations of germ cells using a standard lab setting. This will likely help a wide number of research labs for their studies on germ cells.

Major Concerns:

Page 5, row 164. Please, specify HulaMixer setting

We have specified the settings for cell rotator in the step 2.6.

Page 5, row 199. It seems to me that beside "Figure 1A-C" it would be more appropriate "Figure 1A and 1C".

Modified.

Page 9, row 318. How many micrograms of proteins from purified fractions are needed (as average) for IP and mass spectrometry analyses? This can be of course variable from target to target. However, it would be good to know whether in author's experience, isolation of germ cells from a single mouse is a standard for approaching IP and mass spectrometry experiments.

To exemplify that the amount of protein in one MDR fraction is enough for successful immunoprecipitation, we performed IP from individual MDR-enriched fractions with anti-PIWIL1 antibody. Western blot analysis showed that the material obtained from a single fraction is sufficient for IP, and even for the detection of co-immunoprecipitated PIWIL2. This result can be found in the Figure 3D. We also included a Western blot analysis of whole cell extracts made from selected fractions (around 10% of each fraction loaded to the gel) (Figure 3C). The representative results section (lines 356-362) and figure legends were modified accordingly. According to our experience, the amount of total protein in one fraction, or the amount of immunoprecipitated protein complexes from one fraction, should be sufficient for the identification of proteins by mass spectrometry. However, we do not have our own experience on mass spec analysis after MDR enrichment, and therefore, we left it out from the text.

Page 10-11, rows 380-381. It is not clear to me whether authors have used the MDR protocol to isolate spermatogonia and early meiotic (leptonema/zygonema) cells using juvenile mice with not success, or if the inability to isolate the above cell types refer to the use of adult testes as starting material. I believe that if authors have used the MDR protocol starting from juvenile mice testes with success, this should be described. In describing the MDR protocol applied to juvenile mice, authors should specify how many testes are used, protocol-specific differences relatively to adult testes, germ enrichment in each fraction, and RNA/protein amount collected, as shown in Figures 2B and 3A-B.

We have only used adult mice so far. Because STA-PUT has been successfully used to enrich meiotic cells from juvenile testes, we assume that the MDR could be used for similar approaches. However, we have not tested the performance of MDR protocol in enriching early spermatogenic cells from juvenile testes. The text has been modified to clarify this issue (lines 418-424).

Minor Concerns:

Fig. 4. I would add to the protocol overview the amount of starting material and average amount of RNA and proteins (output) obtained in fractions 1(ES), 2-9 (RS) and 13-16(Spc).

We have included the information about the starting material and average number of cells as well as amount of RNA/proteins obtained per fraction in the Figure.

Reviewer #2:

Manuscript Summary:

In this manuscript, the authors Ros et al. described a protocol named MDR to isolate subpopulations of pachytene spermatocytes, round spermatids and elongating spermatids from mouse testes. Compared to classical methods termed STA-PUT velocity sedimentation by unit gravity and fluorescence-activated cell sorting (FACS), several advantages of this protocol include fewer animals cost, less time usage, as well as no requirement for specialized training or instruments while pertaining good validity. This protocol would be particularly useful in many labs that are not yet equipped with STA-PUT apparatus to study spermatogenesis. However, the manuscript requires substantial improvements to reach the publication level.

Major Concerns:

1. Since this simplified protocol can be applied for isolation from postnatal mouse testes of at least three cell populations, exemplified here by pachytene spermatocytes, round spermatids and elongating spermatids, the abbreviation "MDR" (Modified Density gradient for Round spermatids) sounds not that inclusive. Could it be "SDG" (Simplified Density Gradient Method for Germ Cells)?

This protocol was originally developed specifically for the isolation of round spermatids from which it took the colloquial abbreviation of MDR. In our hands, the protocol performs the best for enriching round spermatids and the purity of round spermatid fractions is superior to the purity of other cell types (see lines 83-84). Therefore, to highlight this, we would like to keep the abbreviation MDR if possible.

2. Lines 295-297: "...This protocol has been optimized particularly for round spermatids...and we routinely obtain highly pure round spermatid fractions (up to 95%)". The STA-PUT method can routinely yield more than 94% purity for steps 1-8 round spermatids (Bellve, A.R. 1993). So, it's not clear what the optimization here indicates.

We are sorry about this unclearly formulated sentence. What we mean is that when we were developing the protocol, we were mostly interested in obtaining enriched round spermatids and therefore, the conditions (the design of the gradient, sedimentation time etc.) were optimized to favor their purity over other cell types. We did not intend to compare it to the STA-PUT method, which yields very similar purity for round spermatids. We have modified the sentence (lines 315-317).

3. It's confusing that either spermatocytes or pachytene spermatocytes are presented in figures and the main text. It can be much clearer because spermatocytes do not indicate any single specific type. In addition to DAPI, immunofluorescent staining should be done to prove the 75% purity for pachytene spermatocytes, in which the commercially available γ H2AX antibody can be used to mark the prominent feature of formation of XY body.

We have included new representative results (Figure 2C and D) to show that the isolated spermatocytes have sex bodies (anti- γ H2AX) and are therefore pachytene spermatocytes. We modified the text throughout the manuscript to specify the type of spermatocytes (including the title). In the same figure, we have also included example images showing staining of round spermatid fractions with anti-DDX4 antibody (chromatoid body staining) and PNA (acrosomal staining) to validate these fractions. A new paragraph was included in the representative results to describe these stainings (lines 329-344).

4. In the Discussion, the authors need to add a paragraph to compare MDR with STA-PUT, pointing out fully what details make the process of the former shorter in completion time and what about its shortcomings. Figure 4 seems inconsistent with the step-wise protocol contents. The authors should compare the timeline of the workflow of MDR to a parallel one of STA-PUT.

We agree with the reviewer that the discussion required some revision to bring out the benefits of the MDR method more clearly. The most important feature of the MDR protocol over STA-PUT is that it offers a considerably simplified strategy, particularly from the standpoint of unequipped research groups. Another advantage is that you can get quite nice fractions from only one mouse, therefore reducing the number of mice. We have obtained highly enriched populations from KO mice with reduced testis size as well. Otherwise it is really quite similar to the STA-PUT in terms of the time scale and the purity of cell fractions. We have now modified the discussion to clarify these issues. We have also revised the Figure 4 (see also response to the reviewer #1), but we did not compare the timelines of the MDR and STA-PUT because we came to the conclusion that these methods are timewise very similar.

5. In the representative results, the authors need to calculate recovered cell numbers of pachytene spermatocytes, round spermatids and elongating spermatids. The STA-PUT method can recover 4.0×10^6 /adult testis of pachytene spermatocytes, $20\text{--}40 \times 10^6$ /adult testis of Steps 1-8 round spermatids, and $25\text{--}50 \times 10^6$ /adult testis of Steps 12-16 round spermatids (elongating spermatids). The authors should make sure MDR methods can isolate enough cells to do downstream experiments.

We counted cells and included the information in the results (lines 346-351). The number of cells obtained by MDR is lower than that obtained by STA-PUT, but it is enough for many downstream analyses (see lines 355-362, and new Figures 3C and D).

Minor Concerns:

1. In step 1.4, should mention the pH of the 1 x KREBS buffer.

We added the pH for 1X KREBS.

2. In step 2.1, should mention the age and weight of mouse testes.

We have specified that the mouse should be adult, more than 7 weeks old, and the weight of an adult testis is 80-120 mg.

3. In step 5.5, should indicate the different cell diameters.

We have modified the text to include the diameters of DAPI-stained nuclei.

4. Line156: please confirm that 3 minutes is enough for dispersing all interstitial cells, the outcome of which from treatment is better to be shown as a photo.

The outcome of the Collagenase digestion (separation of the tubules from each other) will be visualized in the video, and therefore, we suggest not to include a photo to demonstrate this.

5. Line163: During the trypsin digestion, move the tube to the 34°C, 5% CO₂, 95% humidity-incubator? Please explain why not continue the digestion in a 37°C water bath or use a 37°C incubator.

The physiological temperature for testicular cells is 34°C, and therefore, long digestions are usually done at this temperature. However, the 3 minutes digestion time is so short that for convenience, we do it at 37°C (recommendation by the manufacturer) and the digestion time has been optimized for this temperature. Trypsin digestion is done at 34°C because the length of the digestion is longer. We have added a note in the step 2.4. to explain this.

6. Line168: Before filtering the solution, need to stop the trypsin digestion with 10% FBS?

It is true that the Trypsin digestion is usually stopped by FBS. However, we have skipped this step because it did not improve the outcome of the MDR enrichment. We have added a note in the step 2.6. to explain this.

7. Line 317: How many proteins are required for IP and IP-Mass?

To exemplify that the amount of protein in one MDR fraction is enough for successful immunoprecipitation, we performed IP from individual MDR-enriched fractions with anti-PIWIL1 antibody. Western blot analysis showed that the material obtained from a single fraction is sufficient for IP, and even for the detection of co-immunoprecipitated PIWIL2. This result can be found in the Figure 3D. We also included a Western blot analysis of whole cell extracts made from selected fractions (around 10% of each fraction loaded to the gel) (Figure 3C). The representative results section (lines 355-362) and figure legends were modified accordingly. According to our experience, the amount of total protein in one fraction, or the amount of immunoprecipitated protein complexes from one fraction, should be sufficient for the identification of proteins by mass spectrometry. However, we do not have our own experience on mass spec analysis after MDR enrichment, and therefore, we left it out from the text.

8. Many errors in spelling, grammar or format should be corrected.

We have proofread the text and edited the language.

1 Table 2: 0,5 mL? 9,5 mL? - ", "should be "."?

Tables were modified to use a period symbol as decimal separator.

2 Line 45: "The drawback of these techniques it that:..." - "it" should be "is"?

Corrected.

3 Line 85: "thought" should be "through"?

Corrected.

4 Line 363: "interested" should be "interest" ?

The text was modified.