Video Article

Separation of Spermatogenic Cell Types Using STA-PUT Velocity Sedimentation

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Abstract

Mammalian spermatogenesis is a complex differentiation process that occurs in several stages in the seminiferous tubules of the testes. Currently, there is no reliable cell culture system allowing for spermatogenic differentiation *in vitro*, and most biological studies of spermatogenic cells require tissue harvest from animal models like the mouse and rat. Because the testis contains numerous cell types - both non-spermatogenic (Leydig, Sertoli, myeloid, and epithelial cells) and spermatogenic (spermatogonia, spermatocytes, round spermatids, condensing spermatids and spermatozoa) - studies of the biological mechanisms involved in spermatogenesis require the isolation and enrichment of these different cell types. The STA-PUT method allows for the separation of a heterogeneous population of cells - in this case, from the testes - through a linear BSA gradient. Individual cell types sediment with different sedimentation velocity according to cell size, and fractions enriched for different cell types can be collected and utilized in further analyses. While the STA-PUT method does not result in highly pure fractions of cell types, *e.g.* as can be obtained with certain cell sorting methods, it does provide a much higher yield of total cells in each fraction

(~1 x 10^8 cells/spermatogenic cell type from a starting population of 7-8 x 10^8 cells). This high yield method requires only specialized glassware and can be performed in any cold room or large refrigerator, making it an ideal method for labs that have limited access to specialized equipment like a fluorescence activated cell sorter (FACS) or elutriator.

Video Link

The video component of this article can be found at http://www.jove.com/video/50648/

Introduction

Mammalian spermatogenesis is a complex differentiation process that occurs in several stages in the seminiferous tubules of the testes[1]. Briefly, stem-like spermatogonia that reside near the epithelium of the seminiferous tubule divide and differentiate into spermatocytes, which then undergo meiotic divisions. After meiosis is complete, the resulting haploid cells, or round spermatids, undergo spermiogenesis, a differentiation process that involves the shedding of cytoplasm and compaction of the nucleus. Spermatids gradually develop a flagellum and undergo elongation and condensation of the nucleus, producing elongating and then condensing spermatids, respectively. The end products are spermatozoa, which are released into the lumen of the seminiferous tubule and ultimately into the epididymis where they mature further.

Because the process of spermatogenesis relies on special hormonal and molecular conditions in the testes, a reliable *in vitro* culture system for the entire process of spermatogenesis has not yet been developed[2,3]. Culture methods have been developed for creating "primordial germ cell-like cells" and haploid, "round spermatid-like cells" from stem cells, but these methods are not yet able to generate large numbers of these cells and fail to produce later spermatogenic cell types[4,5]. Fortunately, the spermatogenic cell types differ significantly in size, which allows for a single-cell suspension obtained from whole testes to be separated with a liquid gradient. The STA-PUT method, demonstrated here, uses a linear BSA gradient and simple sedimentation to separate spermatogenic cells based on size and mass[6-9].

The STA-PUT method has several advantages over the other two most widely used methods to separate spermatogenic cell types: FACS and elutriation[10-13]. The STA-PUT apparatus requires only several pieces of specialized glassware assembled in a cold room or large refrigerator. Thus, it is less expensive than using a cell sorter or an elutriator. The STA-PUT method yields higher amounts of cells per cell type and testis than can be sorted by FACS in a comparable time frame, although the purity of each cell population is not as high as those obtained with

FACS[11]. Cell sorting utilizing magnetic beads (magnetic activated cell sorting, MACS) has recently been successfully employed for enrichment of spermatogonia from a mixed testicular cell population, but it is currently unsuitable for separating spermatocytes or spermatids due to lack of knowledge of appropriate surface markers[14]. An additional advantage of the STA-PUT method over FACS or MACS is the ability to isolate viable cells suitable for subsequent culture because, in contrast to most FACS protocols, it does not require any DNA or other types of staining. For studies that require large yields of spermatogenic cells types at ~90% purity, the STA-PUT is an ideal method.

Protocol

The STA-PUT protocol involves three stages: 1) Set up of the apparatus and reagents, 2) Preparation of cell suspension from whole testes, and 3) Cell loading, sedimentation, and fraction collection. When performed by a team of two researchers, the protocol takes eight hours on average.

1. Setting up the STA-PUT Apparatus (Figure 1)

***STA-PUT apparatus should be placed in a 4°C large refrigerator or a cold room that can also accommodate a fraction collector, if that method of collection is preferred.

1. The night before (or at least a few hours before) you perform the method, wash all equipment (especially the glassware and tubing) and sterilize with 70% ethanol. Let equipment dry completely before assembling the apparatus as illustrated in **Figure 1**.
2. Secure the two 2 L cylinders (**Figures** 1B and **C**) and the cell loading chamber (**Figure 1A**) to the top platform and connect all with two small pieces of tubing with tube clamps. Clamp all tubes closed. Seal the spout on the right-most 2 L cylinder.
3. Place a small stir bar in the cell loading chamber (**Figure 1A**) and a larger stir bar in the left-most 2 L cylinder (**Figure 1B**) that will contain the 2% BSA.
4. Place the 2 L sedimentation chamber on the platform (**Figure 1D**). Place the metal baffle (**Figure 1F**) directly on top of the opening in the bottom of the sedimentation chamber (**Figure 1D**). This is critical, as the baffle prevents vortexing of the liquid and disruption of the cell gradient during fraction collection. Place the lid on top of the sedimentation chamber.
5. After applying a very small amount of vacuum grease to the ground glass joint of the three-way stopcock (**Figure 1G**), clamp the stopcock to the bottom of the sedimentation chamber, connecting the ground glass joints of the stopcock and the sedimentation chamber.
6. Connect the cell-loading chamber (**Figure 1A**) to the right outlet of the stopcock with tubing. Close the stopcock.
7. Attach the cell fractionation tubing to the left outlet of the stopcock. The fractionation tubing comprises a piece of tubing with a glass Pasteur pipette connected to the open end. A piece of smaller bore tubing is attached to the narrow end of the glass pipette. The narrow pipette restricts the flow of the cell suspension during fraction collecting. Clamp this small tube at the very bottom.
8. Prepare 2 L Krebs (1x) buffer the day of the experiment (**Table 1**). Then, prepare 550 ml 2% BSA in 1x Krebs, 550 ml 4% BSA in 1x Krebs, and 50 ml 0.5% BSA in 1x Krebs. Filter and cool these solutions to 4 °C.
9. Pour the 4% BSA solution in the right 2 L cylinder (**Figure 1C**) and the 2% BSA solution in the left 2 L cylinder (**Figure 1B**). Make sure there are no large bubbles in the tubing that connects these cylinders.
10. Pour Krebs buffer into the cell loading chamber and fill the tubing connecting this chamber with the sedimentation chamber, making sure there are no large bubbles, as these could disturb the gradient. Squeezing or flicking the tubing gently helps to remove the bubbles. Make sure all of the Krebs buffer is in the tube and not in the cell chamber.
11. Allow a small amount of Krebs buffer to flow into the sedimentation chamber and then drain almost all the buffer into the cell fractionation tubing in order to fill the tube and remove any large bubbles.
12. Place the fraction collector directly under the sedimentation chamber. Make sure that all the fraction tubes are in place and covered with plastic wrap to prevent contamination.

2. Isolating Spermatogenic Cells from Whole Testes

1. Dissect testes from *ca*12 adult mice (preferably at least eight weeks old) and place in Krebs buffer at room temperature to wash off any contaminating material. This protocol being demonstrated involves the use of laboratory mice and was executed in compliance with the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.
2. Add 45 mg collagenase to 50 ml Krebs buffer in a conical tube right before you intend to add the seminiferous tubules. Allow this solution to heat up to 33 °C for a few minutes. Split evenly into two 50 ml conical tubes.
3. Decapsulate the testes in a separate plate containing 8 ml Krebs buffer, discarding the tunica albuginea and releasing the seminiferous tubules. The tunica albuginea is the thin membrane that surrounds the seminiferous tubules. The tubules should readily detach from the tunica albuginea. To do this, make an incision in the tunica albuginea, hold this membrane with a pair of forceps, and push the tubules out of and away from the membrane with another pair of forceps.
4. Add 4 ml Krebs buffer containing the tubules to each conical tube containing 25 ml of collagenase solution and incubate, shaking, at 33 °C for 10 min. At the end, the tubules should have a "spaghetti-like" appearance.
5. Allow the tubules to settle for ~5 min to the bottom of the tube. Pour out the supernatant and wash 2x in 25 ml Krebs buffer (at room temperature), allowing the tubules to settle to the bottom of the tube each time. Leave ~5 ml Krebs buffer in each tube.
6. While washing, add 30 mg trypsin to 50 ml Krebs buffer in a falcon tube right before you intend to add the seminiferous tubules. Allow this solution to heat up to 33 °C for a few minutes. Split evenly into two 50 ml conical tubes.
7. Add 25 ml trypsin solution to each of the two tubes containing the tubules. Add 3 µg DNAse (1 µg/10ml) to each tube to prevent cells from clumping. Incubate, shaking, at 33 °C for 10 min.
8. Use a wide bore pipette to agitate the solution containing the tubules, pipetting them in and out approximately 10x. The solution should begin to look more like a single cell suspension.
9. Incubate, shaking, at 33 °C for an additional 10 min. Use a wide bore pipette to disperse the tubules into a single cell suspension, pipetting them in and out approximately 25x. If you still see a lot of tubules or cell clumps, disperse more with the pipette and/or add another 3 µg DNAse to each tube (double initial concentration).

10. Filter the single cell suspension through a 100 µm mesh cell strainer (one for each tube of 30 ml cell suspension). Combine the cell suspensions and count the total number of cells (should be between 7-8 x 10^8 cells). ***DO NOT LOAD MORE THAN 800,000,000 cells onto the gradient.
11. Based on the cell count obtained in step 2.10, pellet the appropriate volume of filtered cells at 450 rcf for 5 min and wash with 30 ml Krebs buffer. Repeat. ***Usually 22 testes will yield more than 800,000,000 cells, but do not load more than this number of cells.
12. Carefully resuspend cells in 25 ml 0.5% BSA containing between 2.5 and 5 µg DNAse (depending on the degree of cell clumping) without creating bubbles. Make sure the cells do not clump. Filter the single cell suspension through a 100 µm mesh cell strainer. The cells are now ready to load. Mix well before loading by inverting the tube a few times.

3. Cell Loading and Sedimentation

1. Make sure that the stopcock is closed, but in the position that will allow liquid to flow from the cell chamber (**Figure 1A**) into the sedimentation chamber (**Figure 1D**).
2. Turn both stir bar plates on to a low setting (approximately 70 rpm) to allow the stir bars to move continuously, but slowly.
3. Pour the cell suspension into the cell chamber (**Figure 1A**) and open the stopcock so that the cells flow slowly into the sedimentation chamber at a rate of 10 ml/min (**Figure 1D**). Close the stopcock. Flow rate can be determined by marking volume intervals on the sedimentation chamber.
4. Pour 5 ml of 0.5% BSA solution into the cell chamber and drain into the sedimentation chamber at a rate of 10 ml/min. Close the stopcock. This step will wash the cells out of the cell chamber. Repeat 4x.
5. Prepare the gradient: Open the clamps between the cell chamber (**Figure 1A**) and the two 2 L cylinders (**Figure 1B and C**), and begin to drain the liquid into the sedimentation chamber at a rate of 40 ml/min (**Figure 1D**) immediately. Adjust the flow rate so it takes approximately 20-30 min to load the BSA gradient into the sedimentation chamber. A thin, undisturbed layer of cells lying on top of the BSA gradient should be visible. Once most of the BSA is loaded, close the stopcock and turn it to the position that will allow liquid to drain from the sedimentation chamber into the fractionation tube.
6. Turn off the stir plates.
7. Allow the cells to sediment for one hour and 45 minutes. DO NOT DISTURB THE APPARATUS DURING THIS TIME. Any mechanical agitation could disturb the gradient.

4. Fractionation and Analysis of Fractions

***As you perform the following steps, be careful not to disturb the gradient. If the BSA gradient is disturbed, the procedure will not work!

1. Once the cells have sedimented, slowly drain 50 ml from the sedimentation chamber into a 50 ml conical tube and set aside. This fraction usually contains unwanted clumps of cells and debris.
2. Attach the fraction tube to the fraction collector. ***It is also possible to collect the fractions by hand if a fraction collector is not available.
3. Collect fractions: Adjust the flow rate with the stopcock so that 10 ml (filling one collection tube) is collected every 45 sec.
4. Cap the fraction collection tubes and spin for 5 min at 450 rcf at 4 °C. Pour off supernatant gently, resuspend cells in remaining liquid, and keep cells on ice.
5. Once all fractions are collected, microscopically analyze the fractions for different cell populations. Different cell types can be distinguished based on cell size and nuclear morphology[8,15] . **Figure 2** illustrates "representative results" for the three pooled fractions that are enriched for (a) somatic and meiotic cells and spermatogonia, (b) round spermatids, and (c) elongating and condensing spermatids.
    - Type A spermatogonia are 12-14µm ca. in diameter and have round nuclei that are homogenous in chromatin composition.
    - Type B spermatogonia are 8-9µm ca. in diameter and have round nuclei that show more dense heterochromatin along the nuclear periphery.
    - Pre-leptotene spermatocytes are 7.5-8.2µm ca. in diameter, have less cytoplasm than Type B spermatogonia, and have nuclei that look similar to those in Type B spermatogonia.
    - Leptotene primary spermatocytes are 8-10 µm ca. in diameter and have nuclei that look similar to those of pre-leptotene spermatocytes, but seem to gain more dense chromatin at the nuclear membrane.
    - Zygotene primary spermatocytes are 10-12 µm ca. in diameter and have nuclei that look similar to those of leptotene primary spermatocytes.
    - Pachytene spermatocytes are anywhere from 12-18 µm ca. in diameter and consist of a thin rim of cytoplasm surrounding a large nucleus. More clumps of dense chromatin can be seen in the nucleus.
    - Round spermatids are 10 µm ca. in diameter and have a round nucleus with a densely-staining chromocenter in the middle.
    - Residual bodies are ~6.5 µm in diameter, are round, and lack a nucleus.
    - Elongating and condensing spermatids are similar in size to round spermatids, but have a unique, sickle-shaped nucleus.

    1. Start with fraction 15 and analyze every five fractions up to 85. Usually, fractions below 15 will be too mixed and clumpy, and fractions above 85 will contain few to no usable cells.
    2. To analyze a fraction, take 5uL of liquid from the fraction collection tube (once cells have been resuspended after centrifugation) and add to 5uL of 8% formaldehyde in Krebs buffer. Allow the fixed cells to sit at room temperature for five minutes.
    3. Add 5uL of 0.1% Triton and DAPI (5 µ/ml Krebs buffer) to the fixed cells. Allow the cells to sit at room temperature for 5 min.
    4. Place 10 µl of the resulting solution onto a slide, cover with a cover slip, and analyze with a fluorescence microscope to determine the purity of each fraction.
    5. Combine fractions that are similar in size and nuclear morphology (see "Representative Results") to create the following populations of cells: meiotic and somatic diploid cells, round spermatids, and condensing/elongating spermatids.

Representative Results

The ideal result from the STA-PUT procedure is a fairly noticeable separation of cells from the testes based on cell size and density. While cells isolated from the testes are sedimenting through the BSA gradient, several distinct bands of cells can be observed. Any clumps of cells tend to sink to the bottom of the gradient and will not contaminate the other fractions. A little further up the gradient will be the large somatic and meiotic cells. Farther up the gradient still will be smaller round spermatids. At the top of the gradient will be condensed spermatids, sperm, and contaminating red blood cells (these appear to be small round cells without a nucleus).

Fractions can be analyzed quickly using a combination of light and fluorescent microscopy **(Figure 2)**[15]. Meiotic, spermatogonial, and somatic diploid cells are the largest cells found in the testes and will contain large nuclei that stain relatively homogeneously with DAPI. Round spermatids are smaller cells with smaller round nuclei, generally with a brightly staining chromocenter. Condensing/elongating spermatids are small cells that often look oblong, as if a small tail is forming. These cells have smaller, compact nuclei that stain brightly with DAPI and are shaped like a sickle. Once cell fractions are combined, purity can be further determined by western blot analysis of the cell lysates **(Figure 3).** Common markers of meiotic cells are the synaptonemal complex 1 proteins Scp1 and Sycp2[16]. Common markers of condensing spermatids are transition proteins (*e.g.* TP1) or protamines[17].

Although each STA-PUT run can be different, usually meiotic cells, spermatogonia and somatic diploid cells will be found in fractions *ca.* 25-40, round spermatids in fractions *ca.* 55-65, and condensing/elongating spermatids in fractions *ca.* 65-75 Due to a smaller difference in size between somatic/meiotic cells and round spermatids, fractions *ca.* 45-50 often have an even percentage of somatic/meiotic cells and round spermatids. Staining with DAPI will help to distinguish these two populations of cells. There is less overlap of the round spermatid and condensing/elongating spermatid fractions due to the larger difference in size between these two populations of cells. Usually, fractions below 15 will contain many large clumps of cells and fractions above 85 will contain few cells and many residual bodies, or membrane-bound cytoplasm that is shed by spermatids.

Generally, when cells from ~22 testes are fractionated with the STA-PUT procedure, it yields *ca.* 10^8 cells/spermatogenic cell type (meiotic/somatic diploid cells, round spermatids, and condensing/elongating spermatids). Fractions that are combined to create the final population of cells should be at least 80% pure for the type of cell in question. If you do not see this degree of purity or higher, there may be a problem with cell separation or the BSA gradient. Also, if there are few cells in the first 20 fractions and an abundance of cells in fractions 80+, or if there is an abundance of cells in the first 20 fractions and hardly any in fractions 70+, the sedimentation time needs to be further optimized. Please see the Discussion for suggestions on how to trouble shoot.

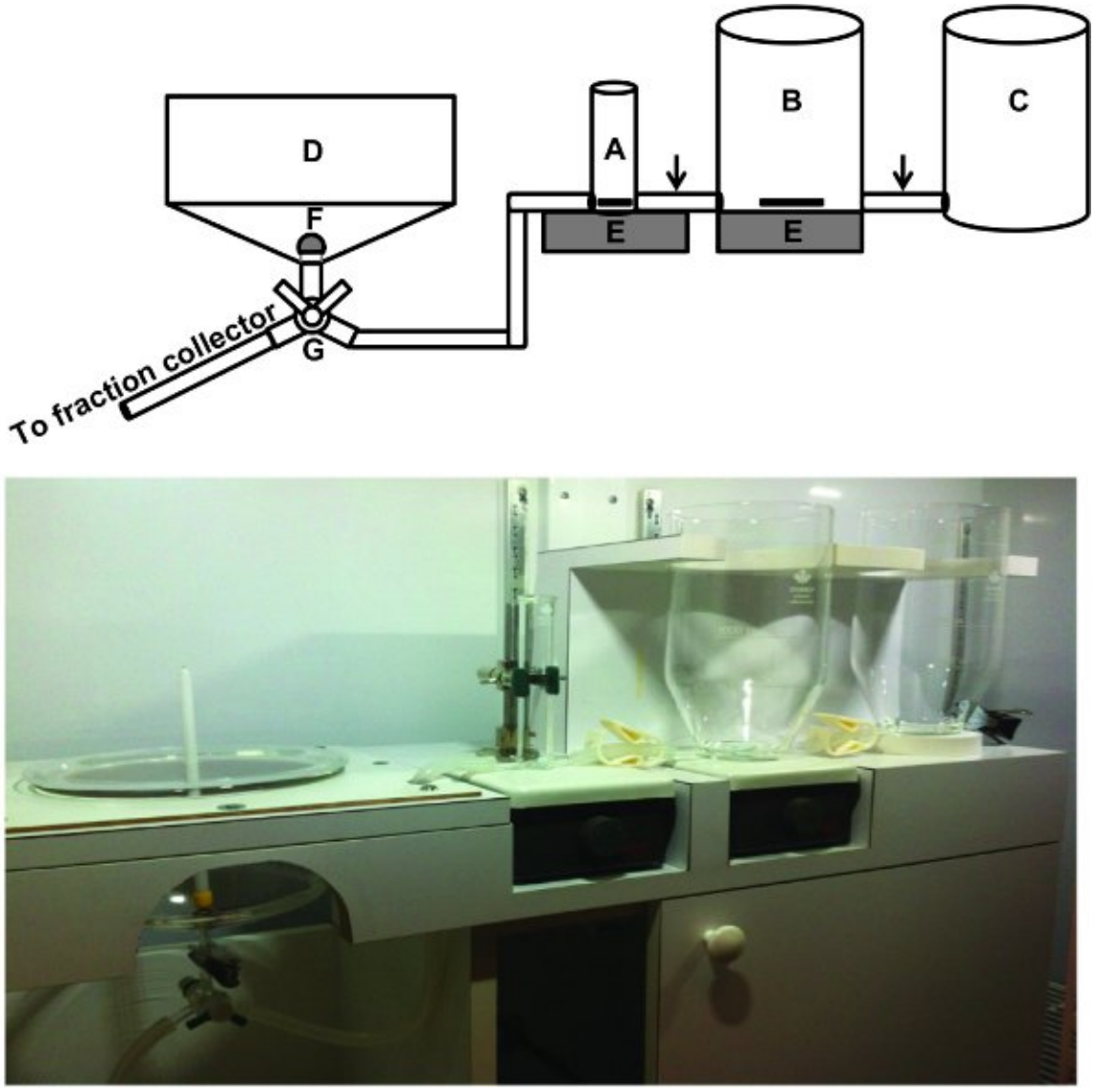

Figure 1. Setting up the STA-PUT Apparatus: A schematic and actual image of the STA-PUT apparatus are shown. All glassware is connected by plastic tubing, including the tube that connects the apparatus to the fraction collector. Arrows indicate location of clamps. A) Cell loading chamber, contains a stir bar; B) 2 L cylinder for 2% BSA, contains a stir bar; C) 2 L cylinder for 4% BSA; D) Sedimentation chamber; E) Stir plates; F) Baffle; G) Stopcock. Click here to view larger image.

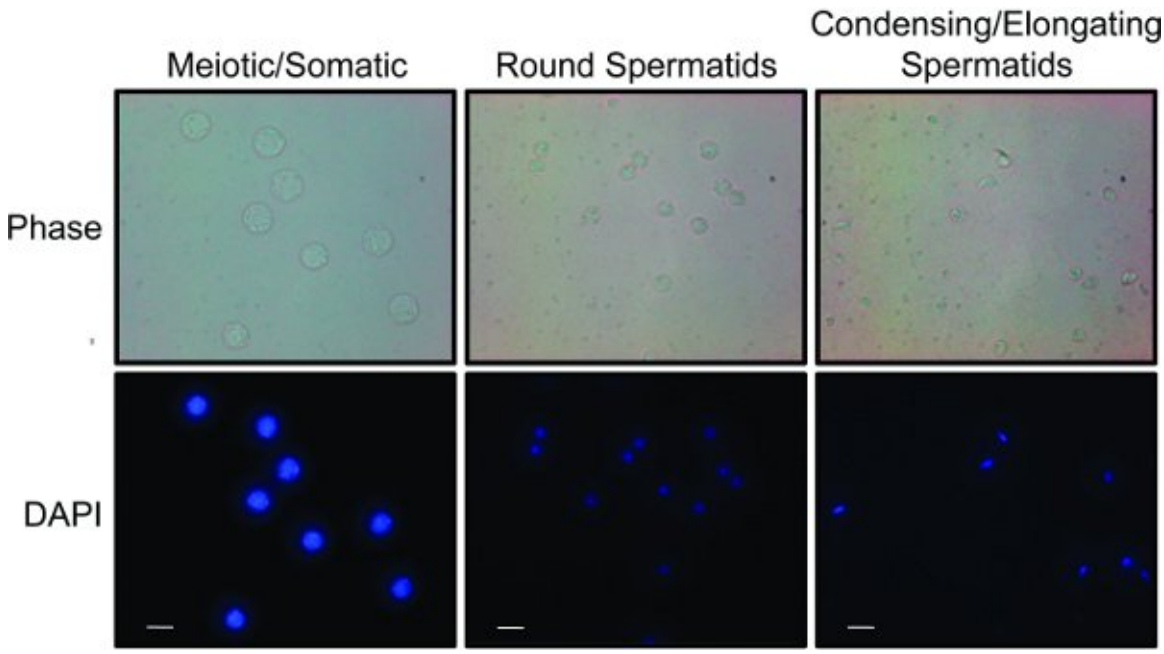

Figure 2. Cell populations obtained from the STA-PUT: Fractions were combined into three separate populations of cells: meiotic and somatic cells, round spermatids, and condensing and elongating spermatids. Each population is stained with DAPI to show differences in nuclear size and morphology. Phase contrast imaging conveys differences in cell size and shape. White bar represents 10 μm. Click here to view larger image.

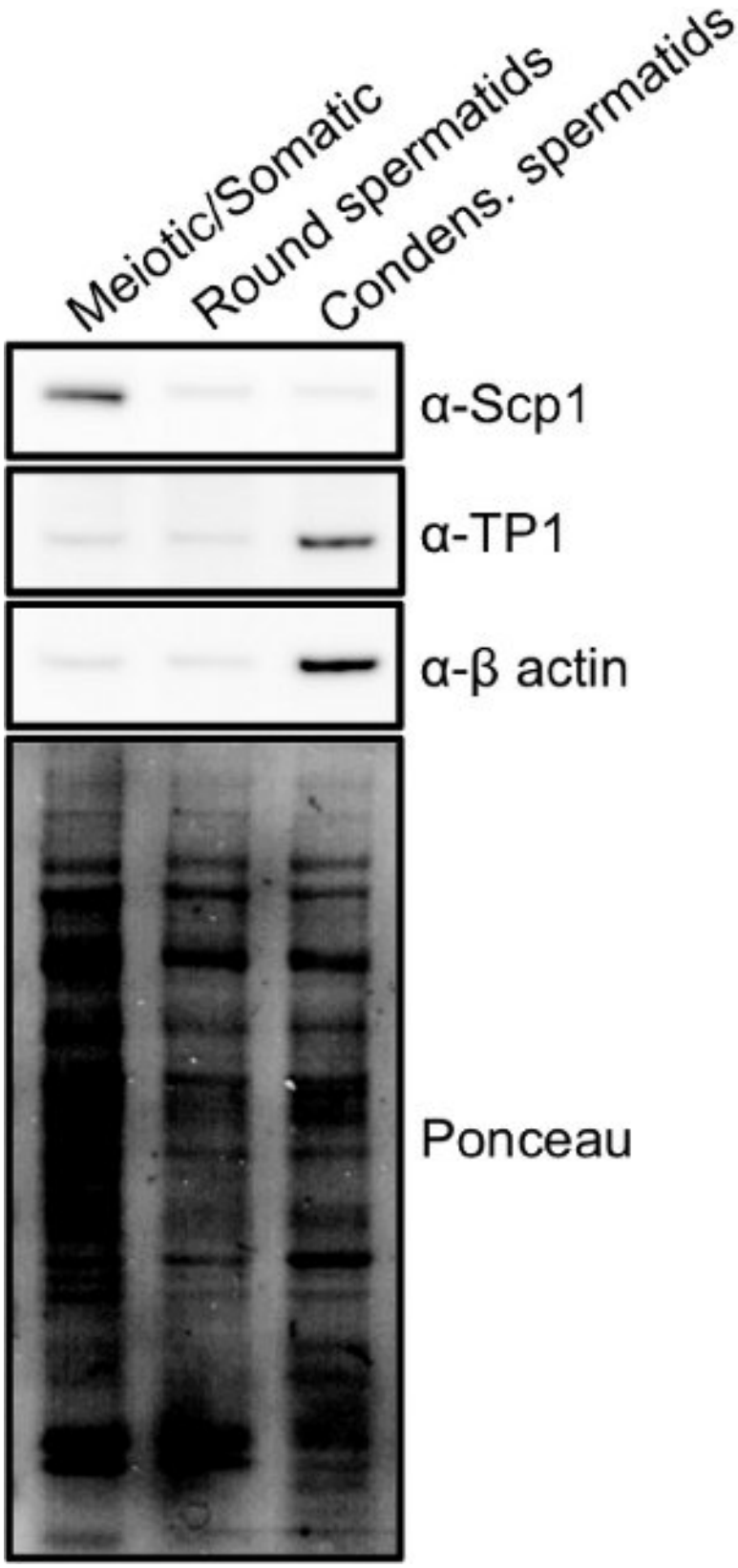

Figure 3. Markers of different cell populations obtained from the STA-PUT: Whole cell extracts were made from each cell population shown in **Figure 1.** and western blot analysis was performed to show protein expression differences for each population. Synaptonemal complex protein 1 (Scp1) is a protein expressed exclusively during meiosis and is found enriched in the meiotic fractions, while the condensing spermatid fraction is enriched for transition protein 1 (TP1), a protein expressed late in spermiogenesis. Click here to view larger image.

Discussion

Those who study spermatogenesis rely on animal models for spermatogenic cell samples, as a reliable cell culture system does not yet exist for generating all spermatogenic cell types[3]. Although spermatogenic cells are readily collected from whole testes, only a mixed population results. This poses a problem for those who wish to study specific subtypes of these cells, such as meiotic cells, round spermatids, and condensing spermatids. Three different methods are currently used to separate these subtypes of spermatogenic cells: STA-PUT, FACS, and elutriation[6-13]. The latter two methods require access to expensive pieces of equipment: a cell sorter and an elutriator, respectively. Although the FACS method yields highly pure populations of the different spermatogenic cell types, the process takes six hours and yields only 0.5-2.0 x 10^6 cells per cell type per two to three testes[11]. Elutriation, like the STA-PUT method, separates cells based on size and density, but requires access to an elutriator, which is more expensive than the STA-PUT apparatus.

The STA-PUT procedure uses a simple BSA gradient to separate spermatogenic cells of different sizes with a fairly high yield (~10^8 cells/population per ~22 testes). Relative to FACS methods, the STA-PUT procedure yields more cells/testis and takes much less time to separate cell types. Compared to FACS and elutriation, the STA-PUT procedure is relatively simple and inexpensive. The STA-PUT requires only a cold room or large refrigerator and a set of specialized glassware, making it an ideal method for labs without access to a cell sorter or elutriator. When performed properly, the STA-PUT can provide an approximately 90% pure population of round spermatids or condensing spermatids.

The STA-PUT method is very useful, but requires optimization at several different steps, especially sedimentation. Sub-optimal separation of cell types can be caused by several different issues, most relating to the BSA gradient. To make a proper gradient, turn on the stir bars in the cell loading chamber and the 2 L cylinder holding the 2% BSA while you are creating the gradient. Also clear all tubing of bubbles and put the baffle in place in the sedimentation chamber before loading the cells. Reducing the flow rate of the BSA into or out of the sedimentation chamber may help. Most importantly, the STA-PUT apparatus should not be disturbed during the creation of the gradient, sedimentation, or fraction collection.

Sub-optimal cell yields can be the result of insufficient number/size of testes, inadequate cell separation during collagenase/trypsin treatment, and cell clumping. If one is unable to obtain the appropriate number of cells for this STA-PUT protocol due to the use of neonatal mice or genotypes that produce small numbers of spermatogenic cells, it may be necessary to use more animals per STA-PUT or to order a STA-PUT glassware kit optimized for smaller volumes and cell numbers (available from ProScience)[18]. To obtain an optimal number of cells (700-800 million), use at least 11 male mice of reproductive age, ideally at least 8-9 weeks old. However, no more than 800 million cells should be loaded into one STA-PUT. If cells have not dissociated into a single cell suspension after trypsin treatment, the DNAse concentration can be increased up to 1 µg/5 ml solution. DNAse is sensitive to repeat cycles of freeze/thaw, and using fresh DNAse each time will result in a more effective dispersion of tubules into a single cell suspension. One can use a wide bore pipette to help break apart cell clumps before filtering through mesh.

One aspect of the STA-PUT method that will require optimization is the amount of time the cells are allowed to sediment through the BSA gradient. One hour and 45 min usually works well, but this time may differ from lab to lab. Usually, different layers of cells can be seen visually throughout the BSA gradient. If there are few cells in the first 20 fractions collected and an abundance of cells in fractions 80+, the sedimentation time may need to be extended. If there are too many cells in the first 20 fractions collected and there is insufficient separation of cell types, the sedimentation time may need to be lowered.

The cells acquired with the STA-PUT procedure can be used for many different types of experiments. The STA-PUT provides ample material for western blot analysis, immunofluorescence, and RNA analysis, although large-scale biochemistry experiments may require combining material from several different STA-PUT runs. When separated from other cell types, haploid spermatids can be cultured and subjected to *in vitro* molecular manipulation for up to three days, which can be easier than *in vivo* treatment or creating a knockout animal[19]. Cells obtained with the described STA-PUT protocol have been cultured for one day without obvious signs of contamination, but if cells are to be used for longer cell culture experiments, equipment should be sterilized with ethanol, all solutions should be filter sterilized, and all steps before cell loading and after fraction collection should be performed in a tissue culture hood. In addition, culture media containing antibiotics should be used. The fact that the STA-PUT method does not require cell fixation makes it an ideal procedure for experiments that require viable cells.

Disclosures

The authors declare that they have no competing financial interests. This protocol being demonstrated involves the use of laboratory mice and was executed in compliance with all relevant guidelines, regulations and regulatory agencies. Animals used in this protocol are maintained under the guidance and approval of the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC protocol #804284).

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