Received: 20 September 2021 | Revised: 17 January 2022 | Accepted: 21 January 2022
DOI: 10.1002/cyto.a.24539

TECHNICAL NOTE

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

Dual DNA staining enables isolation of multiple sub-types of post-replicative mouse male germ cells

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Funding information
Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung, Grant/Award Numbers: 31003A-146293, 31003A-172873; Novartis Research Foundation

Abstract

During spermatogenesis, mammalian male germ cells undergo multiple developmental processes, including meiosis and post-meiotic differentiation (spermiogenesis). To understand the transitions between different cellular states it is essential to isolate pure populations of cells at different stages of development. Previous approaches enabled the isolation of cells from different stages of meiotic prophase I, but techniques to sub-fractionate unfixed, post-meiotic spermatids have been lacking. Here we report the development of a protocol enabling simultaneous isolation of cells at different stages of meiotic prophase and post-meiotic differentiation from testes of adult mice. This approach builds on existing fluorescence activated cell sorting protocols designed to purify cells in different stages of meiotic prophase I. By utilizing the specific spectral properties that two different DNA dyes (Hoechst 33342 and SYTO 16) exhibit when bound to chromatin of different stage male germ cells, we obtain highly pure populations of cells in relatively large numbers. This FACS protocol will enable immunocytological and molecular characterization studies of fractionated meiotic and haploid germ cells from both wild type and genetically mutant animals.

KEYWORDS
germ cell, spermatid, spermatocyte, spermatogenesis

1 | INTRODUCTION

Spermatogenesis represents an extensive differentiation process that occurs over the course of several weeks in the adult mouse [1]. During this process a population of adult stem cells, known as spermatogonial stem cells, are recruited into a periodic differentiation program, undergoing a programmed number of cell divisions while also initiating a cellular differentiation program [2]. This ultimately results in a pool of transit amplifying differentiating spermatogonia that then undergo a final, pre-meiotic, DNA replication before progressing into meiotic prophase. This cell cycle stage is protracted in male mammals, with cells spending a particularly long time in pachynema. Following this stage, cells rather rapidly complete the two meiotic divisions, producing haploid products known as round spermatids. Following another extended time period, round spermatids initiate nuclear elongation and compaction, becoming elongating and elongated spermatids, resulting finally in the production of the mature spermatozoa [3]. Post-meiotic differentiation, referred to as spermiogenesis, is a time period in which many dramatic changes required for the generation of mature sperm occur. This includes the near global removal of histone proteins from the chromatin, silencing of transcription and removal of nearly all the cytoplasm [3]. Purification of cells specifically undergoing these changes is essential to understand the molecular mechanisms underlying them.

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Several approaches have been applied to isolate cell populations from testes for analysis. These approaches have historically relied on two primary features of testicular germ cells for purification: size and shape or the successive appearance of different germ cell types in juvenile animals after puberty. Isolation of cells based upon size and shape using centrifugal elutriation or sedimentation through gradients (for instance of BSA, as in the so-called STA-PUT approach [4–6]) can fractionate certain cell populations to reasonable purity. Others, however, that are present in relatively low abundance, or which are not particularly homogeneous in terms of shape have proven more intractable. Analysis of juvenile animals in theory allows examination of many cell populations during their first appearance in the testis. However, while this process is somewhat synchronous, substantial variation can exist between animals, an effect that becomes a larger problem with increasing age [6, 7]. In addition, while juvenile spermatogenesis is thought to resemble the steady state process occurring in adults, it is known that some aspects of these processes differ [8-10].

As spermiogenesis progresses, cells become increasingly non-round and become smaller in size, thus making their purification more difficult. Recently, approaches synchronizing the process of spermatogenesis by manipulation of retinoic acid signaling in early postnatal animals have been developed, greatly improving the capacity for cellular isolation [11-12]. However, the use of such approaches for cell sorting generally requires the addition of a transgenic reporter construct to cells to fully separate germ cells from somatic cells [13], requiring additional crosses when mutant animal analysis is desired.

Staining cells with DNA dyes prior to fluorescence activated cell sorting (FACS) is a common approach to isolate cells based on their cell cycle stage. The standard vital DNA dye Hoechst 33342 has been used extensively in the mouse testis to separate meiotic from post-meiotic cells, and to sub-fractionate cells within meiotic prophase [14, 15]. This approach, however, is not efficient for the sub-fractionation of different types of haploid spermatids. More recently, a special spectral property of the green fluorescent DNA dye SYTO 16 has been reported in fixed cells [16]. In this study Simard et al., [16] showed that cells containing compacted chromatin, a characteristic of spermatids following their nuclear elongation, show much higher fluorescence with SYTO 16 than round spermatids, despite possessing identical DNA content. This staining approach, however, has thus far not been adapted to live cells, and does not allow for simultaneous isolation of meiotic and post-meiotic cells from the same animal. We developed an approach combining Hoechst 33342 and SYTO 16 along with the cell viability dye DRAQ7 to efficiently isolate several populations of meiotic and post-meiotic male germ cells from genetically unmodified, unperturbed adult mice. This approach provides a rapid and simple approach to obtain cells from both wild type and mutant animals for extensive characterization, with relatively high developmental resolution.

2 | MATERIALS AND METHODS

2.1 | Animals

Eight-week-old C57BL/6JRj/6 mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and allowed to age in our animal facility. All experiments were performed in accordance with Swiss animal protection laws (license 51, Kantonales Veterinäramt, Basel, Switzerland) and institutional guidelines from the Friedrich Miescher Institute for Biomedical Research.

2.2 | Materials and reagents

2.2.1 | Consumables used

15 ml conical tubes (BD Falcon, #352097); 50 ml conical tubes (CellStar, 227261); Disposable transfer pipette (VWR, 612–1747); Shaking water bath; 40-μm nylon cell strainer (BD Falcon, #352340); 30 μm tube-top filters (Sysmex, 04-004-2326); 5 ml round bottom polypropylene tubes (BD Falcon, 352,063); Ten-well diagnostic slides (ThermoFisher Scientific ER-208B-CE-24, 10-well, 6.7 mm).

2.2.2 | Reagents used for generation and staining single-cell suspension

Collagenase Type I (Worthington Biochemical, #LS004196); DNAse I (Sigma DN25-100MG); Gey's Balanced Salt Solution (GBSS) (Sigma-Aldrich, G9779-500ML); 0.25% Trypsin, phenol red (Gibco, 25050014); Hoechst 33342 (10 mg/ml solution in water) (Life Technologies, #H3570); SYTO 16 (1 mM solution in DMSO) (ThermoFisher S7578); DRAQ7 (300 μM) (BioLegend, 424001); FBS (Gibco).

2.2.3 | Reagents used for quality control by microscopy

16% paraformaldehyde (Electronic Microscopy Sciences, 15710); Bovine Serum Albumin (BSA) (Sigma, A9418-5G); mouse anti-γH2AX monoclonal antibody (Millipore 05-636); rabbit anti-SYCP3 polyclonal antibody (abcam ab15093); rabbit anti-TNP2 polyclonal antibody (a kind gift from Stephen Kistler); mouse anti-PRM1 monoclonal antibody (Briar Patch Biosciences MAb-Hup1N-150); VectaShield mounting media with DAPI (Vector Labs H-1200).

2.2.4 | Stock solutions

DNAse I is diluted to 5 mg/ml in water and stored at -20°C.

2.2.5 | Solutions for the enzymatic digestion of testes (made fresh at time of experiments)

Collagenase I solution: (200 U/ml Collagenase I and 5 μg/ml DNAse I dissolved in GBSS), Trypsin solution: (200 U/ml Collagenase I, 0.025% Trypsin and 5 μg/ml DNAse I dissolved in GBSS).

2.2.6 | Solutions for microscopy

Hypotonic buffer (30 mM Tris, pH 8.2, 50 mM sucrose, 17 mM sodium citrate dehydrate); PFA-T (1% paraformaldehyde, 0.15% Triton X-100, pH 9.2); Blocking solution (PBS + 1% BSA).

2.2.7 | Generation of single cell suspensions from mouse testes

The two step enzymatic dissociation of testes was based on the protocol of Gaysinskaya and Bortvin [17].

1. Purification of seminiferous tubules: The tunica albuginea was removed from each testis and seminiferous tubules were briefly washed in GBSS with care to dissociate interstitial cells from tubules yet maintain tubules as intact as possible. Tubules from each testis were then transferred into 15 ml conical tubes and incubated in 6 ml of Collagenase I solution for 3 min at 32°C shaking.
2. Removal of somatic cells: Tubules were allowed to settle to the bottom of the tube for 1–2 min and supernatant was then removed.
3. Generation of single-cell suspension: 6 ml of Trypsin solution was added to each tube and tubules were pipetted up and down using a disposable plastic transfer pipette. Samples were then digested for 12 min at 32°C and then again pipetted up and down 20–30 times before being incubated for an additional 12 min at 32°C. After incubation samples were pipetted up and down a third time, until no obvious cell clumps were visible. 600 μl FBS was then added to each tube to inactivate the trypsin, and cells were filtered through a 40 μm filter into a fresh Falcon tube. When cells from multiple animals were to be sorted at the same time, cells were pooled and filtered into 50 ml conical tubes.
4. Staining cells with DNA dyes: An additional 10 μl (per initial testis) of DNAse I (5 mg/ml) was added to each tube to prevent cell clumping. 15 μl Hoechst 33342 and 8 μl SYTO 16 (per testis) were added to the tube and mixed by inversion. Cells were stained at room temperature, rotating, and protected from light for 40 min up to 2 h.
5. Preparation of cells for FACS: Cell suspensions were centrifuged at 250xG for 10 min at room temperature and supernatants were removed. Pellets were resuspended in 1 ml GBSS per testis, and 2 μl 5 mg/ml DNAse I and 10 μl DRAQ7 were added per testis to the tubes and mixed by gentle inversion. Cells were then strained through a 30 μm filter into 5 ml polypropylene tubes and placed on ice (protected from light) until cell sorting commenced.

2.2.8 | Fluorescence activated cell sorting

Setup of FACS Aria III for cell sorting

A BD FACS Aria III was used for cell sorting. Data analysis was performed using the BD FACSDiva software. The sorter was fitted with a 70 μm nozzle and lasers at 375, 488, 561, and 633 nm. Sheath pressure was set to 70 psi (sheath buffer was composed of PBS without Mg^{++} and Ca^{++}). Sort Mode was set to 4 Way Purity (Yield Mask 0, Purity Mask 32, and Phase Mask 0). Drop Frequency was set to 86,900 Hz (Amplitude of 11.8 and Drop Delay at 44.09). Plate voltage was set to 4500 Volts. The filter sets used for sorting were: (a) Hoechst 33342 Blue: 450/20, (b) Hoechst 33342 Red: 610LP/670LP, (c) SYTO 16: 502LP/530/30, (d) DRAQ7: 755LP/780/60.

Setup of flow cytometry profile and gating strategy for isolation of testicular germ cells

Several working plots as shown in Figure 1 were created: (a) FSC-A (linear scale) versus DRAQ7-A (log scale) (FIgure 1A), (b) Hoechst 33342 Red-A versus Hoechst 33342 Blue-A (both linear scale) (Figure 1B), (c) SYTO 16-A (log scale) versus FSC-A (linear scale) (Figure 1G), (d) FSC-A versus SSC-A (linear scale, Figures 1C, 1E, 1H, 1I, and 1J), (e) Hoechst 33342 Blue-W versus Hoechst 33342 Blue-A (both linear scale, Figures 1D, 1F, 1K, 1L, and 1M). Data from 100,000 cells were acquired to draw the initial gates.

On the FSC-A versus DRAQ7 plot, a gate called P1 ("Alive") excluding cells with high DRAQ7 and very low FSC values was drawn (Figure 1A). All subsequent gates were "children" of the P1 gate.

On the Hoechst 33342 Red-A versus Hoechst 33342 Blue-A plot, gates P2-P4 around populations of interest were drawn as follows: (a) P2 was drawn around the dense region with low Hoechst 33342 Red-A and low Hoechst 33342 Blue-A region representing all haploid spermatids, (b) P3 was drawn around the region containing high Hoechst 33342 Red-A and low to mid Hoechst 33342-Blue-A signal representing cells in early meiotic prophase (Leptotene and Zygotene spermatocytes), (c) P4 around the region containing high Hoechst 33342 Red-A and high Hoechst 33342-Blue-A signal. This region contains cells in late meiotic prophase (Pachytene and Diplotene spermatocytes).

Cells from gate P2 were then displayed on the SYTO 16-A versus FSC-A plot (Figure 1G). Three additional gates (P5-P7) were then drawn on this plot as follows: (a) P5 was drawn around the region containing lowest SYTO 16-A and high FSC-A signal, representing round spermatids, (b) P6 was drawn around the region containing highest SYTO 16-A and high FSC-A signal containing early elongating spermatids, (c) P7 was drawn around the region containing high SYTO 16-A and low FSC-A signal, containing late elongating spermatids.

For each individual subpopulation (P3-P7) plots of FSC-A versus SSC-A were created and gates around the regions containing highest density of cells were drawn to isolate homogeneous subpopulations. For each further isolated subpopulation (P8, P9, P10, P14, P16) additional plots of Hoechst 33342 Blue-W versus Hoechst 33342 Blue-A were created and gates (P11, P12, P13, P15, P17) around the dense population present in each plot were drawn (especially removing outliers with higher Hoechst 33342 Blue-W signal, which contain potential doublets).

Sorting of testicular germ cells

The FACS Aria III was run using the profiles established in the previous section. Samples were run with a flow rate of less than 25,000

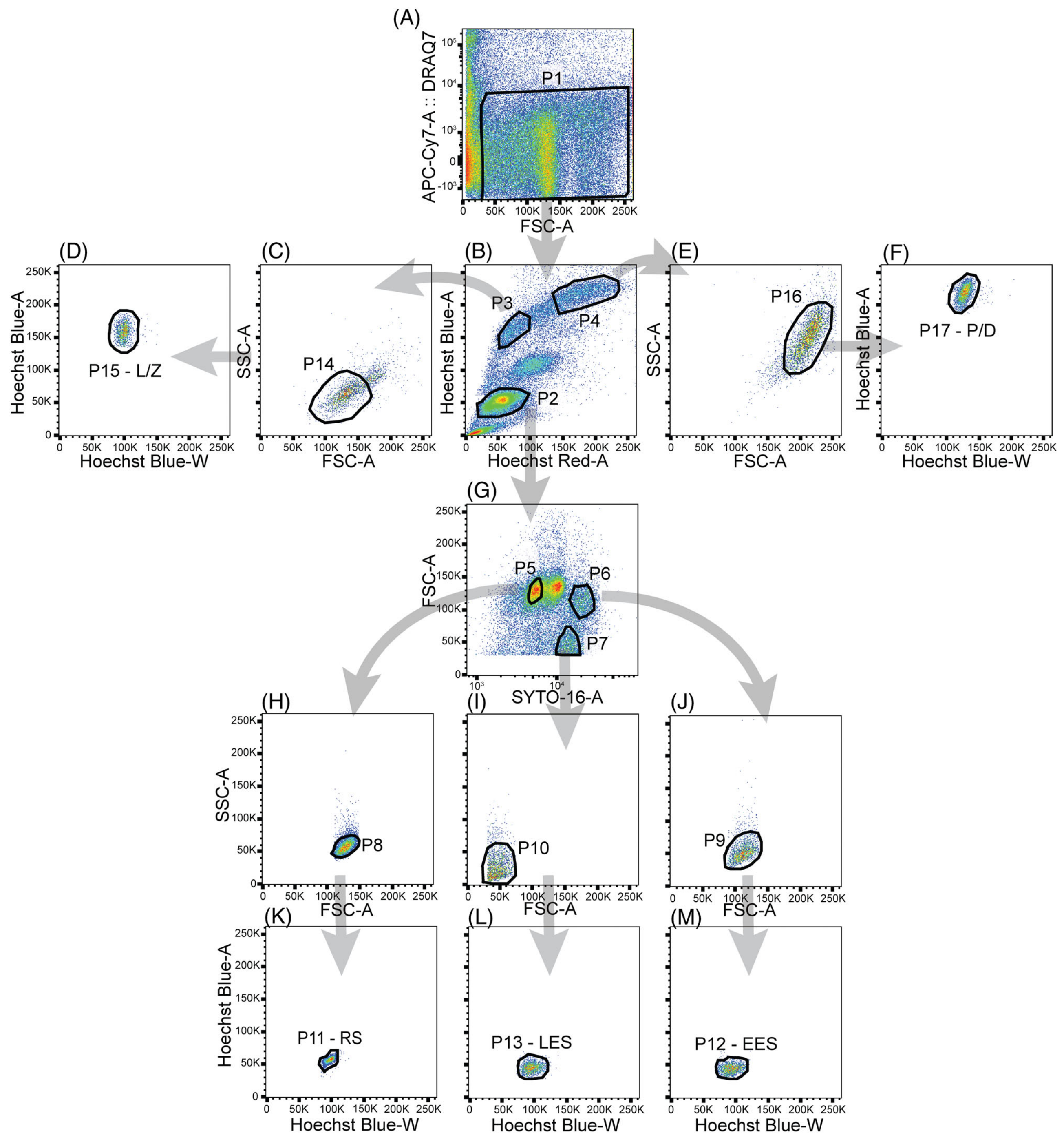

FIGURE 1 Gating strategy for sorting of post-replicative testicular germ cells. Figure panels show gating plots with data from a representative sort of testicular cells from a wild type adult (3–6-month-old) mouse [Color figure can be viewed at wileyonlinelibrary.com]

events per second (to maintain a relatively high efficiency of sorting). Samples were collected into 1.5 ml Eppendorf tubes, pre-treated overnight with PBS + 2.5% BSA at 4°C (from which the liquid was removed, leaving 50 μl for sorting).

2.2.9 | Quality control by immunofluorescence

To ensure that cells are truly pure, it is recommended to fix 5000–10,000 cells onto slides and examine them by immunofluorescence microscopy using appropriate cellular markers (adapted from Peters et al. 1997 [18]).

For meiotic spermatocyte populations

A 20 μl volume of hypotonic buffer was added to 5 μl of each cell population and incubated at room temperature for 10 min. A 30 μl droplet of PFA-T was placed into each well of a 10-well slide. 10 μl of hypotonically treated cells were then added into each well. Cells were then incubated 1–2 h at room temperature or overnight at 4°C in a humid chamber, after which the lid of the chamber was removed, and

cells were allowed to fully dry onto slides. Slides were then either processed immediately for analysis of stored in −80°C for later analysis. Each well was washed two times with 30 μl of PBS in rapid succession, followed by a third wash of 5 min at room temperature. Cells were blocked for 20 min at room temperature in a humid chamber with blocking solution. Cells were then incubated for 1 h at room temperature (or overnight at 4°C) with 1:500 anti-SYCP3 and 1:5000 anti-γH2AX diluted in blocking solution. Cells were then washed three times for 5 min each with 30 μl PBS at room. Cells were then incubated 30 min at room temperature with fluorescently conjugated secondary antibodies (diluted 1:500 in blocking solution). Cells were then washed three times for 5 min each with 30 μl PBS at room temperature. Slides were then mounted with 4 μl of VectaShield mounting media containing DAPI per well and covered with a 50 mm coverslip. Slides were sealed with nail polish and stored at 4°C until imaging on a fluorescent microscope with appropriate filter sets.

For haploid spermatid populations

A 30 μl droplet of PFA-T was placed into each well of a 10-well slide and then 5 μl of each FACS-sorted cell suspension was added to each well. Cells were incubated in a humid chamber 1–2 h at room temperature or overnight at 4°C. The lid of the chamber was then removed cells were allowed to dry completely onto slides. Slides were then analyzed immediately or placed in −80°C for later analysis. Each well was washed two times with 30 μl of PBS in rapid succession, followed by a third wash of 5 min at room temperature. Samples were blocked for 20 min with blocking solution at room temperature in a humid chamber. Cells were incubated 1 h at room temperature (or overnight at 4°C) with 1:1000 rabbit anti-TNP2 and 1:500 mouse anti-PRM1 diluted in blocking solution in a humid chamber. Cells were washed three times for 5 min each with PBS. Cells were incubated for 30 min at room temperature with appropriate secondary antibodies diluted 1:500 in blocking solution in a humid chamber. Cells were washed 3 times for 5 min each with PBS. Slides were mounted with 4 μl of VectaShield mounting media containing DAPI per well and covered with a 50 mm coverslip. Slides were sealed with nail polish and stored at 4°C until imaging on a fluorescence microscope with appropriate filter sets.

3 | RESULTS AND DISCUSSION

3.1 | Generation of single-cell suspension and sorting of cells

As noted by Gaysinskaya, et al. [15], the generation of high-quality single cell suspensions is essential to obtain reproducible, highly pure subpopulations of testicular germ cells. We began by using the procedure described in Gaysinskaya, et al. [15] and Gaysinskaya and Bortvin [17]. This protocol uses a sequential digestion of testicular tissue with two enzymes: collagenase followed by trypsin. Treatment of seminiferous tubules with collagenase removes the interstitial somatic cells surrounding the germ cell-containing tubules. We found that the supernatant from tubules treated extensively with collagenase contains elongating spermatids in addition to somatic cells. We speculate that these cells are generally less well attached to the structure of the tubule and are thus more easily released through breaks in tubules generated during tissue processing. For this reason, to maximize the number of elongating spermatids available for purification, we made two modifications to the original protocol. First, prior to treatment with trypsin, we attempt to maintain tubules as intact as possible to provide minimal locations for elongating spermatids to escape. Second, we have reduced the duration of collagenase treatment compared to the original protocol. These two modifications lead to consistent generation of single cell suspensions, while maintaining relatively large numbers of differentiated spermatids.

Spermatids are the most abundant cell population in the testis [1]. When sorting both meiotic and post-meiotic cells simultaneously, it is difficult to obtain similar numbers of cells of different fractions. We have chosen to pool cells from early prophase (leptonema and zygonema) and late prophase (pachynema and diplonema) into only two populations, as this results in similar quantities of material from spermatocyte and spermatid sorts. If higher resolution analysis of meiotic prophase I is required, we recommend using the protocol developed by Gaysinskaya, et al. [15], which results in more subpopulations of spermatocytes, but substantially fewer cells per subpopulation.

We consistently observe that cells containing haploid DNA content (as determined by Hoechst 33342) possess three distinct levels of SYTO 16 (Figure 1G), a result also previously observed with fixed cells [16]. To maximize the purity of cells obtained in this protocol, we do not isolate cells with intermediate SYTO 16 and high FSC-A levels, as these cells, while consisting mostly of round spermatids, contain a substantial portion of contaminating elongating spermatids. If cell numbers are extremely limiting and purity is less essential these cells could be used as an additional source of round spermatids.

We have chosen to use DRAQ7 to distinguish live from dead cells in this protocol. This is because the fluorescence spectra for this dye is highly distinct from that of Hoechst 33342 and SYTO 16. We have used a 755LP/780/60 filter for detection of DRAQ7 signal. Other filters (such as 690LP/730/45) may also be used for this dye to capture more of the emitted light. It would also be possible to utilize other cell viability dyes (such as propidium iodide), provided that their fluorescence spectra do not overlap with those of the two DNA dyes used for sorting.

Speed during cell preparation prior to FACS is key to maintaining a large portion of living cells during sorting (as judged by exclusion of the vital dye DRAQ7 in this protocol). The total duration to generate a single cell suspension stained with Hoechst 33342 & SYTO 16 from a mouse is approximately 2 h, and we can generally sort enough cells for many molecular analyses in around 1 h. Staining with these two DNA dyes reaches equilibrium at around 40 min at room temperature and does not substantially change even if staining is left up to 2 h. Analysis of cells re-stained with DRAQ7 following sorting reveals only a minimal increase in cell death associated with sorting itself. We have, however, observed a marked increase in the percentage of DRAQ7 positive cells when stained cells are left on ice for a

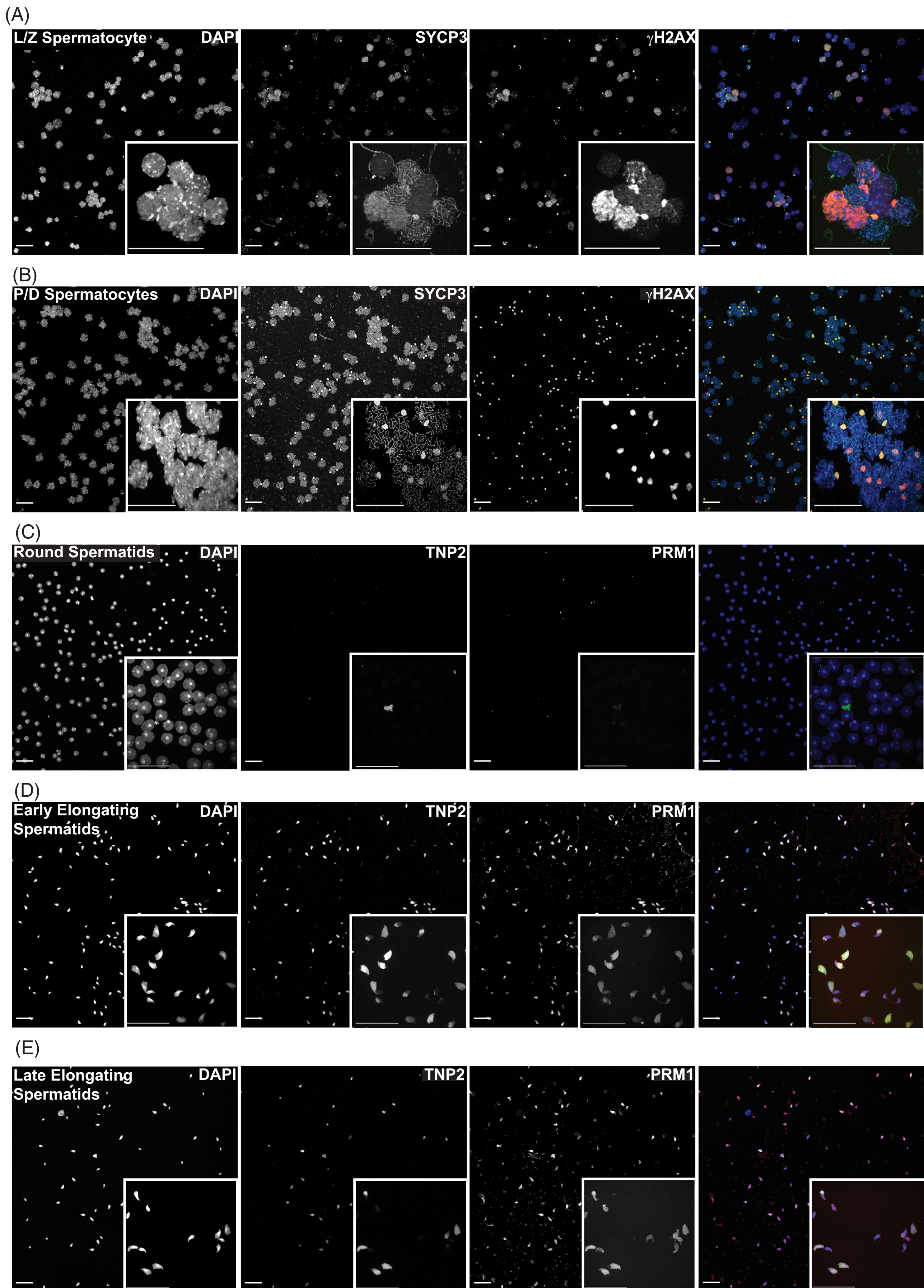

FIGURE 2 Legend on next page.

TABLE 1 Quantification of the purity of cell populations

Population sorted	% L/Z spermatocyte	% P/D spermatocyte	% Round spermatid	% Early elongating spermatid	% Late elongating spermatid	% Other
Leptotene/zygotene spermatocyte (L/Z)	**89.26** (**0.75**)	7.24 (0.77)	0.77 (0.18)	0.21 (0.19)	0.12 (0.20)	2.41 (0.15)
Pachytene/diplotene spermatocyte (P/D)	4.93 (1.97)	**92.86** (**1.76**)	1.05 (0.35)	0.35 (0.36)	0.22 (0.38)	0.59 (0.43)
Round spermatid (RS)	0.00 (0.00)	0.47 (0.21)	**95.40** (**0.62**)	1.76 (0.34)	1.04 (0.65)	1.33 (0.95)
Early elongating spermatid (EES)	0.00 (0.00)	0.13 (0.22)	1.30 (0.50)	**85.88** (**1.37**)	11.96 (0.97)	0.73 (0.43)
Late elongating spermatid (LES)	0.00 (0.00)	0.21 (0.19)	0.71 (0.51)	20.84 (5.62)	**77.29** (**5.51**)	0.95 (0.82)

Note: Cell purities from three independent sorts of wild type (C57BL/6) adult (3–6-month-old) male mice were calculated. For each population at least 250 cells were classified (based on nuclear morphology and marker expression) as belonging to one of the five sorted populations or to other cell types (other) if morphology did not match. Values are presented as mean percentage with *SD* in parentheses. The bold values refer to the main cell types obtained within a designated fraction of sorted cells.

protracted period. These germ cells also tend to clump, leading to possible nozzle clogs during sorting. For these reasons we recommend that if cells from multiple (i.e., more than two) animals are to be sorted on the same day, the start of processing should be staggered, such that no cells are left for more than 3 h as a suspension on ice.

3.2 | Assessment of population purity by immunofluorescence

The general purity of cell fractions isolated from adult testes can be determined based on the size and shape of the nuclei in these cells. The addition of antibodies for specific markers can speed the analysis of fraction purity and help to refine characterization of ambiguous cells. The expected results for cells purified using this approach are as follows. Leptotene/Zygotene spermatocytes (P15) possess a medium sized nucleus with a relatively diffuse DAPI signal. Marker analysis shows small spots or fibers of SYCP3 and foci of γH2AX throughout the nucleus (Figure 2A) [19]. Pachytene/Diplotene spermatocytes (P17) contain a large nucleus with multiple lobes of DAPI signal and more clearly defined spots of heterochromatin. SYCP3 is present in distinct thick fibers and γH2AX covers a specific region of the nucleus (the XY body) in these cells (Figure 2B). Round spermatids (P11) possess a clear nuclear morphology defined by a small round nucleus, frequently with a single DAPI-dense chromocenter in the center (Figure 2C). Early elongating spermatids (P12) have small crescent-shaped nuclei and show strong positive signal for TNP2 (Figure 2D). Late elongating spermatids (P13) also possess a crescent-shaped nucleus, though it is smaller than that seen in early elongating spermatids, and they are strongly positive for PRM1 (Figure 2E). The size differential between these populations can be challenging to judge by eye, thus use of transition protein staining (which is weak or negative in late elongating spermatids) serves as a very useful diagnostic [20,21]. We have successfully used both a rabbit polyclonal anti-TNP2 antibody (a kind gift from Stephen Kistler) and a commercially available goat polyclonal anti-TNP2 antibody (Santa Cruz Biotechnology [sc-21,106], diluted at 1:200) for staining transition proteins.

Analysis of the purity of cells from each population was determined from three independent sorts of wild type C57BL/6J mice (Table 1). We observed good reproducibility and high levels of purity for all five populations. Early and late elongating spermatids showed the relatively lowest level of purity (with the contaminants largely coming from the other species of elongating spermatids in both populations), likely owing to the continual nature of differentiation as spermatids undergo nuclear elongation.

4 | CONCLUSIONS

The approach presented here augments existing testicular germ cell purification methods by improving separation of post-meiotic spermatids while also allowing simultaneous isolation of meiotic spermatocytes. The methodology is rapid, inexpensive, and suitable for sorting using many different FACS setups, as the dyes utilized possess

FIGURE 2 Quality control for sorting of male germ cells. (A,B) Representative staining of meiotic spermatocyte populations with antibodies against synaptonemal complex protein 3 (SYCP3) and phosphorylated H2A.X (γH2AX), a marker of DNA damage. (C–E) Staining of post-meiotic spermatid populations with antibodies against transition protein 2 (TNP2), a marker specific to early elongating spermatids and protamine 1 (PRM1), a marker found in both early and late elongating spermatids. Scale bars represent 50 μm [Color figure can be viewed at wileyonlinelibrary.com]

fluorescent properties compatible with standard filter sets. Some alteration of gating strategy may be necessary when using other FACS machines as behavior of cell populations has been shown to differ between flow cytometers (even of the same model) [20].

ACKNOWLEDGMENTS

We thank W. Stephen Kistler (University of South Carolina) for the anti-TNP2 antibody. Laurent Gelman and Steven Bourke (FMI Facility for Advanced Imaging and Microscopy) and the FMI animal facility provided excellent assistance. Research was supported by the Novartis Research Foundation and the Swiss National Science Foundation (31003A-146293; 31003A-172873). Open access funding provided by Universitat Basel.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Mark E. Gill developed the protocol together with Hubertus Kohler who provided FACS expertise. Mark E. Gill performed the sorting and imaging experiments. Mark E. Gill wrote the manuscript. Antoine H. F. M. Peters edited the manuscript and coordinated the project.

ETHICS STATEMENT

All experiments described here were performed in accordance with the Swiss animal protection laws (license 51, Kantonales Veterinäramt, Basel, Switzerland) and institutional guidelines.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/cyto.a.24539.

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REFERENCES

1. Russell LD, Ettlin RA, Sinha AP, Clegg ED. Histological and histopathological evaluation of the testis. Clearwater, FL: Cache River Press; 1990.
2. de Rooij DG. The nature and dynamics of spermatogonial stem cells. Development. 2017;144:3022–30.
3. Gill ME & Peters AHFM. Chromatin structure, composition and function during spermatogenesis encyclopedia of reproduction. In M. K. Skinner, editor. Vol. 3. Academic Press: Elsevier; 2018. p. 85–89. https://doi.org/10.1016/B978-0-12-801238-3.64431-8
4. Grabske RJ, Lake S, Gledhill BL, Meistrich ML. Centrifugal elutriation: separation of spermatogenic cells on the basis of sedimentation velocity. J Cell Physiol. 1975;86:177–89.
5. Meistrich ML. Separation of mouse spermatogenic cells by velocity sedimentation. J Cell Physiol. 1972;80:299–312.
6. Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J Cell Biol. 1977;74:68–85.
7. Nebel BR, Amarose AP, Hacket EM. Calendar of gametogenic development in the prepuberal male mouse. Science. 1961;134:832–3.
8. Yoshida S, Sukeno M, Nakagawa T, Ohbo K, Nagamatsu G, Suda T, et al. The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. Development. 2006;133:1495–505.
9. Busada JT, Velte EK, Serra N, Cook K, Niedenberger BA, Willis WD, et al. Rhox13 is required for a quantitatively normal first wave of spermatogenesis in mice. Reproduction. 2016;152:379–88.
10. Guo J, Sosa E, Chitiashvili T, Nie X, Rojas EJ, Oliver E, et al. Single-cell analysis of the developing human testis reveals somatic niche cell specification and fetal germline stem cell establishment. Cell Stem Cell. 2021;28:764–78.
11. Snyder EM, Davis JC, Zhou Q, Evanoff R, Griswold MD. Exposure to retinoic acid in the neonatal but not adult mouse results in synchronous spermatogenesis. Biol Reprod. 2011;84:886–93.
12. Hogarth CA, Evanoff R, Mitchell D, Kent T, Small C, Amory JK, et al. Turning a spermatogenic wave into a tsunami: synchronizing murine spermatogenesis using WIN 18,446. Biol Reprod. 2013;88:40.
13. Romer KA, de Rooij DG, Kojima ML, Page DC. Isolating mitotic and meiotic germ cells from male mice by developmental synchronization, staging, and sorting. Dev Biol. 2018;443:19–34.
14. Bastos H, Lassalle B, Chicheportiche A, Riou L, Testart J, Allemand I, et al. Flow cytometric characterization of viable meiotic and postmeiotic cells by Hoechst 33342 in mouse spermatogenesis. Cytometry A. 2005;65:40–9.
15. Gaysinskaya V, Soh IY, van der Heijden GW, Bortvin A. Optimized flow cytometry isolation of murine spermatocytes. Cytometry A. 2014;85:556–65.
16. Simard O, Leduc F, Acteau G, Arguin M, Gregoire MC, Brazeau MA, et al. Step-specific sorting of mouse spermatids by flow cytometry. J Vis Exp. 2015;106:e53379.
17. Gaysinskaya V, Bortvin A. Flow cytometry of murine spermatocytes. Curr Protoc Cytom. 2015;72:1–24.
18. Peters AH, Plug AW, van Vugt MJ, de Boer P. A drying-down technique for the spreading of mammalian meiocytes from the male and female germline. Chromosome Res. 1997;5:66–8.
19. Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, et al. Recombinational DNA double-strand breaks in mice precede synapsis. Nat Genet. 2001;27:271–6.
20. Meistrich ML, Mohapatra B, Shirley CR, Zhao M. Roles of transition nuclear proteins in spermiogenesis. Chromosoma. 2003;111:483–8.
21. Balhorn R, Weston S, Thomas C, Wyrobek AJ. DNA packaging in mouse spermatids. Synthesis of protamine variants and four transition proteins. Exp Cell Res. 1984;150:298–308.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Gill ME, Kohler H, Peters AHFM. Dual DNA staining enables isolation of multiple sub-types of post-replicative mouse male germ cells. Cytometry. 2022;101:529–36. https://doi.org/10.1002/cyto.a.24539