Multispecies purification of testicular germ cells

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ABSTRACT

Advanced methods of cellular purification are required to apply genome technology to the study of spermatogenesis. One approach, based on flow cytometry of murine testicular cells stained with Hoechst-33342 (Ho-FACS), has been extensively optimized and currently allows the isolation of 9 germ cell types. This staining technique is straightforward to implement, highly effective at purifying specific germ cell types and yields sufficient cell numbers for high throughput studies. Ho-FACS is a technique that does not require species-specific markers, but whose applicability to other species is largely unexplored. We hypothesized that, due to the similar cell physiology of spermatogenesis across mammals, Ho-FACS could be used to produce highly purified subpopulations of germ cells in mammals other than mouse. To test this hypothesis, we applied Ho-FACS to 4 mammalian species that are widely used in testis research - *Rattus norvegicus*, *Cavia porcellus*, *Canis familiaris* and *Sus scrofa domesticus*. We successfully isolated 4 germ cell populations from these species with average purity of 79% for spermatocytes, and 90% for spermatids and 66% for spermatogonia. Additionally, we compare the performance of mechanical and chemical dissociation for each species, and propose an optimized gating strategy to better discriminate round and elongating spermatids in the mouse, which

can potentially be applied to other species. Our work indicates that spermatogenesis may be uniquely accessible among mammalian developmental systems, as a single set of reagents may be sufficient to isolate germ cell populations from many different mammalian species, opening new avenues in the fields of development and male reproductive biology.

INTRODUCTION

Spermatogenesis is a complex developmental process in which early spermatogonial stem cells differentiate into spermatozoa in the seminiferous tubules of the testes. The study of this fascinating process has produced critical insights into stem cell biology [1], developmental gene regulation [2], adaptive evolution [3] and fertility [4]. With over 30 different distinct cell types in the vertebrate testis, there is exceptional diversity in the expression profiles of cells within a single individual, which can become confounding when studying expression differences among individuals or developmental stages [5]. This has compelled researchers to develop methods for effective male germ cell enrichment and isolation, such as StaPut velocity sedimentation, elutriation, magnetic-activated cell sorting (MACS), whole testis collection during first wave of spermatogenesis and fluorescence-activated cell sorting (FACS) with Hoechst-33342 (Hoechst).

StaPut and elutriation are fairly efficient techniques that allow separation of different germ cells based on their size and density. When applied to mouse, StaPut yields about 10^8 cells/population from 22 testes with 90% purity, whereas approximately 10^7 cells/population can be obtained by elutriation of two testes with 80-95% purity rate [6, 7]. In both methods, the fractionation step that collects purified cells from different BSA or Percoll gradients is labor intensive (3-4 hours) and requires proficiency from practice as well as specific equipment. Also, both techniques are unsuitable for detailed molecular studies during meiosis as they can only separate one type of meiotic cell subpopulation at a time and fail to yield sufficient purity [8, 9]. MACS, which separates desired germ cell populations by conjugating the germ cells with a known surface marker antibody primed with magnetic beads, may circumvent this issue by performing purification in parallel with population-specific antibodies. However, only spermatogonia and spermatids are proven to have established surface markers for successful enrichment [6]. Furthermore, antibody-assisted purification has limitations in that it be necessary to develop species-specific reagents for each marker, and antibody-assisted purification typically does have the sensitivity to discriminate between cells at slightly different stages of a quantitative developmental process. Collecting mouse testis samples at specific days post-partum (dpp), timed for the first appearance of different germ cell-types during first wave of spermatogenesis, is also used to enrich specific germ cell populations [10]. Given that the testis size is very small at those time points, and that samples comprise a mixture of all testicular cells, this approach is experimentally challenging and fails to detect intrinsic biological variations among individual cells. Importantly, evidence from

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different studies suggests that the first wave is regulated differently from adult spermatogenesis [10-12].

FACS of Hoechst stained (Ho-FACS) murine male germ cells can discriminate 9 germ cell-types [8, 13-16]. Hoechst-33342 is a vital dye that binds preferentially to poly(d[AT]) sequences in the minor groove of DNA, with secondary binding taking place at higher ratios. These two DNA binding sites show varying binding energies and consequent spectrum shifts in relation to chromatin amount and structure [17, 18]. It has been proposed that this spectral shift could be used to discriminate between cells with similar DNA content but different chromatin properties [19-21]. Indeed, Ho-FACS of male germ cells exhibits a pattern that reflects changes in DNA content (blue fluorescence) and chromatin structure (red fluorescence) throughout spermatogenesis. In fact, red fluorescence shifts resulting from progressive chromatin de-condensation during meiotic prophase allow the resolution of different meiotic subpopulations [13, 15]. Spermatogonial stem cells are an exception and represent a side population due to BCRP1-dependent dye efflux, which is switched off after the spermatogonial stages [13]. Therefore, measuring Ho intensity as a function of blue and red fluorescence is representative of 3 cellular properties: ploidy, chromatin structure/accessibility, and dye efflux caused by ABC transporter activity [13, 15, 22, 23]. With over 95% purity of sorted populations [15] and an average of 10^7 cells/ population from two testes in less than two hours, this technique has proven highly efficient and less labor intensive. Although the actual FACS session requires specialized sorting equipment (UV laser) and a skilled operator, many research facilities provide cell-sorting services.

Recently, there is a growing interest in applying genomic technology to germ cells, especially in an evolutionary context [24, 25]. In that sense, purified cells can be used for numerous applications ranging from studying gene regulation, nucleosome mapping, epigenetics, development in germ cells and many more [26-28]. To unravel the complexity of germ cells at a genomic level, researchers need an efficient and high-throughput purification technique that can be applied easily, to many species. Given that Ho-FACS is not based on a species-specific molecular signature (e.g. an antigen), and that the cellular machinery for spermatogenesis is similar across all vertebrates, we hypothesized that separation of different germ cell types by Ho-FACS could be applied to other species. To test this hypothesis, we applied Ho-FACS to four species that are highly valued by the testis research community: *Rattus norvegicus* (rat), *Cavia porcellus* (guinea pig), *Canis familiaris* (dog) and *Sus scrofa domesticus* (miniature pig).

Our results provide detailed descriptions on how Ho-FACS performs with an optimized gating strategy that includes a cell viability gate with propidium iodide (PI) staining and a DNA content gate, at cell enrichment for 4 primary types of germ cells in each of the 4 species that we investigate. Each of our target spermatogenic germ cell types could be distinguished by Ho-FACS of the diploid mammalian species. We also demonstrate the use of a mechanical testis dissociation protocol in comparison to species-specific conditions for enzymatic dissociation, and present an optimized FACS gating strategy based on cell shape, size and complexity to distinguish elongated and round spermatids in mouse.

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Collectively, we offer the first proof of principle that flow cytometry can be applied transversally across mammalian species to isolate Hoechst stained male germ cells in different developmental stages.

MATERIALS AND METHODS

Animals

C57BL/6 male mice (Jackson Laboratory), Sprague Dawley male rats (Harlan Bioproducts), guinea pigs and miniature pigs were raised in animal facilities at Washington University in St. Louis.. Dog testes were collected at Hillside Animal Hospital (St. Louis, MO), from animals scheduled for castration, and were transported to the lab on ice for immediate processing. Prior to surgery, dogs are routinely injected with lidocaine and bupivacaine to help with the recovery process. All testis samples were obtained from sexually mature animals (Mice: 8 - 12 weeks, Rats: 70 days, Dogs: 12-24 months, Guinea pigs: 3 months; and Mini pig: 6 months) and procedures were conducted in compliance with regulations of the Animal Studies Committee at Washington University in St. Louis

Collection and processing of testicular tissue

Fresh testes from each species were de-capsulated, rinsed in 1X Phosphate buffered saline (PBS; Thermo Scientific #AM9625) and cut to the size of mouse testis (approximately 1.5cm X 0.7cm). These tissue fragments were used without further processing for dissociation and FACS sorting or fixed for histology. For immunofluorescence, tissue was fixed in 4% Paraformaldehyde (PFA; VWR #15710) overnight at 4°C and washed with 70% EtOH at least 3 times. Testes sections used for Hematoxylin-Eosin (HE) staining were collected in Modified Davidson's solutions (24h at room temperature with gentle rotation; Electron Microscopy Sciences #64133-50), fixed in Bouin's solution (24h at room temperature with gentle rotation; Sigma #HT101128) and washed with 70% EtOH until any remaining yellow color of Bouin's fixative was completely removed.

Immunofluorescence and Hematoxylin-Eosin Staining

Fixed testes samples were processed in an ethanol series, embedded in paraffin and 5µm sections were cut. Slides were de-paraffinized with xylene and rehydrated to PBS through sequential ethanol washes with decreasing alcohol concentrations. Standard HE staining was performed according to HE protocol adapted from Belinda Dana (Department of Ophthalmology, Washington University in St. Louis School of Medicine) with Hematoxylin 560 (Surgipath #3801570) and 1% Alcoholic Eosin Y 515 (Surgipath #3801615) for overall morphological evaluations. Immunofluorescence staining was performed after antigen retrieval (boiling in citric acid buffer for 20min) and tissue permeabilization/blocking (0.5% triton x-100 + 2% goat serum in 1X PBS for 1h at room temperature). Primary [anti-P-H3(ser10); AbCam, #Ab5176] and secondary (goat anti-rabbit ALF 633; Life Technologies #A21071) antibodies were diluted (1:100; 1:500 respectively) in antibody dilution buffer (1X PBS + 1% Tween 20 + 1% BSA)

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and incubated overnight at 4°C and 4h at room temperature, respectively, in a humid chamber. After secondary antibody incubation, sections were stained with Hoechst-33342 (1:500; Life Technologies, #H3570), washed with 1X PBS and mounted with ProLong Diamond Antifade Mountant (Life Technologies #P36961). For comparative purposes with FACS sorted germ cells, only Hoechst fluorescence is shown from these sections.

Testis dissociation and Hoechst Staining

Two different types of testicular dissociation protocols were used in this work: enzymatic and mechanical. The latter was performed using a Medimachine system (BD™ Biosciences; Cat. #340588) in line to the method previously described for rodents in [29]. A multispecies enzymatic dissociation protocol was designed based on the procedure described in [8] for mouse, as described below, and species-specific adjustments were made in terms of enzymes used, their relative concentrations, and incubation time and temperatures (See supplementary data). Except for mini pig, whose species-specific adjustments were made according to [30], enzymatic dissociation conditions were adopted following the Worthington references for reproductive tissue dissociation[31].

Enzymatic dissociation of testicular tissue (multispecies protocol):

Preparation of solutions (fresh; prior to testes collections): Collagenase type I (120U/mL; Worthington Biochemical, #LS004196) + Cycloheximide (0.1mg/mL; Amresco #94217) in 1X Dulbecco's Modified Eagle Medium (DMEM; Life Technologies #31053). Trypsin (50mg/mL; Worthington #LS003708) in 10mM HCl. DNase I (1mg/mL; Roche #10104159001) in 50% glycerol.

1- Testis enzymatic digestion: Testes (mouse) or testes fragments (rat, dog, and frog) were placed in 15mL conical tubes containing 3mL of DMEM/Collagenase I/CHX solution, and 10µL of DNase I solution. The tube was shaken vigorously until the testicular tubules started to disperse and then agitated horizontally at a speed of 120 for 15 min at 33°C. Temperature and agitation speed were the same for all subsequent incubation steps.

2- Somatic cell removal: Tubules were decanted for 1 min vertically at room temperature and the supernatant was discarded to remove somatic cells.

3- Seminiferous tubule digestion: 2.0 mL of DMEM/Collagenase I/CHX, 50µL of Trypsin and 10µl of DNase I solutions were added and the tube was inverted several times. After 15 min incubation period, the tubules were gently pipetted up and down for 3 min using a plastic disposable Pasteur pipet with wide orifice. Then, 30µL of trypsin and 10µL of DNase I solution were added and the tube was inverted several times, followed by another 15 min digestion period.

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4- Staining with Hoechst: 400µL of Fetal Bovine Serum (FBS; Thermo Scientific #10082139) was added and mixed by inverting to inactive trypsin, followed by addition of 5µL of Hoechst-33342 (Hoechst; Life Technologies, #H3570) and 10 µL of DNase I. The cell suspension was incubated for 15 min, then passed through two 40µm 1X DMEM pre-wetted disposable filters and stored on ice and protected from light until ready for FACS processing (not more than 30min).

Mechanical dissociation of testicular tissue:

Two to three testis fragments of ~2-3 mm^3 were placed in a petri dish containing 100 µL of 1x DMEM (Life Technologies #31053), cut with a scalpel and transferred to a pre-wetted disposable disaggregator Medicon with 50-µm separator mesh (BD™ Biosciences; Cat # 340591). Tissue was processed for 5 minutes and resulting cell suspension was recovered from the Medicon unit with a 3 mL disposable syringe, passed through two 40µm 1X DMEM pre-wetted disposable filters, and transferred back to the Medicon unit for 5 more minutes of processing. The resulting single cell suspension was transferred to a 1.5 mL tube and stained with 10 µL of Hoechst and 2 µL of PI for 30 minutes at room temperature in the dark. Samples were then filtered again (40 µm filter) and kept on ice in the dark until FACS processing.

Fluorescence Activated Cell Sorting (FACS)

Cells were sorted and analyzed by a Beckman Coulter MoFlo Legacy cell sorter, using Summit Cell Sorting software, similarly to the descriptions of [8]. Hoechst was excited using a ultra-violet laser and triggered for scatter by a 488nm blue laser. To detect Hoechst's wide emission spectrum, the U.V. laser was paired with a 463/25nm band pass filter for detecting Hoechst Blue and a 680nm LP band pass filter for Hoechst Red. A 555DLP dichroic was also used to distinguish blue from red emission wavelengths. Samples were analyzed using a 70-micron nozzle and the sorting flow rate was set to 1,000-2,000 events/second. A minimum of 500,000 events were detected before proceeding to gating. Two different gating strategies were used. For cell suspensions prepared by enzymatic tissue dissociation without PI staining (rat and dog: Fig. S1), a sequential cell gating strategy was applied: debris was excluded based on FSC vs SSC plot, then singlets gated by adjusting threshold for forward scatter pulse width and finally red/blue Hoechst fluorescence was used to detect different spermatogenic germ cell populations. For samples obtained by mechanical dissociation (Fig.5) or enzymatic dissociation with PI staining (Guinea pig and Mini pig) two intermediate gating steps were introduced as previously described by Gaysinskaya and co-workers [14] for the mouse and depicted in figure 4: debris was excluded based on FSC vs SSC plot, then singlets gated by adjusting threshold for forward scatter pulse

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width. Live single cells, negative for PI, were gated based on PI red fluorescence and FSC and plotted in histograms of cell counts in relation to measurements of Ho blue fluorescence. 3 peaks with increasing Ho fluorescence could be detected representative of haploid (1C), diploid (2C) and tetraploid (4C) cells. This DNA content gate was used to refine populations of spermatocytes and spermatids, which were then discriminated by finally plotting the function of Ho-blue and red fluorescence intensity. Spermatogonial stem cells were identified from PI negative cells as a direct measurement of Ho fluorescence since they represent a side population resulting of Ho efflux and therefore Ho-blue is not representative of DNA content in these cells. Single stained cell suspensions for Ho and PI were used to set optimal photomultiplier tube (PMT) voltages.

Each testis was processed for 45 minutes to 1.5 hours to collect an average of 6.0 X 10^6 cells for each subpopulation. Cells were collected into 1 mL of 1X DMEM + 10% FBS in 5 mL polypropylene round-bottom tubes or 1.5mL tubes that were pre-coated with FBS. To concentrate the samples and remove dead cells and cellular debris, sorted cells were pelleted by centrifugation (600xg at 4°C for 10min) and washed in 1mL of ice-cold 1X PBS.

Microscopic evaluation of purified cells

To identify the cell types gated in each FACS sorted population we evaluated chromatin structure and cell morphology microscopically, based on Hoechst fluorescence. During the wash step after FACS, 100 µL of sorted cells were collected, fixed in 4% PFA and stored at 4°C in the dark. Slides were mounted with 20 µL of fixed cells from each population and visualized in upright confocal or light microscopes. To quantify cell purity, images were obtained from a minimum of 5-15 random fields and/or at least 100 cells (when available) were counted to estimate contamination with other cell types. To avoid human errors, cells were counted independently by two researchers and an estimated average cell count was reported.

RESULTS

Efficiency of tissue dissociation protocol is crucial for cell sorting with Hoechst staining

We isolated two testes from each animal in the study: 10 mice, 4 rats, 3 dogs, 2 guinea pigs and 1 miniature pig (herein minipig). In order to confirm a normal adult testis phenotype of the collected specimens, we performed Hematoxylin and Eosin (HE) staining of tissue sections from one testis, and then submitted the other testis for FACS. A microscopy analysis of the HE slides shows the expected tissue architecture and organization of normal adult male testes and highlights some general differences across species (Fig. 1). Mammals have a tubular testicular arrangement, with spermatogenesis progressing from the periphery towards the lumen, and show interspecific variability of germ cell morphology.

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The success of cell sorting protocols depends on the quality of inputted single-cell suspensions, and is therefore directly affected by the efficiency of tissue dissociation. Here, we evaluated the use of different protocols for testis dissociation, enzymatic and mechanical, in each of the species studied. We applied an enzymatic dissociation protocol optimized for mouse testis to all species, referred to as the "multispecies" protocol, and defined species-specific protocols by adjusting incubation temperatures and times, trypsin concentrations and/or introducing the use of hyaluronidase to improve digestion of connective tissues (Methods). To control for technical and biological variables, the experiments were performed simultaneously in tissue sections of the same testis for both biological replicates, except for minipig. Overall, species-specific dissociation protocols performed better as evaluated by the separation of distinct clusters obtained on the FACS profiles (Figure S1). The main goal of a tissue dissociation protocol is to reduce the amount of manipulation and time while retaining the viability of the dissociated cells. We therefore tested the applicability of mechanical dissociation testicular dissociations in different species using a method original described for rodents [29]. To evaluate the performance of our approach, we estimated the mean percentage of cells that passed the gates during FACS and compared these values with the ones obtained for mouse. The proportion of cells from total counts (Fig. 2) is indicative of the sample quality and a reflection of the efficiency of tissue dissociation protocols. The average percentage of cells passing the debris filter was comparable between species, with mechanical dissociation performing similarly or better when compared to enzymatic dissociation with species-specific protocol. Notably, the percentages estimated for all species are directly influenced by the high stringency of the debris filter applied during FACS. These results suggest that cell sorting with Hoechst staining seems very sensitive to sample quality, validating our approach of designing species-specific enzymatic dissociation protocols that are effective in generating good single-cell suspensions. More importantly, mechanical dissociation with Medimachine (BD™ Biosciences) provides a standard method for testicular dissociation that reduces the processing time and is applicable to different mammalian species.

Male germ cell-types of different mammalian species can be discriminated by Ho-FACS

We first isolated four different populations (Spg; Spc I; Spc II and Spd) from dog and rat testicular cell suspensions obtained from species-specific enzymatic dissociation (Figure S1). The gates for sorting were defined based on the cluster of cells observed and taking into account the expected location of the subpopulations in terms of Ho red and blue fluorescence: i) spermatogonia (Spg; side population)- low Hoechst blue and red fluorescence; ii) primary spermatocytes (Spc I; 4N eu- to heterochromatin) – high Hoechst blue and a wide range of low to high Ho red fluorescence; iii) secondary spermatocytes (Spc II; 2N eu- to heterochromatin)- intermediate Hoechst blue and a wide range of low to high Ho red fluorescence; iv) spermatids (Spd; 1N compacted chromatin with structural variations resulting from histone to protamine transition) – low Hoechst blue and a narrow range of Ho red. Moreover, it appears

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that the chromatin of the dog germ cells is generally more compact throughout spermatogenesis as the clusters of cells show a trend of lower red Hoechst fluorescence. To identify the germ cell-types and quantify the purity of the sorted subpopulations, we performed a microscopy evaluation of cell morphology and chromatin structure based on Hoechst fluorescence (Methods; Figure 3). Immunofluorescence in tissue sections with Hoechst was used as reference for the pattern of Hoechst staining in different germ cells (Fig. 3A). Spg are small, round shaped cells with distinct pericentric heterochromatin. Spermatocytes are larger granulated cells with Spc II populations defined by the detection of binucleated cells or cells in diakinesis. Spd are small haploid cells that can be round or elongated in shape. Despite the similar size, round spermatids can be clearly distinguished from Spg by the presence of localized chromocenters. Purity was estimated based on this analysis, indicating 74%-85% purity of specific cell-types passing through each gate (Table S1)except for the dog Spg population (46%), due to the close proximity of the eSpd and Spg populations in fluorescence space (Figure S1). For the Spc I gates, most contamination was with preLeptotene spermatocytes. In the rat, this could have resulted from the absence of clearly defined pre-meiotic and meiotic spermatocytes subpopulations during FACS.

Then, to ensure the viability of the cells being and to refine to purity of the populations obtained, we applied a gating strategy similar to that described for the mouse in Gaysinskaya, et al. [15] (Figure 4). This strategy includes a viability gate based on propidium iodide (PI) staining as well as a DNA content gate where a histogram obtained based on Ho-blue fluorescence shows peaks representative of haploid (1C), diploid (2C) and tetraploid (4C) cells. Figure 5 shows the cytograms, as a function of Ho-blue and red fluorescence, generated during Ho-FACS of single-cell suspensions obtained from testicular tissue of all the different species by mechanical dissociation. Although we see some expected interspecific variation in the pattern of the FACS profiles, we can clearly distinguish at least four subpopulations of male germ cells for all species. The different cell populations were sorted by applying the gating strategy described in figure 4 and purities were estimated (Table 1) by a similar morphological analysis as described above (Fig.3). Looking at the relative frequency of cells passing through each gate (Table 1) a similar higher frequency of spermatids was detected for all species, however, the abundance of other germ cell types varies amongst species. These observations were expected and presumably reflect interspecific differences in testis composition and the technical challenge of making standardized settings for subpopulation gating. Interestingly, while this gating strategy generally improved the purity of germ cell populations isolated from the rat and dog (Table 1 and Table S1), the guinea pig and mini pig Spg populations showed contamination with other cell types and an overall lower level of purity. Altogether, our results suggest that Ho-FACS, combined with PI staining, of testis single-cell suspensions can be used to isolate germ cells from rat, guinea pig, dog and mini pig, further strengthening our hypothesis that this method can potentially be applied as a generalized procedure for isolation of germ cells in different mammalian species.

Round and elongating spermatids can be separated by Ho-FACS based on cell shape and size

During Ho-FACS of the different mammalian species, it was notable that Ho-red and blue fluorescence alone could discriminate round and elongated spermatid populations in the dog sample, it was insufficient to further refine this population in the remaining species (Figure 5, Table 1). Given that round (rSpd) and elongating (eSpd) spermatids are molecularly very distinct in terms of transcription activity as well as the differentiation occurring in the latter during spermiogenesis, we sought to evaluate a different strategy to isolate different mouse spermatid subpopulations by FACS. It has been previously suggested that rSpd and eSpd could be gated based on high forward scatter (FSC high) and low forward scatter (FSC low), respectively [13]. Interestingly, we observed that gating based on the FSC parameter alone introduced some contamination in the sorted populations. Microscopy quantification of purity of sorted populations based on cell morphology and Ho fluorescence revealed and enrichment of ~62% for eSpd and 84% for rSpd (Fig. S2). Gating for events with low FSC and high VS low side scatter (SSC), we increased purity levels to 92% of eSpd and 86% rSpd (Fig. S2). Finally, we observed that the lowest levels of contamination could be obtained by the combination of FSC and SSC gating followed by Hoechst red/blue fluorescence. It seems that eSpd can be isolated gating for low FSC&SSC with 83-92% enrichment range, whilst rSpd appear to have higher FSC&SSC values and can be separated with 86-95% accuracy (Fig. 6; Table 1). Importantly, this gating strategy is based on cell size, shape and complexity and thus potentially applicable to Ho-FACS of any species undergoing spermiogenesis during gamete development.

DISCUSSION

One of the major challenges in male reproductive biology has been to design a method to differentiate and isolate subtypes of developing germ cells with a high percentage of recovery and low contamination with other cell-types. Since the first reports over a decade ago, flow cytometry of Hoechst stained murine male germ cells has been slowly revisited and optimized to isolate pre-meiotic (spermatogonia), meiotic (preleptotene, leptotene, zygotene, pachytene, diplotene and secondary spermatocytes) and post-meiotic (round and elongating spermatids) stages [8, 13-16, 32]. This technique is based on measurements of chromatin amount and structure detectable using the fluorescent Hoechst-33342 DNA dye. Flow cytometry of testicular cell suspensions from non-mouse mammalian species using different dyes, staining protocols and flow cytometry parameters for analysis have revealed similar profiles in terms of DNA ploidy/stainability (Reviewed in [33]). We reasoned that, for species sharing similar chromatin dynamics (2N- 4N- 2N- 1N) and structure throughout spermatogenesis, major populations of germ cells in different stages could be isolated by Ho-FACS. Here we propose a Ho-FACS standardized protocol for germ cell purification that is fast, straightforward and applicable to mammalian species beyond mouse (Figure 4).

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Using three rodent species (mouse, rat and guinea pig) and two non-rodent species (dog and mini pig), we show that the general resolution of distinct cell populations is maintained across mammals and allows the isolation of at least four developmental stages: spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids. The purity of these subpopulations was slightly reduced when compared to previous works for the mouse [13, 15], but shows good enrichment of expected cell-types (Table 1). It is important to highlight that higher purities of early and mid-late Spc I (93-97%) have been described for FACS sorting of germ cells with >2h of PI staining [34], suggesting that longer incubation periods increase the power of discriminating germ cells based on DNA-binding dyes in flow cytometry. Moreover, this could also explain a lower percentage of Spc I cells detected in the guinea pig and rat samples. Nonetheless, a reduced processing time is crucial to preserve the physiology of ex-vivo cells and, in that regard, a combination of Ho and PI staining for 30 minutes seems to be sufficient for a good enrichment of different male germ cell types (Fig. 4). The presence of eSpd was the major source of contamination within the Spg gates and resulted from their close special proximity in Hoechst plots, reaching the highest values in the guinea pig and mini pig FACS. One possible way to circumvent this issue would be to stain germ cells with a membrane permeable marker for the acrosome, allowing to gate cells for the presence of this spermatid-specific structure. In fact, Spg is the most challenging population to isolate based on Ho staining. Spermatogonial stem cells show a unique Ho fluorescence pattern and represent a side population as a result of BCRP1-dependent dye efflux [13]. Other methods such as MACS using Spg-specific makers would be more suitable to isolate spermatogonial stem cells for studies focusing in this particular cell type. Nonetheless, a sample preparation method achieving an enrichment of even 50% spermatogonia is likely to have an important impact as useful tool in the world of germ cell genomics, especially in single cell studies.

Future work would also include the optimization of this protocol to discriminate other cell types in different mammals. Here, we describe an optimized gating strategy based on cell size, shape and complexity to differentiate rSpd and eSpd in the mouse (Fig. 6 and Fig. S2), suggesting that the isolation of populations enriched for these germ cells can be achieved for other mammalian species. Also, discrimination between different meiotic stages, already resolved for mouse [13-15], would broaden the scope of the application of this technique in the field of male reproductive biology.

Overall, we provide the first evidence supporting the applicability of Ho-FACS as a transversal method to isolate male germ cells in different developmental stages across mammalian species. As a proof of principle, our work has major implications for several types of studies in developmental biology. First, it provides the tools to investigate the dynamics of germ cell development in different species individually, which would benefit research of understudied mammalian species such as domesticated animals [35]. Furthermore, using the same experimental procedure in different species reduces noise and eliminates sources of variables which often challenge comparative studies. In the "omics" era, with the growing interest in applying genome technology to address questions about epigenetics,

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regulation and protein diversity throughout spermatogenesis [11, 12, 24-28, 36-39], this technology could be used to comprehensively tackle different aspects of germ cell development with an evolutionary perspective.

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AUTHORS CONTRIBUTIONS

ACL and MJ obtained and processed tissue. ACL, MJ, AU and JR performed histology and immunofluorescence assays. ACL and MJ analyzed the data. ACL and MJ wrote the paper with input from all authors. AML and DFC supervised the project.

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Table 1. Statistics of Ho-FACS of male germ cell suspensions obtained by mechanical dissociation

			% cells in DNA content gate			Purity of sorted germ cell populations (%)					
Species	**% Singlets**	**% Live cells**	**1C**	**2C**	**4C**	**Spg**	**Spc I**	**Spc II**	**Spd**	**rSpd**	**eSpd**
Mouse	98.4	92.5	34.7	3.9	3.72	74	82	87.5	95.2	95*	92*
Rat	95.7	93.8	37.7	5.3	5.4	83	81	82	87	.	.
Guinea Pig	96.2	92.1	39.3	7.6	5.8	48	68.7	85	87	.	.
Dog	97.9	86.5	16.4	3	0.5	78	.	87	.	91	81
Mini Pig	95.9	93.2	26.9	6.4	3.5	49	52	82	92	.	.

* Obtained by enzymatic dissociation and gated based on FSC&SSC parameters (see text)

Figures

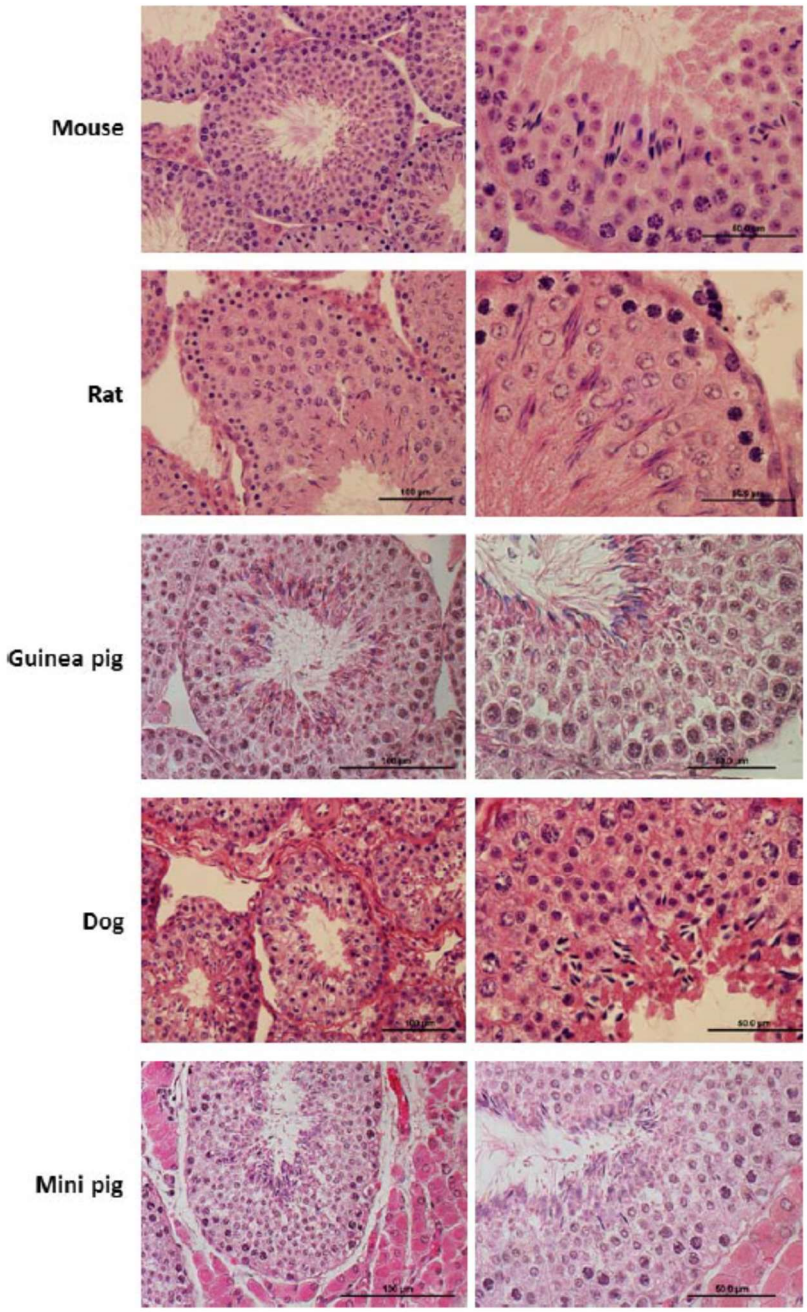

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Figure 1. Hematoxylin and Eosin staining of testicular cross-sections of collected specimens.

For each animal studied in the paper (n=10 mice, n=3 dogs, n=4 rats, n=2 guinea pigs, n= 1 mini pig) we processed testis fragments for histology and for FACS. Here we present representative H&E staining from each species. In each subject, histological examination of testicular cross-sections shows the presence of all germ cell-types in different developmental stages, confirming that the specimens were sexually mature and presented a normal testicular phenotype. Left panel represents higher magnification of the section displayed in the right panel (40X).

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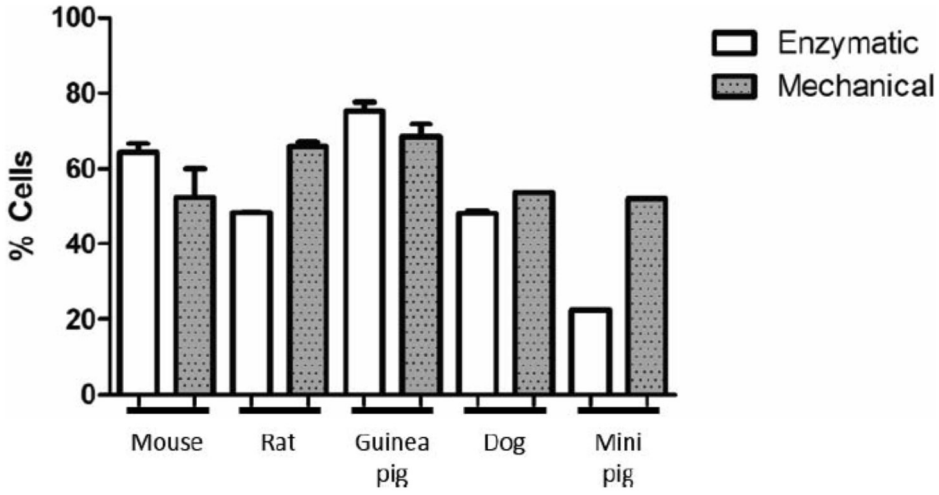

Figure 2. Evaluation of testis dissociation protocols by flow cytometry.

In order to evaluate the efficiency of mechanical and enzymatic dissociation protocols in different species we estimated the percentage of live cells from total number of cells. The quality of mechanical single-cell suspensions was comparable to the species-specific enzymatic dissociation protocol indicating that this method can be used to quickly obtain single cell suspensions of different mammalian species. Datasets were obtained from variable numbers of experimental replicates (mouse: 10; dog: 3; rat: 4; guinea pig: 2; and mini pig: 1) using FlowJo® software v10 (Tree Star Inc.). Histograms were generated with GraphPad Prism (version 5.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com), plotting the calculated mean values with standard deviation.

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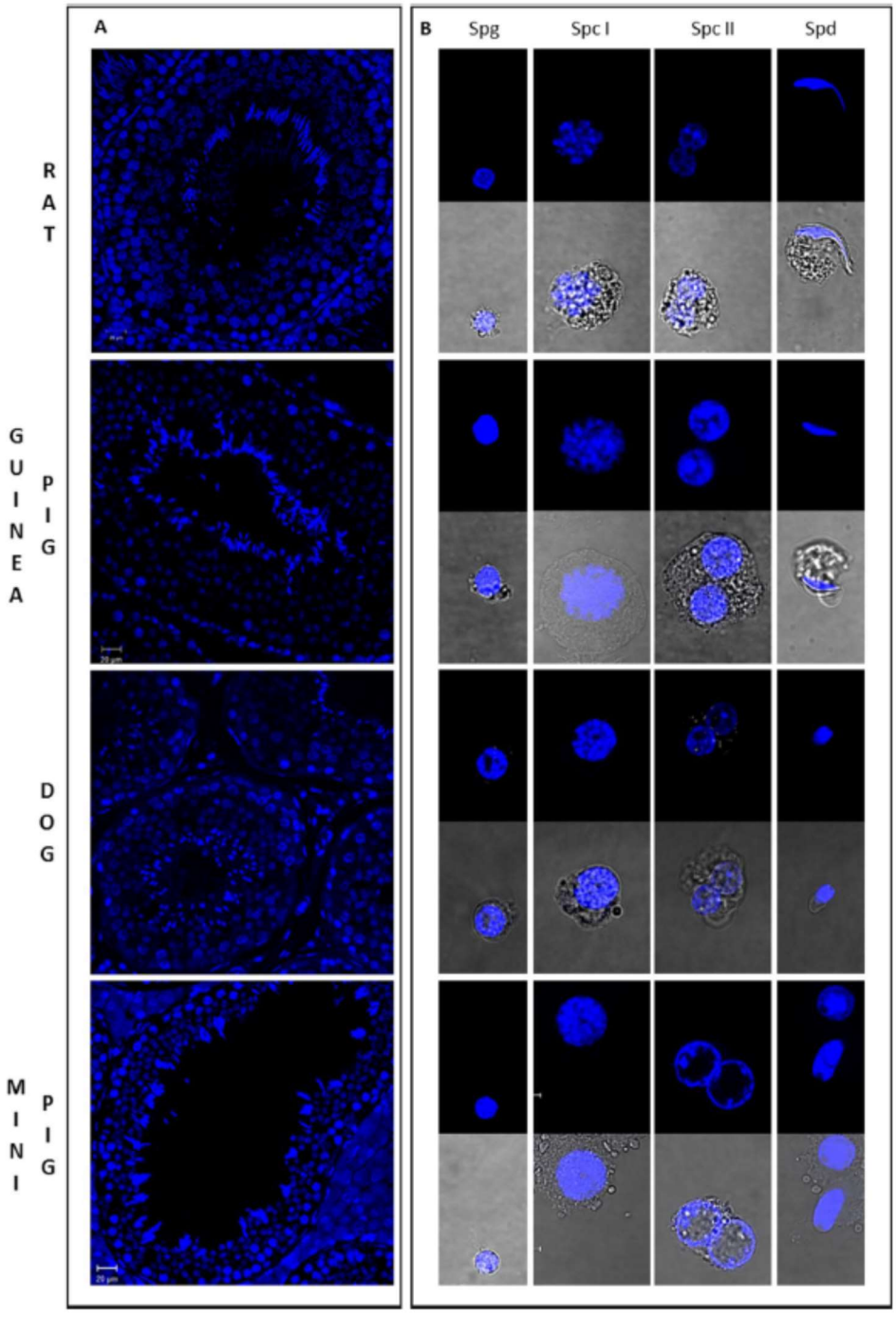

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Figure 3. Microscopic evaluation of germ cell populations isolated from mammalian testes by Ho-FACS.

Immunostained cross-sections of dog and rat testes (A) were used as reference for the classification of isolated germ cells in respect to chromatin structure marked with Hoechst (blue). Morphological evaluation of chromatin structure was performed based on cell shape and size, allowing the identification of different germ cell-types (B). Spg are small round cells with compact heterochromatin whereas Spc I and Spc II show larger and more complex cells with more diffuse chromatin (primary spermatocytes) and/or binucleated cells (secondary spermatocytes). Spd gates comprise cells in different states of spermiogenesis, ranging from round to completely elongated spermatids.

Spg: spermatogonia; Spc I: primary spermatocytes; Spc II: secondary spermatocytes; and Spd: spermatids panels indicate the designated FACS gate.

Slides were visualized in a confocal microscope. For each isolated population, Hoechst fluorescence of sorted cells was visualized after FACS and images were collected under a 63X magnification lens, with (lower panel) or without (upper panel) white light transmission.

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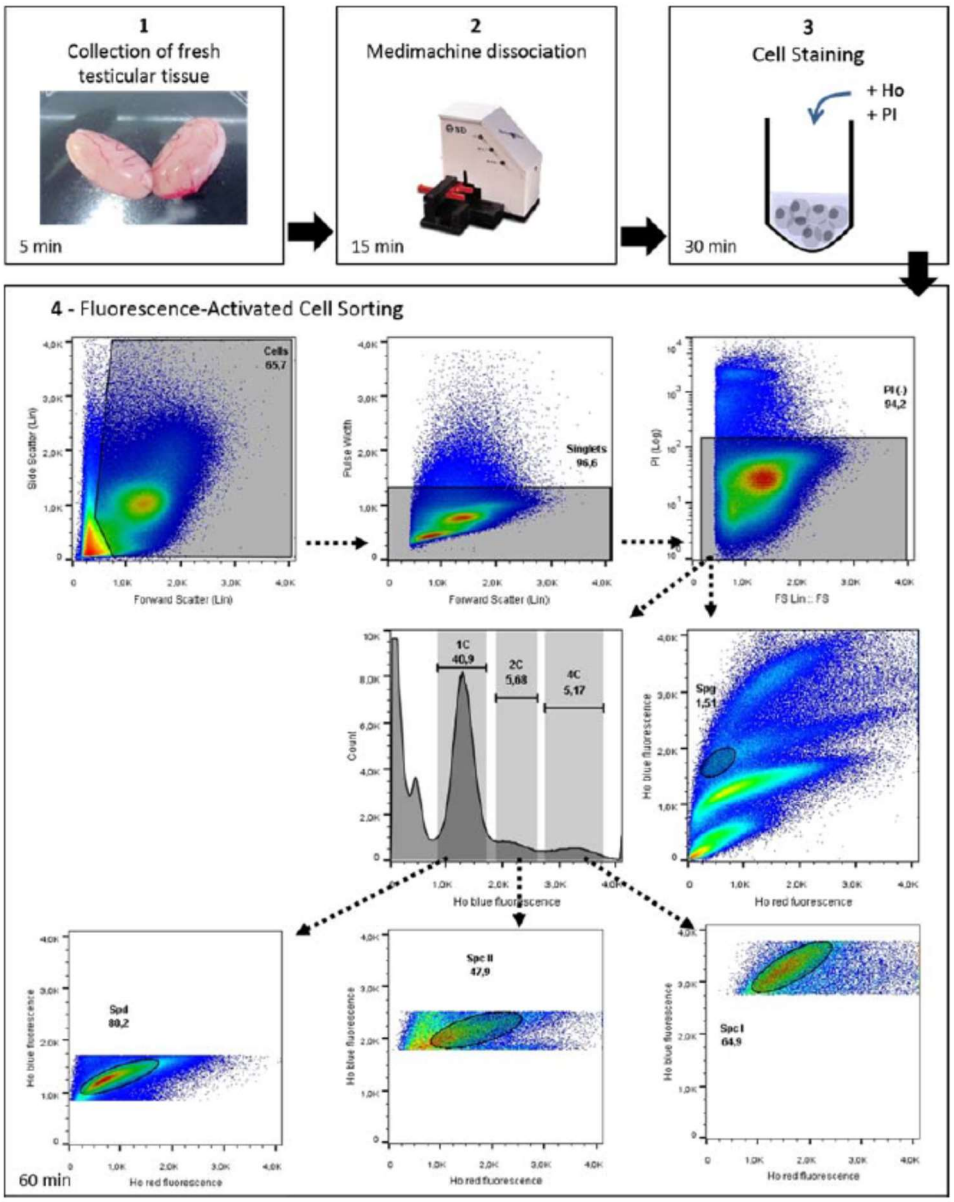

Figure 4. Workflow of Ho-FACS isolation of mammalian male germ cells

This image summarizes the steps for germ cell isolation of mammalian germ cells, represented here by the application of this protocol to rat testis.

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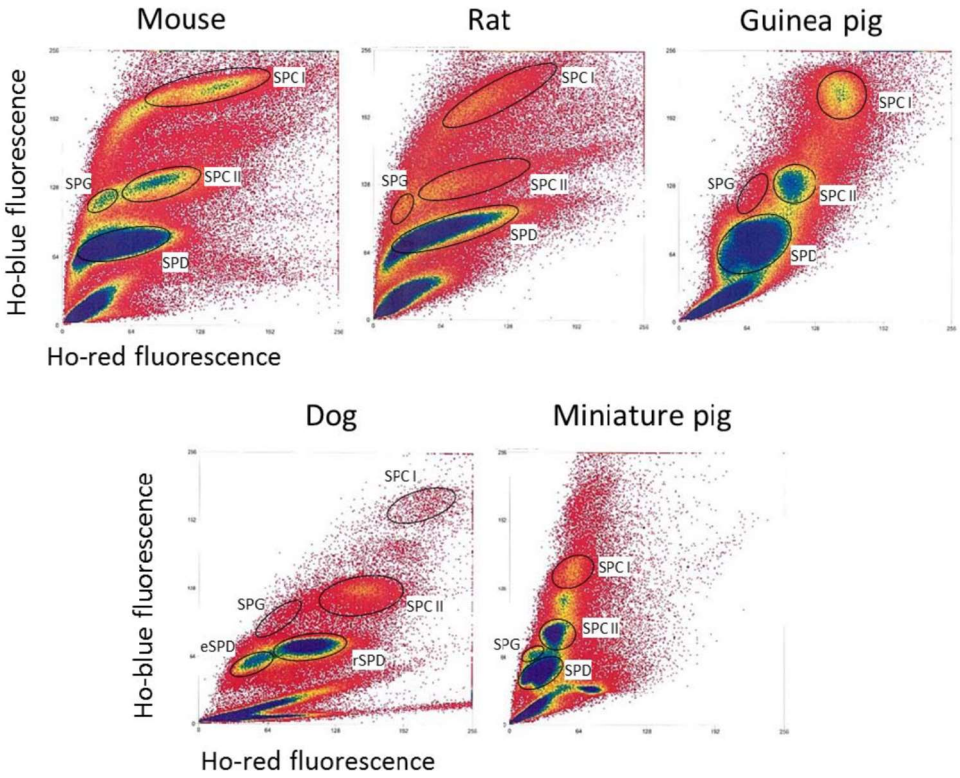

Figure 5. Interspecific comparison of Ho-FACS plots of testicular single-cell suspensions

The binding of Hoechst to DNA results in different FACS patterns depending on chromatin compaction and quantity. Plots represent the ratio of blue and red Ho fluorescence obtained by flow cytometry after testis mechanical dissociation and staining of germ cells of the mouse, rat, guinea pig, dog and mini-pig. Gating (round circles) was defined based on observed cell clusters and expected location of populations in relation to Ho fluorescence. A minimum of 4 populations were identified and sorted for all species. SPG: spermatogonia; SPC I: primary spermatocytes; SPC II: secundary spermatocytes; SPD: spermatids; eSPD: elongating spermatids; rSPD: round spermatids.

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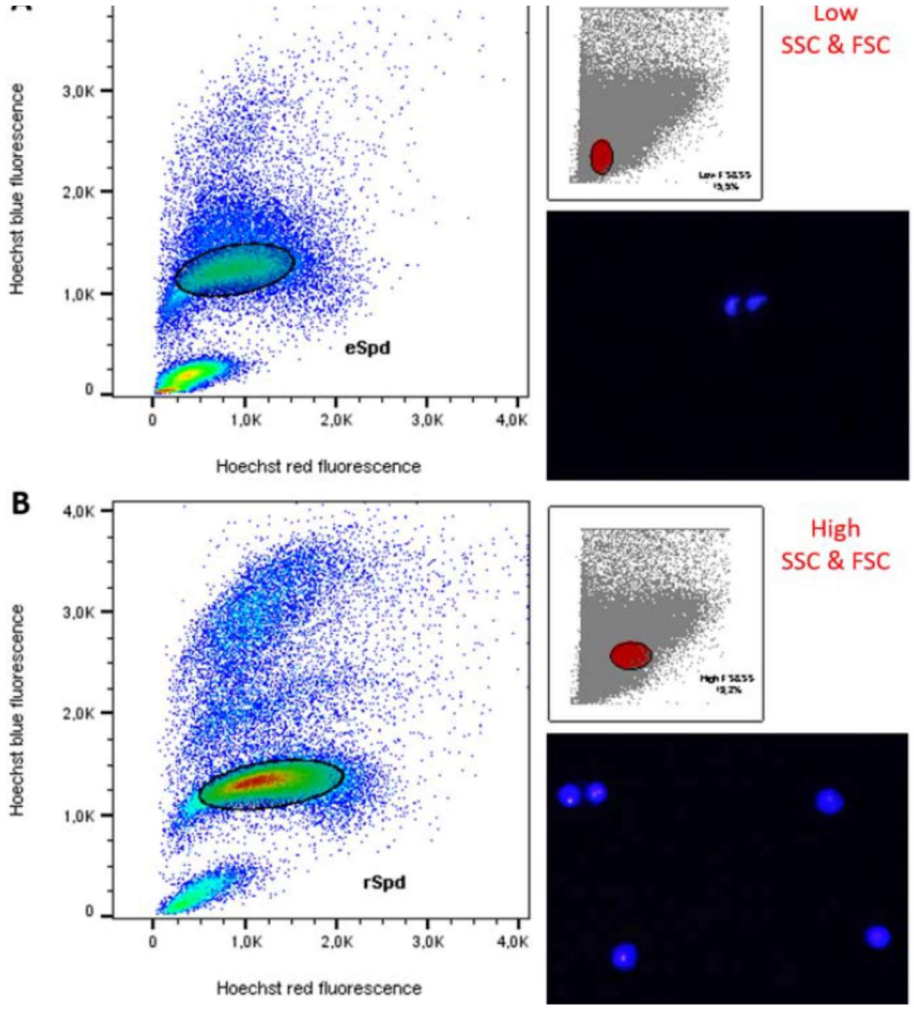

Figure 6. Gating strategy to discriminate round and elongating spermatids.

Cell shape and complexity influence the ratio of forward (FSC) and side scatter (SSC) parameters measured by flow cytometry. The smaller windows in both images show the parent gate (red full circle) based on FSC and SSC. Gated cells then clustered as functions of Hoechst blue/red fluorescence with the pattern expected for haploid cells with condensed chromatin, defining the gates for sorting. Morphology of sorted cells was evaluated microscopically based on Hoechst fluorescence and confirms the enrichment of the expected cell-types in each population. Therefore,

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elongating spermatids are smaller and less complex showing lower ratios of FSC and SSC (A), whereas round spermatids present higher FSC and SSC (B).

Cell images were obtained by light microscopy with a UV lamp (16X magnification lens). Plots were generated using FlowJo® software v10 (Tree Star Inc.).

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Supplementary data

Species-specific alterations of enzymatic dissociation protocol

In order to evaluate the effect of testis dissociation protocols in FACS, tissue sections of all specimens were also dissociated using species-specific protocols based on the procedure described as multispecies protocol with the following modifications:

Rat and Guinea pig: Trypsin stock concentration was adjusted to 1mg/ml and Hyaluronidase (1mg/ml; Sigma-Aldrich #H6254) stock solution was added to the 1X DMEM/Collagenase I. The last incubation time in step 4 was adjusted to 20 min.

Dog: Collagenase stock concentration was adjusted to 0.2% by dissolving 20mg Collagenase type I in 10ml 1X DMEM. The first incubation time in seminiferous tubule digestion was adjusted to 30min.

Mini pig: 0.1% Collagenase type 4 and 0.1% Hyaluronidase were added to 1X DMEM and trypsin concentration adjusted to 0.25%. All incubations were carried out at 37°C.

Supplementary figure 1. Ho-FACS plots of cell suspensions obtained using enzymatic dissociation protocols.

Sample preparation has direct implications in the success and results obtained by flow cytometry. In this figure, plots reflect measurements of Hoechst fluorescence of stained germ cells isolated from testes of rat, guinea pig, dog and mini pig, using an enzymatic dissociation multispecies protocol, optimized for mouse, or species-specific enzymatic dissociation protocols (See Methods and Supplementary data). In general, adjusting the enzymatic dissociation according to the species being process resulted in a higher abundancy of male germ cells for each population. Percentages indicate the proportion of cells within the gates in relation to the total number of live cells. Smaller

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windows show the parent gates leading to the plots generated as a function of Ho-blue and Ho-red fluorescence intensities.

Spg: spermatogonia; Spc I: primary spermatocytes; Spc II: secondary spermatocytes; Spd: spermatids. Plots were obtained using FlowJo® software v10 (Tree Star Inc.).

Supplementary figure 2. Optimization of a gating strategy to isolate round and elongating spermatids.

In order to discriminate between round (rSpd) and elongating spermatids (eSpd) we defined the parent gates (circles and squares with cells labeled red) to reflect differences in cell shape (A) or complexity (B). Gates for sorting were then defined by the expected pattern of Hoechst blue/red fluorescence for spermatids. Cell populations gated for high or low FSC were enriched 62% for rSpd and 84% for eSpd respectively (A). Within a range of low FSC, gating for higher or lower SSC increased the enrichment to 86% and 92% of rSpd and eSpd, respectively, in the sorted population (B). Morphology of sorted cells was evaluated based on Hoechst fluorescence and images were acquired by light microscopy with a UV lamp (16X magnification lens.). Plots were generated using FlowJo® software v10 (Tree Star Inc.)

Table S1. Purity of cell populations isolated by species-specific enzymatic dissociation of rat and dog testes.

Species	Spg	Spc I	Spc II	Spd
Rat	81%	74%	79%	85%
Dog	46%	81%	81%	82%

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