Video Article

Simple and Efficient Technique for the Preparation of Testicular Cell Suspensions

Rosana Rodríguez-Casuriaga[1], Gustavo A. Folle[2], Federico Santiñaque[2], Beatriz López-Carro[2], Adriana Geisinger[1,3]

[1]Departamento de Biología Molecular, Instituto de Investigaciones Biológicas Clemente Estable (IIBCE)

[2]Servicio de Citometría de Flujo y Clasificación Celular (SECIF), Instituto de Investigaciones Biológicas Clemente Estable

[3]Sección Bioquímica, Facultad de Ciencias, Universidad de la República

Correspondence to: Rosana Rodríguez-Casuriaga at r.rodriguezcasuriaga@gmail.com, Adriana Geisinger at adriana.geisinger@gmail.com

URL: http://www.jove.com/video/50102
DOI: doi:10.3791/50102

Keywords: Cellular Biology, Issue 78, Medicine, Biomedical Engineering, Anatomy, Physiology, Cell Separation, Flow Cytometry, Cytological Techniques, Meiosis, Spermatogenesis, Cell Biology, Flow cytometry, FACS, testis, meiosis, cell suspension, rodent, cell culture, animal model

Date Published: 8/4/2013

Citation: Rodríguez-Casuriaga, R., Folle, G.A., Santiñaque, F., López-Carro, B., Geisinger, A. Simple and Efficient Technique for the Preparation of Testicular Cell Suspensions. *J. Vis. Exp.* (78), e50102, doi:10.3791/50102 (2013).

Abstract

Mammalian testes are very complex organs that contain over 30 different cell types, including somatic testicular cells and different stages of germline cells. This heterogeneity is an important drawback concerning the study of the bases of mammalian spermatogenesis, as pure or enriched cell populations in certain stages of sperm development are needed for most molecular analyses[1].

Various strategies such as Staput[2,3], centrifugal elutriation[1], and flow cytometry (FC)[4,5] have been employed to obtain enriched or purified testicular cell populations in order to enable differential gene expression studies.

It is required that cells are in suspension for most enrichment/ purification approaches. Ideally, the cell suspension will be representative of the original tissue, have a high proportion of viable cells and few multinucleates - which tend to form because of the syncytial nature of the seminiferous epithelium[6,7] - and lack cell clumps[1] . Previous reports had evidenced that testicular cell suspensions prepared by an exclusively mechanical method clumped more easily than trypsinized ones[1] . On the other hand, enzymatic treatments with RNAses and/or disaggregating enzymes like trypsin and collagenase lead to specific macromolecules degradation, which is undesirable for certain downstream applications. The ideal process should be as short as possible and involve minimal manipulation, so as to achieve a good preservation of macromolecules of interest such as mRNAs. Current protocols for the preparation of cell suspensions from solid tissues are usually time-consuming, highly operator-dependent, and may selectively damage certain cell types[1,8] .

The protocol presented here combines the advantages of a highly reproducible and extremely brief mechanical disaggregation with the absence of enzymatic treatment, leading to good quality cell suspensions that can be used for flow cytometric analysis and sorting[4], and ulterior gene expression studies[9] .

Video Link

The video component of this article can be found at http://www.jove.com/video/50102/

Protocol

1. Preparation of Cell Suspensions

1. Sacrifice the specimen to be used following the recommendations of the specialized committees such as IACUC or equivalent (in Uruguay, National Commission for Animal Experimentation [CNEA]). In our case, an overdose of pentobarbital was administrated.
2. Dissect the testes following standard approved procedures and place them in a 96 mm glass Petri dish on ice, containing 10 ml of ice-cold DMEM supplemented with 10% fetal calf serum.
3. Remove the tunica albuginea and cut the decapsulated testes into square pieces of 2 - 3 mm on each side.
4. Those pieces are then processed in a Medimachine, an automated mechanical grinder where the tissue is disaggregated inside a disposable unit containing a perforated stainless-steel screen and a metal rotor. To do so, place 1 ml of cold supplemented DMEM and 4 - 5 of these pieces in a 50 µm disposable unit, switch on the disaggregator, and process for 50 sec following the simple instructions from the manufacturer.
5. Recover the resulting cell suspension from the disaggregation unit using a 3 - 5 ml syringe without needle.
6. Filter through a 50 µm nylon mesh, previously soaked with 0.5 ml supplemented DMEM.
7. Filter the suspension again using a soaked 25 µm nylon mesh, and place on ice.

8. Count in a Neubauer chamber and adjust cellular concentration to 1 - 2 x 10^7 cells/ml with supplemented DMEM. At least 4 x 10^7 cells/gram of testis material are usually obtained.
9. Finally, add NDA (2-naphthol-6,8-disulfonic acid, dipotassium salt) to a final concentration of 0.2% in order to prevent cell clumping.
10. Optional: check cell viability of the testicular cell suspensions with a commercially available viability kit for animal cells, following manufacturer's instructions.

2. Flow Cytometric Analysis

We have used a Becton-Dickinson FACSVantage flow cytometer equipped with a Coherent argon ion laser tuned to emit at 488 nm for the analysis of cells stained with high concentrations of propidium iodide (PI). (The issue of PI entrance into unfixed cells under stress has been addressed elsewhere[9,10]).

1. For PI staining, add fluorochrome at a final concentration of 50 µg/ml to the cell suspension, and incubate for 10 min at 0 °C in the dark.
2. Laser power is set to 100 mW and a 575/26 band pass filter is used to collect PI-emitted fluorescence in FL2.
3. We perform FC measurements with a 70 µm nozzle. For sorting spermatocyte populations, set sorting mode in Normal-R or Normal-C, using 3 sorted drops as envelope. Keep sample and collection tubes at 3 - 4 °C by using a refrigeration unit. Adjust sample differential to analyze cells at a rate of 500 to 1,500 per second.
4. Use CellQuest software (BD) to analyze the following parameters: forward scatter (FSC-H); side scatter (SSC-H); total emitted fluorescence or pulse-area (FL2-A); and duration of fluorescence emission or pulse-width (FL2-W).

Alternatively, we have employed the vital dye Hoechst 33342 to a final concentration of 5 µg/ml and incubated for 10 min at 37 °C in the dark. Cell analysis was performed by means of a MoFlo Cytometer (DakoCytomation) equipped with a UV excitation wavelength laser operating at 25 mW and using a 70 µm nozzle. Data manipulation was performed with Summit v4.3 software (DakoCytomation).

Representative Results

An example of a well disaggregated cell suspension from rat testes prepared with the protocol described here is shown in **Figure 1**.

In comparison to enzymatic treatments[6,8] and to previously described mechanical disaggregation methods[2], the one presented here is much faster, involves less handling, is easily reproducible (not operator-dependant), and renders scarce cell debris (especially compared to other mechanical methods) and very few multinucleates (which have been described to form as a consequence of extensive tissue manipulation[1,2]). Moreover, as opposed to enzymatic treatments, it avoids the use of RNAses, trypsin and collagenase, that could favor macromolecules degradation.

On the other hand, although previous reports had evidenced that testicular cell suspensions prepared by an exclusively mechanical method clumped more easily than trypsinized ones[1], the application of the present method rendered very little clumping in the cellular suspensions, as can be seen in **Figure 1**. In that sense, we have found the inclusion of NDA very efficient in preventing cell clumping, and avoiding nozzle clogging during subsequent flow studies. **Figure 1** also reveals the scarcity of cell debris, which is also obvious in the FC histograms depicted in **Figure 2**.

Besides, cell suspensions prepared with this method show an adequate representation of the different testicular cell types. This was concluded by comparing the cellular composition of testicular cell suspensions prepared by the method presented here with data reported from cell counts in cross sections of seminiferous tubules, and with cell suspensions prepared using other methods as well (**Table 1**).

FC histograms obtained for suspensions prepared by the present protocol and stained either with Hoechst 33342 (**Figure 2A)** or PI (**Figure 2B**) do not substantially differ from previously reported ones[8], also supporting the assumption that the procedure does not selectively damage any specific cell type.

Testicular cell types based on DNA content	Intact testis[a] (%)	Medimachine - prepared suspensions[b] (%)	Trypsinized suspensions[a] (%)
	A) *Mus musculus*		
C	66.2	62.5	79.6
2C	16.4	18.4	7.3
4C	17.9	18.7	8.7
Others	-	-	4.6
	B) *Cavia porcellus*		
C	66.5	65.5	N/D
2C	11.0	11.5	N/D
4C	22.5	23.0	N/D

[a] Cell counts were assessed by microscopical observation.

[b] Cell counts were assessed by flow cytometry.

Table 1. Relative percentages of adult testicular cell populations differing in their DNA content for cell suspensions from mouse (A) and guinea pig (B) prepared by the hereby presented method, compared to intact testis and - for mouse - to trypsin-prepared suspensions (Modified from Geisinger and Rodríguez-Casuriaga, Cytogenet. *Genome Res.* **128**, 46 (2010)). DNA contents are: C (round and elongating spermatids, spermatozoa), 2C (testicular somatic cells, spermatogonia, secondary spermatocytes), and 4C (primary spermatocytes and a few proliferating spermatogonia in G2 stage).

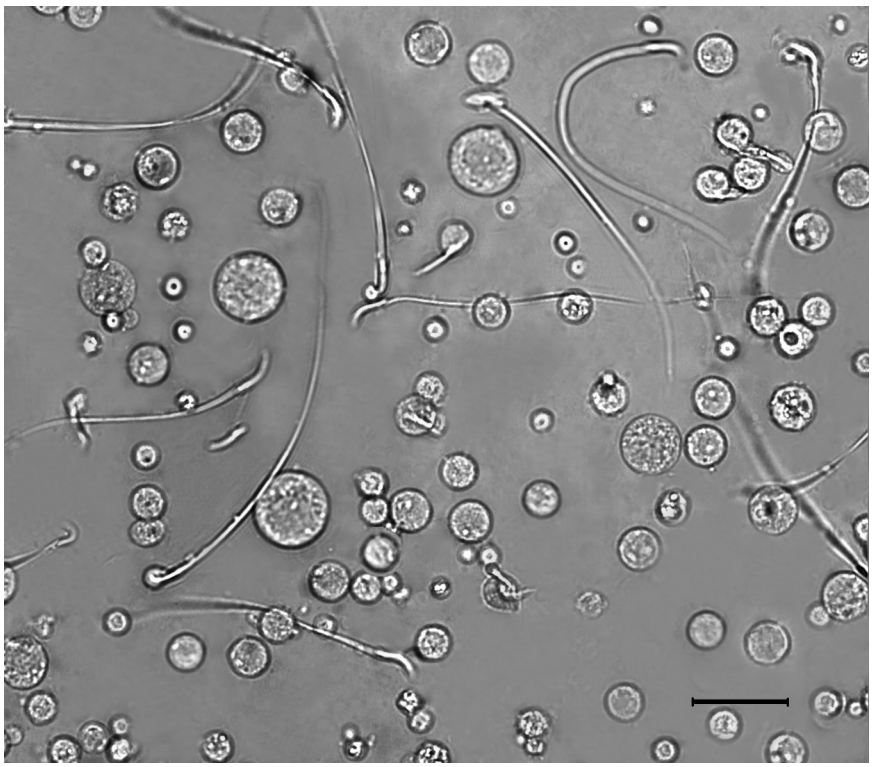

Figure 1. Partial view of a cell suspension from adult rat testis prepared by the present protocol and visualized by phase contrast microscopy. As can be seen, cell cytoplasms are well preserved. The bar corresponds to 25 µm. Reproduced from Rodríguez-Casuriaga, *et al., Biol. Proced. Online.* **11**, 184 (2009).

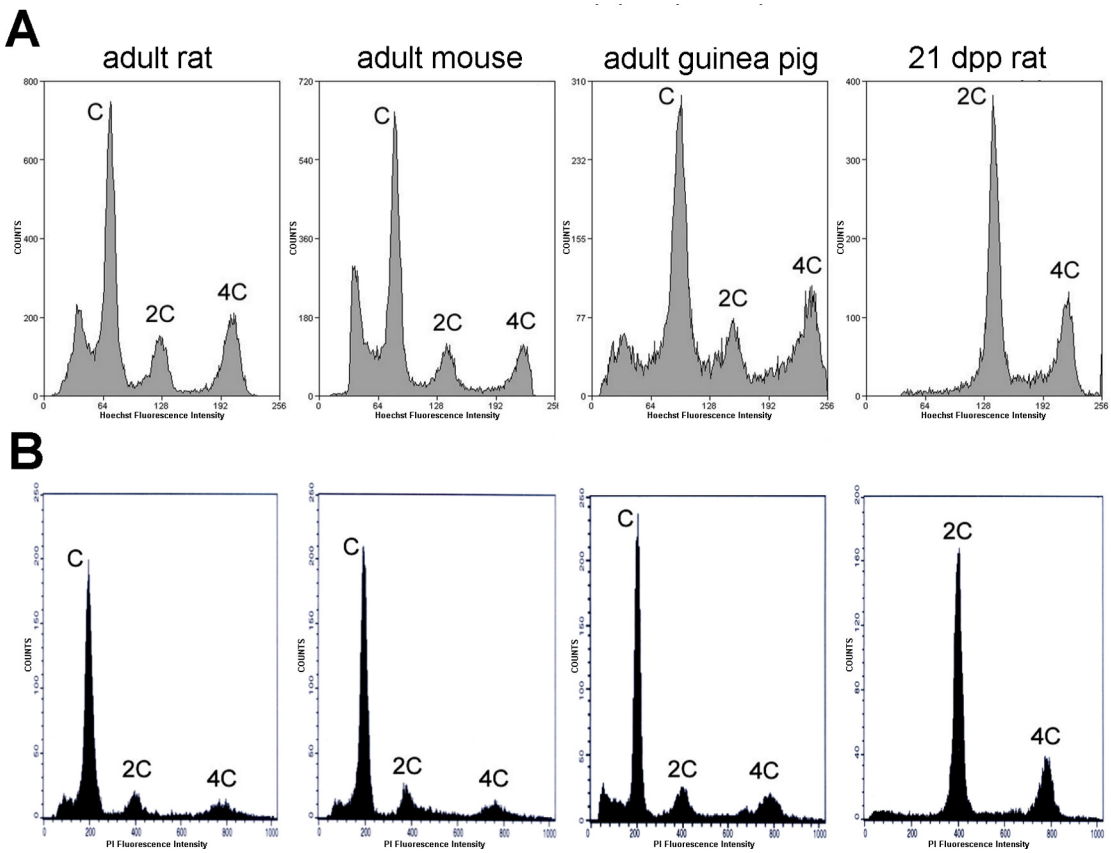

Figure 2. FC DNA content analysis of testicular cell suspensions from adult rats, mice, guinea pigs, and from 21 days post partum (dpp) rat pups, stained with the vital dye Hoechst 33342 (A) or with PI (B). In all the cases C, 2C and 4C cell populations can be easily disclosed in the histograms obtained with both dyes, as well as the apparently sub-haploid peak to the left of the C population. This latter peak has been demonstrated to contain the spermatozoa, which appear as a separate, less stained subpopulation due to their condensed chromatin state[12]. Note the scarcity of debris in all graphics. This is especially evident in the 21 dpp (juvenile specimens) profiles, which lack spermatids and spermatozoa. Modified from Geisinger and Rodríguez-Casuriaga, *Cytogenet. Genome Res.* **128**, 46 (2010). Click here to view larger figure.

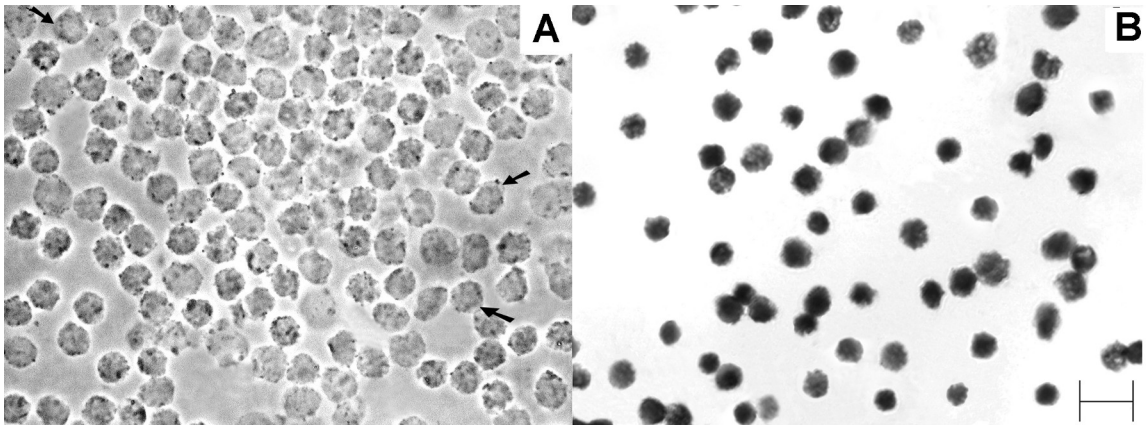

Figure 3. 4C cell population sorted from a testicular cell suspension of adult rat. Sorted cells were deposited onto clean poly-L-lysine-treated slides and observed under phase-contrast microscopy **(A)** or bright field after Giemsa staining **(B)**. Bar = 20 µm. Reproduced from Geisinger and Rodríguez-Casuriaga, *Cytogenet. Genome Res.* **128**, 46 (2010).

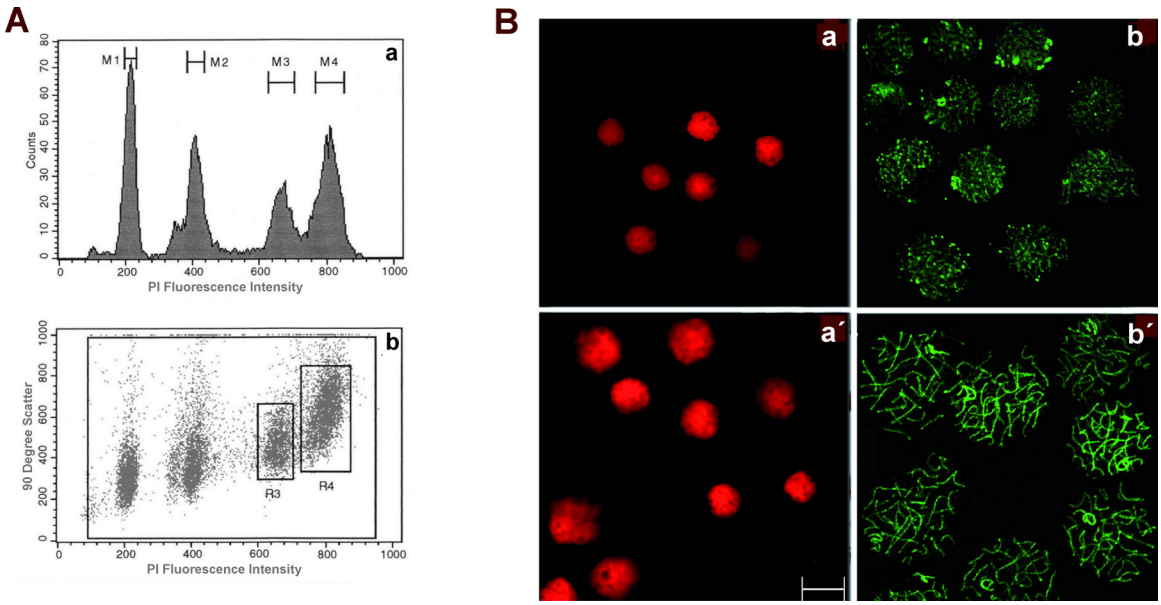

Figure 4. A) FC analysis of an adult guinea pig testicular cell suspension prepared with the protocol described here. **(a)**, histogram; **(b)**, dot plot. Note the two subpopulations in the 4C cell population. **B)** Analysis of sorted cells from 4C R3 **(a,b)** and R4 **(a',b')** regions. **(a,a')**, Epifluorescence microscope images of PI-stained cells. Note that nuclei from R3 region are comparatively smaller. Bar: 10 µm. **(b,b')**, Laser confocal microscopy of immunocytochemical reactions on spread cells using an antibody against Sycp3 (Synaptonemal complex [SC] protein 3, a lateral element component). The picture allows the conclusion that R3 fraction corresponds to early (lepto/zygotene) meiocytes, in which simple axes and short stretches of SCs can be seen, while R4 contains pachytene meiocytes, with completely assembled SCs. Modified from Rodríguez-Casuriaga, *et al.*,*Cytometry A*. **79**, 625 (2011). Click here to view larger figure.

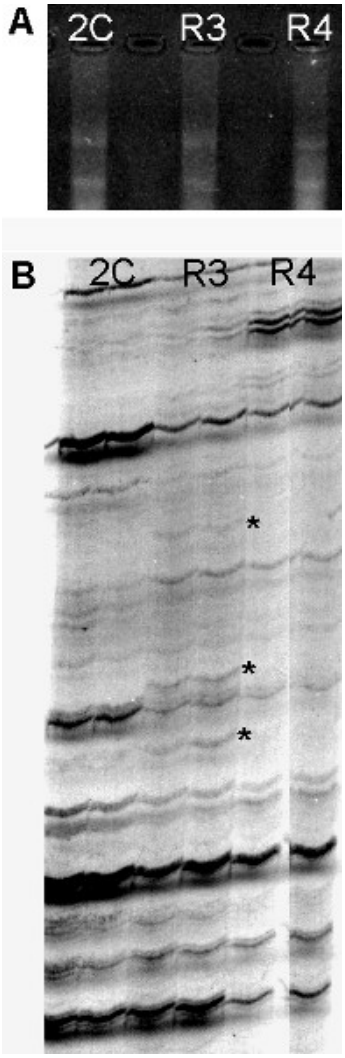

Figure 5. A) Agarose gel electrophoresis of total RNAs extracted from spermatogenic flow-sorted cell fractions 2C, R3 and R4 (explanation on the two latter fractions is in Figure 4). Cell suspensions were prepared with the protocol described here. **B)** Autoradiogram of a denaturing polyacrylamide electrophoresis gel showing differential cDNA bands obtained by means of the "mRNA differential display" method (RNAimage; GenHunter Corporation, Nashville, TN) for one of the primer combinations from the kit. mRNAs from the same three cell populations as in **A)** were compared. All experiments were performed in duplicate (reverse transcription and PCR) following the instructions from the manufacturer, and run in adjacent lanes. Asterisks indicate differential cDNA bands for R3 (early meiotic prophase cells) that appear in duplicate experiments (presumably corresponding to differentially expressed genes). Reproduced from Rodríguez-Casuriaga *et al., Cytometry A.* **79**, 625 (2011).

Discussion

The optimized method described here enables the preparation of cell suspensions from rodent testicular tissue in a very fast and reproducible way, avoiding enzyme and detergent treatment and maintaining good cell integrity and type proportions. The brevity of the procedure (the 15 min span includes testis dissection, tissue cutting, and processing), minimal handling involved, and absence of enzymatic treatments are some of the main advantages. All these would account for the good preservation of short life macromolecules, which is critical when a representative sample of compounds present in the original cell population is required.

Concerning the use of either PI or Hoechst 33342, we have observed no evident differences in the profiles resulting from flow cytometric analysis of testicular cell suspensions with the employment of one or the other dye. The dye choice will rather depend on the user's preferences and availabilities, and on planned further use. On one side, PI is cheaper, and of widespread application as does not require the use of flow cytometers and sorters having a UV laser. On the other, although we have successfully used PI-stained cells for ulterior sorting and differential gene expression studies (**Figure 4**), Hoechst 33342 or another vital dye with low cytotoxicity levels could be the preferable choices for applications where full cell viability is required, such as germ cell culture and/or transplantation[4].

We have been able to sort specific cell populations achieving over 95% purity either for selected testicular populations with different DNA content[4] (**Figure 3**), or even for subpopulations with the same DNA content but different chromatin condensation levels (**Figure 4**). This latter

can be accomplished as long as the subpopulations of interest can be individualized in the cytometric profiles, particularly in the dot plots. For instance, up to now we have been able to sort different stages of guinea pig meiocytes I[9], but not to discriminate and sort subpopulations within the 2C population simply by staining the DNA. Sorted cells have rendered good quality RNA, allowing downstream differential gene expression analyses[9] (**Figure 5**).

As can be seen in the protocol description, it is very simple, easily reproducible, and does not require especially skilled personnel. We consider it can be adopted for a wide variety of applications involving flow cytometry or not. The former range from simple testicular content analysis for the rapid checking of spermatogenic advance, to others with preparative aims such as flow purification of specific cell populations for ulterior molecular studies, as shown here.

Acknowledgements

This work was partly supported by CSIC (I+D project C022) and PEDECIBA. The authors want to thank Mariela Bollati and Valentina Porro from the Cell Biology Unit (Institut Pasteur de Montevideo) for their generous collaboration concerning the MoFlo cell sorter, and Merial-Montevideo for gently providing all the guinea pig specimens used in this project. **Figure 1** was reproduced with permission of BioMed Central; **Figures 2** and **3**, and **Table 1** with permission of S. Karger AG, Basel; and **Figures 4** and **5** were reproduced or adapted with permission of John Wiley & Sons, Inc (copyright owned by Wiley-Blackwell, 2011).

References

1. Meistrich, M.L. Separation of spermatogenic cells and nuclei from rodent testes. In: Prescott DM, editor. *Methods of Cell Biology.* **Volume 15**. *Academic Press, New York.*, 15-54 (1977).
2. Lam, D.M.K., Furrer, R., & Bruce, W.R. The separation, physical characterization, and differentiation kinetics of spermatogonial cells of the mouse. *Proc. Natl. Acad. Sci. USA.* **65**, 192-199 (1970).
3. Romrell, L.J., Bellve, A.R., & Fawcet, D.W. Separation of mouse spermatogenic cells by sedimentation velocity. *Dev. Biol.* **19**, 119-131 (1976).
4. Geisinger, A. & Rodríguez-Casuriaga, R. Flow cytometry for gene expression studies in mammalian spermatogenesis. *Cytogenet. Genome Res.* **128**, 46-56 (2010).
5. Getun I.V., Torres, B., & Bois, P.R. Flow cytometry purification of mouse meiotic cells. *J. Vis. Exp.* **50**, pii: 2602. doi: 10.3791/2602 (2011).
6. Meistrich, M.L. Separation of mouse spermatogenic cells by velocity sedimentation. *J. Cell Physiol.* **80**, 299-312 (1972).
7. Meistrich, M.L., Bruce, W.R., & Clermont, Y. Cellular composition of fractions of mouse testis cells following velocity sedimentation separation. *Exp. Cell Res.* **79**, 213-227 (1973).
8. Malkov, M., Fisher, Y., & Don, J. Developmental schedule of the postnatal rat testis determined by flow cytometry. *Biol. Rep.* **59**, 84-92 (1998).
9. Rodríguez-Casuriaga, R., Geisinger, A., Santiñaque, F., López, B., & Folle, G. High-purity flow sorting of early meiocytes based on DNA analysis of guinea pig spermatogenic cells. *Cytometry A.* **79**, 625-634 (2011).
10. Davey, H.M. & Hexley, P. Red but not dead? Membranes of stressed Saccharomyces cerevisiae are permeable to propidium iodide. *Environ. Microbiol.* **13**, 163-171 (2011).
11. Rodríguez-Casuriaga, R., Geisinger, A., López-Carro, B., Porro, V., Wettstein, R., & Folle, G.A. Ultra-fast and optimized method for the preparation of rodent testicular cells for flow cytometric analysis. *Biol. Proced. Online* **11**, 184-195 (2009).
12. Spanò, M. & Evenson, D.P. Flow cytometric analysis for reproductive biology. *Biol Cell.* **78**, 53-62 (1993).

