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Rapid preparation of rodent testicular cell suspensions and spermatogenic stages purification by flow cytometry using a novel blue-laser-excitable vital dye

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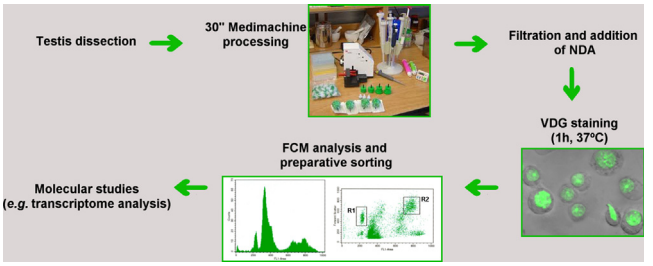
GRAPHICAL ABSTRACT



ABSTRACT

Availability of purified or highly enriched fractions representing the various spermatogenic stages is a usual requirement to study mammalian spermatogenesis at the molecular level. Fast preparation of high quality testicular cell suspensions is crucial when flow cytometry (FCM) is chosen to accomplish the stage/s purification. Formerly, we reported a method to rapidly obtain good quality rodent testicular cell suspensions for FCM analysis and sorting. Using that method we could distinguish and purify early meiocytes (leptotene/zygotene stages, L/Z) from more advanced ones (pachytene, P) in guinea pig, which presents an unusually high content of early stages. Here we present an upgrade of that method with improvements that enabled the obtainment of high-purity meiotic substages also from mouse testis, namely:

- Shortening of the mechanical disaggregation time to optimize the integrity of the suspension.
- Elimination of the 25 μm-filtration step to ensure the presence of large P cells.
- Inclusion of a non-cytotoxic, DNA-specific, 488 nm-excitable vital fluorochrome (Vybrant DyeCycle Green [VDG], Invitrogen) instead of Hoechst 33342 (requires UV laser, which can damage nucleic acids) or propidium iodide (usually related to dead/damaged cells). As far as we know, this is the first report on the use of this fluorochrome for the discrimination and purification of meiotic prophase I substages.

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Methods

Spermatogenic stages purification by FACS using a blue-laser-excitable vital dye

Heterogeneity of mammalian testis is a major difficulty for the understanding of spermatogenesis bases, since pure or enriched cell populations representing the different stages of sperm development are required for most molecular analyses [1]. Diverse strategies such as Staput [2,3], centrifugal elutriation [1] and flow cytometry (FCM) [4–6] have been used to obtain enriched or purified testicular cell populations for subsequent differential gene expression studies. Cells must be in suspension for most enrichment/purification approaches. Ideally, the cell suspension should represent as much as possible the original tissue, lack cell clumps [1], and have a high proportion of viable cells as well as few multinucleates, which tend to form as a consequence of the syncytial nature of the seminiferous epithelium [7,8].

We are hereby presenting an upgrade of a previously reported method for the rapid preparation of rodent testicular cell suspensions [9,10]. The original protocol was used in combination with either the UV-excitable Hoechst 33342 vital dye, or with propidium iodide (PI). Although the conditions formerly used with PI (mechanical stress, high dye concentration and long exposure time) favored its entrance to the cells in the suspension, this fluorochrome would not be the first choice for preparative cell sorting as it is usually excluded from intact cells.

The optimization of the method with a blue-laser-excitable DNA-specific vital dye (Vybrant DyeCycle Green [VDG], Invitrogen) has had a direct effect on the integrity of the purified fractions, turning them more suitable for downstream expression profiling. Besides, the inclusion of VDG afforded very interesting cytometric profiles with a significant number of cell populations distinguishable in the dot plots, even in species with no peculiar abundancy of certain stages such as L/Z in mouse [11]. Within 2C cells, different subpopulations can also be distinguished, but their identities are yet to be determined.

Method detail

Animals

CD-1 Swiss mice were employed in this work. The age range analyzed was 10–27-day-old juveniles, and 40-day-old young adults. 5–7 specimens were used in each case, and analyzed individually to evaluate specimen variability and sorting consistency. All experimentation procedures were performed in accordance with the National Law of Animal Experimentation 18,611 (Uruguay).

Preparation of the cell suspension

(1) Kill the specimen to be used following the recommendations of the specialized committees (in Uruguay, National Commission for Animal Experimentation [CNEA]; experimental protocol 001/02/2012). In our case, an overdose of pentobarbital was supplied, except for the youngest specimens in which cervical dislocation was performed.
(2) Dissect the testes following standard procedures, and place them on ice in a 60 mm glass Petri dish containing 5 mL of ice-cold DMEM supplemented with 10% fetal calf serum (ice-cold freshly prepared DPBS can be used instead, in this and subsequent steps).
(3) Remove the tunica albuginea and cut the decapsulated testes into pieces of 2–3 mm on each side.
(4) Process pieces in a Medimachine (BD Biosciences), an automated electro-mechanical solid-tissue disaggregator [9,10]. Tissue pieces are disaggregated inside a disposable grinder 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 the disposable unit, switch on the disaggregator, and process for 30 s.
(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) To prevent cell clumping add NDA (2-naphthol-6,8-disulfonic acid, dipotassium salt) to a final concentration of 0.2%.
(8) Repeat step 6, and place on ice.
(9) Finally, take an aliquot to count in a Neubauer chamber and adjust concentration to $3–5 \times 10^6$ cells/mL with supplemented DMEM or DPBS. At least $4–5 \times 10^7$ cells/g of testis material are usually obtained. Viability can be checked by Trypan blue exclusion. To do so, mix 100 μL of cellular suspension, 200 μL of DPBS and 300 μL of 0.4% Trypan blue, and count in a Neubauer chamber. This method usually renders above 90% viability.

Flow cytometric analysis

Any flow sorter equipped with a standard 488 nm blue laser can be used for the analysis and separation of cells stained with VDG (Invitrogen). We have used a FACSVantage (BD Biosciences) flow sorter equipped with a Coherent argon ion laser tuned to emit at 488 nm. VDG is a DNA-specific vital dye with non-cytotoxic reported effects that allows cytometric analysis based on DNA content using a blue laser as excitation source. Interestingly, it also renders differential chromatin staining patterns that resemble those obtained with Hoechst dyes under a fluorescence microscope (see Fig. 1).