Flow Cytometry and Spermatogenesis

Subjects: Immunology

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Flow cytometry has become an invaluable tool for the analysis of testicular heterogeneity, and for the purification of stage-specific spermatogenic cell populations, both for basic research and for clinical applications.

spermatogenesis   flow cytometry   FACS   male infertility

1. Biology of Spermatogenesis and Main Difficulties for Its Molecular Study

Mammalian spermatogenesis is the process of male gamete formation, a differentiation process that in normal conditions initiates at puberty and can last as long as the individual. It takes place in the testes, which bear at least seven somatic cell types, and at least 26 morphologically distinct germ cell stages[1].

Spermatogenesis consists of the successive occurrence of three main phases: (i) the proliferative phase of spermatogonia; (ii) the meiotic phase; and, (iii) the terminal differentiation phase or spermiogenesis (Figure 1). All three phases are essential for the reproductive health of the individuals, and consequently for the species survival.
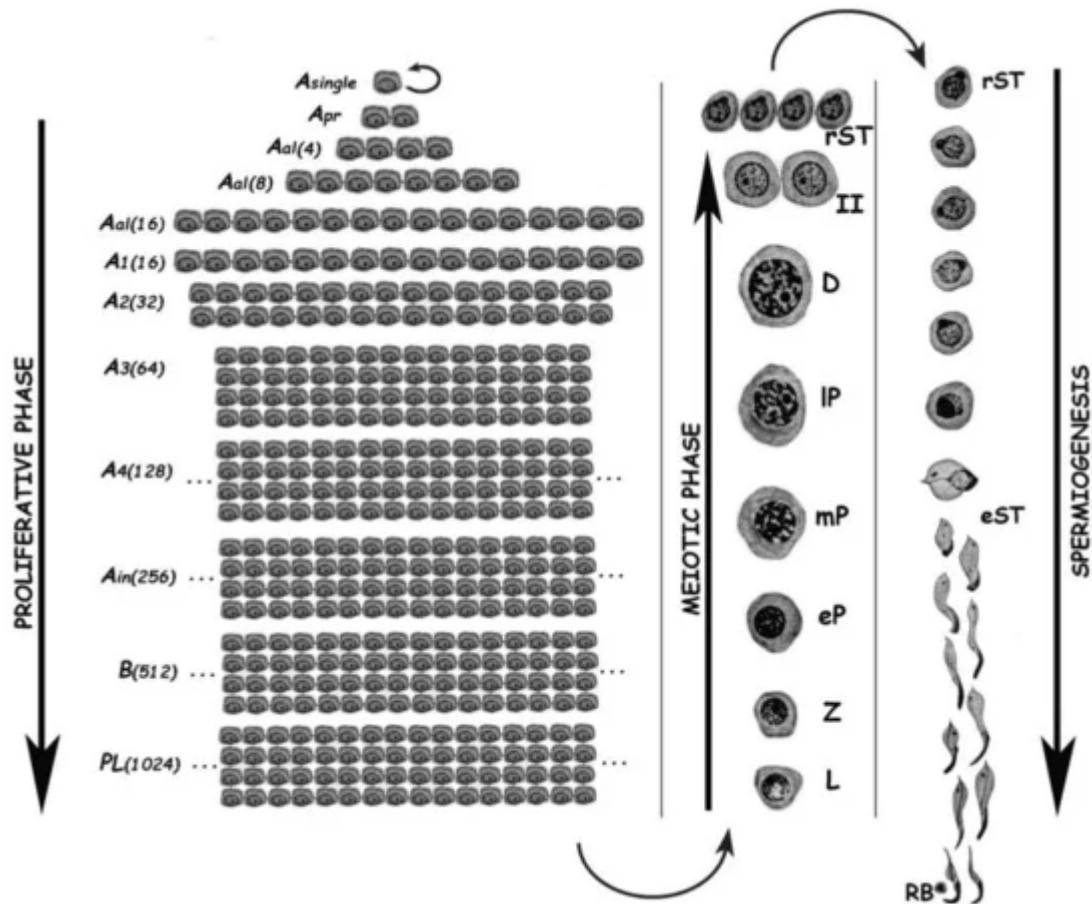
Figure 1. Schematic representation of the different germ cell stages along spermatogenesis of the rat. The three main phases of the spermatogenic process are indicated. A_{single}, A single spermatogonium; A_{pr}, A paired spermatogonia; A_{al}, A aligned spermatogonia; A_1–A_4, type A spermatogonia 1–4; A_{in}, intermediate spermatogonia; B, type B spermatogonia; PL, pre-leptotene spermatocytes; L, leptotene spermatocyte; Z, zygotene spermatocyte; eP, early pachytene spermatocyte; mP, medium pachytene spermatocyte; lP, late pachytene spermatocyte; D, diplotene spermatocyte; II, secondary spermatocyte; rST, round spermatids; eST, elongating spermatids; RB, residual body. In the proliferative phase, the numbers of spermatogonia derived from an A single are indicated in parenthesis.

The proliferative phase depends on the presence of spermatogonial stem cells (SSCs) that amplify themselves to maintain the SSC pool (self-renewal), but also give rise to progenitor spermatogonia (committed to differentiation). For instance, in the mouse SSCs are derived from gonocytes and reside in the population of A_{single} (A_s) spermatogonia. The latter initiate mitotic proliferation either to produce new A_s spermatogonia by complete cytokinesis, or to give rise to chains of A_{paired} (A_{pr}), and $A_{aligned}$ (A_{al}) spermatogonia. These represent undifferentiated spermatogonia that are connected by intercellular bridges as a consequence of incomplete cytokinesis. A_{al} spermatogonia, as well as a few A_{pr} spermatogonia, differentiate into A_1 spermatogonia without division, and then proliferate mitotically five times to sequentially form A_2, A_3, A_4, intermediate, and type B spermatogonia, collectively termed differentiated spermatogonia. Afterwards, type B spermatogonia divide into two primary diploid spermatocytes that enter meiosis[2].

In the meiotic phase, ploidy halving is accomplished through a single round of DNA replication followed by two cellular divisions. During the first meiotic division (meiosis I) homologous chromosomes segregate, and primary spermatocytes (4C, 2n) give rise to secondary ones with 2C DNA content but already haploid (1n). Secondary spermatocytes enter meiosis II, and separation of sister chromatids takes place, generating the round spermatids (1C, 1n), which initiate spermiogenesis. Notably, the reduction in ploidy is of fundamental importance for gametogenesis in all sexually reproducing organisms, as at the time of fertilization fusion of the male and female gametes leads to the restoration of the species chromosome number. In addition to its reductive nature, meiosis is also very peculiar regarding the exchanges of genetic material that take place between homologous chromosomes during prophase I. Homologous chromosomes (i.e., of maternal and paternal origin within each pair) align, and then synapse via a highly specialized proteinaceous structure—the synaptonemal complex (SC)—that assembles during prophase I, enabling the closeness required for homologous recombination[3][4]. Due to the importance of the unique events that take place during meiotic prophase I (formation of the SCs, alignment and pairing, recombination), this has been the most extensively studied meiotic stage. As it is a very long stage, it has been divided into different substages to facilitate its study: leptotene (L), zygotene (Z), pachytene (P), diplotene (D), and diakinesis (see Figure 1). The assembly of the SC starts in L, homologous pairing takes place in Z, and recombination (crossing-over) is the hallmark of P. During D, the SCs disassemble[5]. The eventual alteration of these events often leads to spermatogenic arrest and infertility[6][7][8][9].

Spermiogenesis is the third and final phase of spermatogenesis. Along this post-meiotic stage, round spermatids go through a series of profound morphological and functional changes, giving rise to mature spermatozoa (see Figure 1). In the mouse, spermatids can be morphologically classified as steps 1–8 round spermatids, and steps 9–16 elongating ones[10]. Particularly in the chromatin, the main change in spermatids is the replacement of most histones by transition proteins first, and then by protamines.

 Cellular heterogeneity represents a major drawback for the identification of molecular factors, and the unveiling of molecular mechanisms underneath gamete formation. Studies seeking these goals usually require the isolation of cells from the developmental stage of interest. The lack of an effective in vitro culture system[11][12] has also hampered spermatogenic stage-specific molecular studies.

2. The Spermatogenic Process from a Flow Cytometric Perspective

As mentioned above, spermatogenesis comprises several stages from spermatogonia to elongated spermatids, which present a wide range of variation in their cellular size, shape, inner complexity, chromatin structure, and DNA content. Most these parameters can be detected and measure-assigned by FCM, thus explaining why this powerful technology came to stay in this research area[13]. Table 1 summarizes some important features of the most relevant testicular cell types that are useful in FCM.

Table 1. Principal characteristics of testicular cells from adult rodents.

	Approximate Cellular Size (μm)	Cellular/Nuclear Shape	Inner Complexity	Chromatine Structure	DNA Content
Type A spermatogonia	12–14	Ovoid nuclei	Very low	Homogenous euchromatin	2C-4C *
Type B spermatogonia	8–10	Round nuclei	Low	Heterochromatin along nuclear periphery	2C-4C *
PreLeptotene spermatocytes	7.6–8.2	Round nuclei	Low	Heterochromatin along nuclear periphery	2C-4C *
Leptotene spermatocytes	8–10	Round nuclei	Low	Condensed chromosomes forming thin filaments	4C
Zygotene spermatocytes	10–12	Round nuclei	Medium	Clumps of dense chromatin, mainly at the nuclear periphery	4C
Pachytene spermatocytes	12–18	Large round nuclei; thin rim of cytoplasm; cell volume increases along the stage	High	Abundant clumps of dense chromatin; shorter and thicker chromosomes	4C
Round spermatids	10	Round cells with round nuclei	Very low	Homogenous euchromatin; densely stained chromocenter in the middle	1C
Elongating/elongated spermatids	4–8	Sickle-shaped nuclei	Low-Medium	Increasingly compacted chromatin	1C
Leydig cells	10–12	Polyhedral cells with eccentrically located ovoid nuclei; abundant citoplasmic lipid droplets.	Very high	Prominent nucleoli; abundant peripheral heterochromatin	2C
Sertoli cells	Height (from basal to apical surface): 90; Base: 30	Columnar, Irregular, with apical and lateral invaginations; oval-shaped nuclei	High	Dark nucleolus; central condensation area	2C

Information was extracted from references[14][15], and our own experience. * 4C DNA content in these cells corresponds to the G2 cell cycle phase. Inner complexity level assignment was performed based mainly on observation of flow cytometry (FCM) side scatter parameter.

Regarding DNA content, testicular cell suspensions can be stained with a fluorescent dye and analyzed by FCM. The choice of dyes that stoichiometrically bind DNA allows the discrimination of cell populations with different ploidy based on their fluorescence intensity. In the spermatogenic context, with many cell types differing in their DNA content, this sole parameter enables a gross first simplification concerning testis heterogeneity. Three main groups of events can be distinguished: C (round spermatids, elongating spermatids), 2C (several types of G1 spermatogonia, secondary spermatocytes, testicular somatic cells), and 4C (different stages of primary spermatocytes, G2 spermatogonia) (see the histogram in Figure 2B). In adult specimens, a fourth population (elongated spermatids, sperm) can be observed, bearing an apparently sub-haploid DNA content[16]. Different fluorochromes with distinct action mechanisms have been employed for the DNA content-based discrimination of testicular cell populations (for a revision, see[13]). When the aim is to classify cells for downstream molecular applications, then the vital dye Hoechst 33342 (Ho342) has been the most commonly used dye[17][18][19][20][21][22][23][24].Ho342 is excited in the UV range and emits in blue and red; a combination of both fluorescences enables the resolution of various testicular cell populations (Figure 2A).On the other hand, we have developed an alternative sorting protocol for testicular cell populations based on the vital dye Vybrant DyeCycle Green (VDG) (Figure 2B). As VDG is excited with a blue laser (thus avoiding the potential damage to nucleic acids caused by UV light), it represents an advantage for downstream applications where nucleic acid integrity is an important issue[25][26].
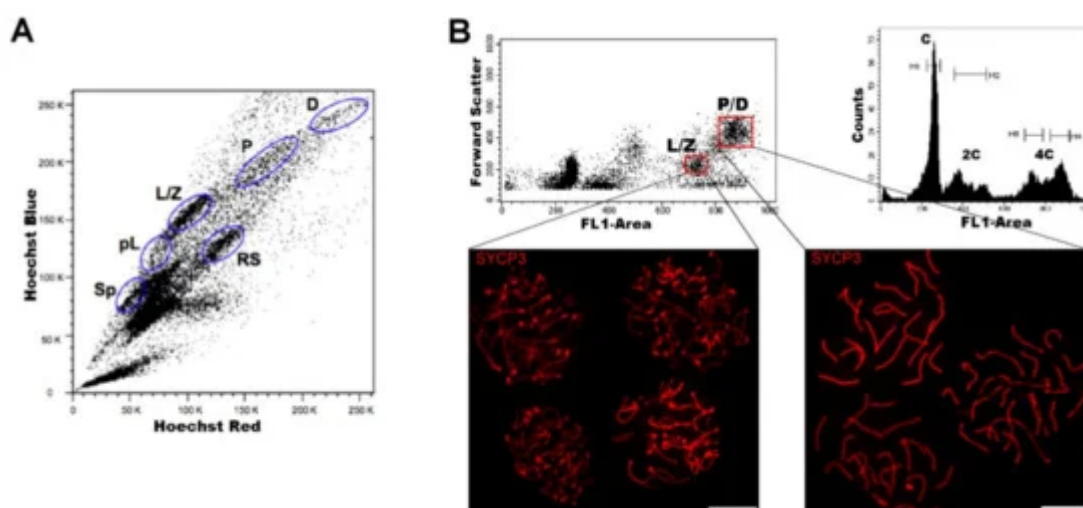

Figure 2. FCM analysis of mouse testicular cell suspensions employing two different DNA dyes. (**A**) Representative Hoechst 33342 (Ho342) FCM profile obtained from the analysis of adult mouse testis, based on emitted blue and red fluorescence. The various spermatogenic cell populations that can be distinguished, and are indicated, are: spermatogonia (Sp), pre-leptotene (pL), leptotene–zygotene (L/Z), pachytene (P), diplotene (D), and round spermatids (RS). This image is reproduced from reference[27] with permission of *J. Vis. Exp.* (**B**)

Representative FCM profiles from the analysis of a 22-day-old mouse testicular cell suspension stained with Vybrant DyeCycle Green (VDG). A Forward Scatter vs. FL1-Area (VDG fluorescence intensity) dot plot and its corresponding histogram are shown. Histogram peaks corresponding to C, 2C and 4C cell populations, and sorting gates within the 4C population (delimited in red) in the dot plot, are indicated. Note the absence of the apparently sub-haploid peak in the histogram, as the profile is from a juvenile animal. Examples of immunolocalizations of SYCP3 (synaptonemal complex (SC) protein 3, a lateral-element–SC component) on cellular spreads from sorted cells, in order to confirm the purity of the sorted fractions, are shown below. As can be seen, cells coming from the selected gates correspond to different stages of the first meiotic prophase: L/Z, in which simple axes (L) and short stretches of SCs (Z) are present; or P/D with completely assembled (P), or disassembling (D) SCs. Bars correspond to 10 µm.

Regarding cellular size, developing male germ cells exhibit a wide size range, with elongated spermatids in the lower extreme (~4–5-µm-long head), and late prophase I spermatocytes in the upper one (almost 20 µm diameter). Forward light scattering (FSC-H) allows size-based discrimination. Furthermore, as the various testicular cell types exhibit different granularity, cellular complexity or granularity is another important aspect in aiding the distinction of stages orcell types present in highly heterogenic testicular cell suspensions, which is accomplished through side scatter parameter (SSC-H, also named 90-degree scattering).

The combination of these three parameters gives rise to multi-parametric analyses with higher information input than when considering just one, thus enabling an advance of one more step towards the distinction of different cell types and stages[13].

Where certain cell types cannot be solely identified or purified based on ploidy, different strategies have been employed such as the introduction of fluorescent labels [e.g. 97,99]. Specific antibody-dependent identification and sorting of cell types has been also a very common strategy for the identification and isolation of cell types that are hardly distinguishable or not distinguishable at all in the cytometric profiles, such as specific populations of spermatogonia, or certain somatic cell types [e.g. 100].

3. FCM as an Analytical Tool of Spermatogenesis

A well-recognized advantage of FCM is its high quantitative analytical power and statistical weight. Depending on the equipment, hundreds or even thousands of events per second can be analyzed. Reproductive biologists have harnessed the analytical power of FCM for multi-parametric analyses of testicular cell suspensions. They have taken advantage of the various differences between cell types and stages, which translated to FCM profiles, allow the assessment of spermatogenic advance. Multi-parametric FCM analyses have been historically employed to study postnatal development of the male gonad in different species [28][29][30][31][32][33][34][35][36][37][38][39][40].

FCM profiling of testicular cell suspensions has also proved useful in the diagnosis of human male infertility[41], in assessing sperm quality[42], in the study of testicular cancer[43], and in the characterization of reproductive disease

mouse models [44], among other analytical applications.

4. FCM as a Preparative Tool in Spermatogenic Studies

Whenever the available FCM equipment is also a sorter, any well-defined population in the dot plots can be chosen and classified by fluorescence activated cell sorting (FACS) for downstream studies. The strategies for the distinction and subsequent sorting of spermatogenic cell populations have been varied, ranging from multiparametric analyses that rely on differences in DNA content and light scattering, to the employment of specific antibodies against the stages of interest.

FCM allows the discrimination of a higher number of spermatogenic cell types and enables the obtainment of highly pure cell populations via FACS[28][45]; therefore, it presents important advantages for transcriptomic studies. Therefore, flow-sorted spermatogenic cell populations have been employed for the obtainment of highly reliable transcriptomic profiles, including scarce cell populations that are hard to obtain with other methods, such as early meiotic prophase stages[17][46][47]. FACS has also complemented single-cell approaches in various studies, either for the enrichment in certain cell types[23][48][49][50][51], for the study of different types of spermatogonia[48][52][53][54][55], or as a way to complement random cell picking (unsorted) for more accurate cell-type assignment [23][56]. FACS has been also combined with the synchronization of spermatogenesis[20][49].

FACS-sorted spermatogenic cell populations have been also employed in studies addressing the understanding of chromatin accessibility, histone modifications, DNA methylomes, and three-dimensional (3D) chromatin structure, during mouse and/or
human spermatogenesis[18][21][22][24][57][58] .

In addition, in vitro recapitulation of the spermatogenic process has been a precious goal among reproductive biologists for at least six decades, as it would facilitate, among others, studies on the requirements of the process in a controlled in vitro environment; research that is difficult and/or unethical to perform directly in vivo; studies on the molecular mechanisms of
pathologies such as testicular cancer or male infertility; fertility restoration or preservation, by the production of haploid male germ cells from undifferentiated germ cells isolated from infertile adult patients, or from pre-pubertal cancer patients before the application of gonadotoxic treatments, respectively[11][59][60][61]. As SSCs are responsible for the continuous production of spermatogonia that sustain spermatogenesis [62], the generation of in vitro culture systems to efficiently maintain and expand SSCs is fundamental for progress towards the above-mentioned goals. FACS, based on specific markers, presents important advantages for the obtainment of high purity SSCs, as it provides morphological data of cells, allows the simultaneous detection of multiple surface markers, and is informative on eventual quantitative differences of biochemical markers between cellular subpopulations [e.g. [51][63][64][65][66][67][68].

5. Conclusions and Perspectives

Being such a heterogeneous tissue, mammalian testis has represented a challenge in the attempt to unravel the molecular bases of spermatogenesis. FCM has significantly contributed to the field in allowing to identify and eventually purify a high number of specific cell types. As more advances are developed, we foresee that the FCM has a lot more to contribute in this area of knowledge, as there is still a lot of ground for potential complementation between FCM and complementary approaches.

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