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Advanced immunostaining approaches to study early male germ cell development

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ABSTRACT

Mammalian male germ cell development takes place in the testis under the influence of a variety of somatic cells and an incompletely defined paracrine and endocrine influences. Since it is not recapitulated well in vitro, researchers studying spermatogenesis often manipulate the germline by creating transgenic or knockout mice or by administering pharmaceutical agonists/antagonists or inhibitors. The effects of these types of manipulations on germline development can often be determined following microscopic imaging, both of stained and immunostained testis sections. Here, we describe approaches for microscopic analysis of the developing male germline, provide detailed protocols for a variety of immunostaining approaches, and discuss transgenic fluorescent reporter lines for studying the early stages of spermatogenesis.

1. Development of the male mouse germline

The male germline is established in mice as primordial germ cells (PGCs) that colonized the fetal testis at ~embryonic day (E)10.5 become prospermatogonia (also termed gonocytes) following sex determination at ~E11 (DiNapoli and Capel, 2008; McLaren, 2003). Over the next several days, prospermatogonia proliferate and become surrounded by somatic Sertoli cells to form the nascent testis, or seminiferous cords. By ~E15.5, fetal prospermatogonia stop dividing and become mitotically arrested in G_0 of the cell cycle until after birth (Vergouwen et al., 1991; Western et al., 2008). At postnatal days (P)1-2, prospermatogonia resume mitosis and transition into type A spermatogonia. This initial spermatogonial population is heterogeneous as early as P3, and can be characterized as either undifferentiated (A_{undiff}) or differentiating (A_{diff}) (Kluin et al., 1984; Niedenberger et al., 2015; Yoshida et al., 2006). This heterogeneity is evident in neonatal spermatogonia based on differences in morphology (Drumond et al., 2011; Kluin and de Rooij, 1981), abundance of specific mRNAs (Yoshida et al., 2004, 2006; Hermann et al., 2015), expression of protein fate markers (Niedenberger et al., 2015; Hermann et al., 2015; Busada et al., 2014, 2015), and the ability to seed spermatogenesis in recipient testes following transplantation (McLean et al., 2003). Over the next few days the numbers of spermatogonia continue to increase, resulting in formation of the foundational pool of spermatogonial stem cells (SSCs), undifferentiated progenitors that are poised to differentiate, and STRA8+/KIT+ differentiating spermatogonia (Niedenberger et al., 2015; Busada and Geyer, 2015; Yang and Oatley, 2014).

Timing during the progression of spermatogenesis is remarkably precise, such that specific cell types appear on predictable days during the 'first round of spermatogenesis' that begins in the neonatal mouse testis at ~P3, with some strain variation. Type A spermatogonia initiate the differentiation program as A_1 spermatogonia form in response to all-trans retinoic acid (ATRA, reviewed in (Busada and Geyer, 2015; Griswold, 2016)). Following several divisions (A_{2-4}, In, B), male germ cells enter meiosis as preleptotene spermatocytes as early as ~P8, and successively become leptotene (~P10), zygotene (~P12), and pachytene spermatocytes (~P14). The first haploid round spermatids are formed by ~P20, which then undergo dramatic morphogenetic changes during spermiogenesis to form condensed spermatids, which are released from the seminiferous epithelium as testicular sperm by ~P35 (Oakberg, 1956; Clermont and Trott, 1969; Bellve et al., 1977). Based on this defined temporal appearance of specific identifiable germ cell subtypes, the developing testis provides an excellent model system to study the progression of spermatogenesis.

Researchers can use mice to study the physiologic roles of various gene products, signaling pathways, and environmental influences on early germ cell development in vivo by generating transgenic and knockout models and by treating mice with hormones, agonists/antagonists, inhibitors, and various toxicants. However, there are small numbers of germ cells in the developing testis, and it is difficult to isolate them with high levels of purity. Therefore, brightfield and fluorescent-based microscopy provide the best means to determine the outcomes

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of these manipulations on the various phases of germline development. In this article, we will describe current methods and tools for imaging and immunostaining prospermatogonia and spermatogonia, review transgenic models for germ cell imaging, and discuss needs that we feel should be addressed by researchers in the field in the future.

2. Harvesting and preparing fetal and neonatal testes for imaging

The removal of fetal and neonatal mouse testes must be done carefully using fine tip forceps and micro-dissection spring scissors. The attached epididymis serves as a convenient means to grasp the tissue while avoiding nicking or crushing delicate testicular tissue. A stereoscope is not required, but can be useful for dissecting fetal testes and for removing epididymal tissue as well as the overlying connective tunica vaginalis and albuginea. Testes can then be used for a variety of downstream applications such as isolation of total testicular DNA, RNA, or protein, germ or somatic cell isolations, or fixation for various imaging modalities, as described below.

2.1. Fixation for imaging

Light microscopy has been used in numerous studies to carefully characterize the various stages of male germ cell development, and specific morphologic criteria have been assigned to germ cell types at each stage of their development. These include characteristic positioning within the seminiferous epithelium as well as differences in nuclear diameter and chromatin appearance (Kluin et al., 1984; Drumond et al., 2011; Kluin and de Rooij, 1981; Chiarini-Garcia and Russell, 2001). However, these accurate determinations are only feasible in sections from samples that have been properly prepared. Testicular morphology is best maintained for light microscopy following thorough fixation, and there are many different types of fixatives that have been used in the literature. The most commonly used fixative for histological analysis is Bouin's solution, which contains paraformaldehyde, picric acid, and acetic acid. The acetic acid component causes characteristic condensation of nuclear chromatin, resulting in subtle differences that can be used to define different germ cell types. Bouin's solution rapidly and thoroughly penetrates tissue, and for mouse testes immersion fixation for ≤24 h is sufficient (P0–4 ≈ 2 h, P5–12 ≈ 4–6 h, P13-adult ≈ 12–24 h). Following fixation, the tissue must be thoroughly washed in 1X PBS to remove as much of the picric acid as possible prior to routine paraffin embedding, sectioning, and staining.

While fixatives such as Bouin's solution preserve tissue organization and cellular structure exceedingly well, they often do a rather poor job of retaining epitopes for subsequent antibody-based immunohistochemical analyses. In addition, picric acid autofluoresces, which makes Bouin's-fixed samples generally unsuitable for fluorescence-based immunostaining. If immunostaining is the desired goal, then one testis (or a portion of one testis) should be immersion-fixed in 4% PFA (see supplemental file, protocol 1) using the incubation times outlined above for Bouin's fixation. Following fixation in 4% PFA, testes washed in 1X PBS and then either dehydrated in ethanol for paraffin embedding or incubated in sucrose prior to cryosectioning.

2.2. Seminiferous cord and tubule whole mount preparation

Testis sections provide a limited two-dimensional view of germ cells within the testis cords, either in longitudinal or cross-sections, and spatial organization within the cords is often lost. Therefore, testis whole ("in toto") mounts are useful to examine relationships between adjacent germ cells and assess the length of interconnected chains of spermatogonia. These are prepared by first detunicating testes and carefully cutting them into thirds, and testis cords can be gently teased apart using forceps. These pieces are then permeabilized prior to immunostaining; we follow a standard immunostaining protocol (see supplemental file, protocol 2), with the caveat that all incubation times are increased to facilitate their penetration into interior cells within this thicker tissue (see supplemental file, protocol 3).

3. Immunostaining

Specific proteins can be detected within isolated cells or a tissue using a variety of immunostaining approaches. These provide a relative comparison of steady-state protein abundance, determination of subcellular localization, and identification of which cell type (s) express that protein. This technique is dependent upon a specific antibody-antigen interaction, and specificity should be verified by using appropriate negative controls such as no primary antibody control, pre-binding the antibody to the immunizing peptide or recombinant protein (if available), and verifying that antibody recognizes a single band on western blot in a lysate from the tissue of interest. Most applications employ indirect immunostaining, in which a primary antibody is applied to tissue followed by incubation with a secondary antibody that is raised against the host species of the primary antibody and is conjugated to a fluorophore or other molecule enabling detection.

3.1. Immunohistochemistry (IHC) on paraffin sections and indirect immunofluorescence (IIF) on cryosections

Each of the staining techniques (IHC and IIF) has distinct advantages and disadvantages. Paraffin-embedded tissues and labeled slides can be stored at room temperature indefinitely, and enormous numbers of formaldehyde-fixed human normal and diseased samples are available through the pathology departments of most hospitals for retrospective analysis. An advantage of IHC on paraffin-embedded sections is that cellular morphology is well-maintained. However, epitopes are often lost in these samples (usually causing reduced sensitivity), and the use of 3,3′-diaminobenzidine (DAB)-based chromogen labeling makes co-labeling proteins with antibodies from different hosts extremely difficult, and these images cannot be combined to provide a 3-dimensional *Z*-stack (as with confocal imaging). IIF on fixed cryosections does have the advantage of increased sensitivity, but sections are less amenable to histological analysis and careful characterization of nuclear morphology, which has proven quite useful for discriminating between different spermatogonial subtypes (Drumond et al., 2011; Kluin and de Rooij, 1981; Chiarini-Garcia and Russell, 2001). We have optimized reliable protocols for DAB-based IHC on Bouin's- and PFA-fixed paraffin-embedded testis sections (see supplemental file, protocol 4) as well as IIF on PFA-fixed cryosections (see supplemental file, protocol 2).

3.2. Co-immunostaining with IIF

Simultaneous staining for up to three different proteins provides distinct advantages for studying spermatogenesis, as it allows researchers to mark all germ cells (using DDX4 or TRA98, for example), identify specific spermatogonial fate (e.g. undifferentiated spermatogonia with GFRA1 or differentiating spermatogonia with KIT) and then detect a third protein of interest. This allows for assignment of novel protein expression in specific cell types as well as straightforward analysis of changes in the germ cell populations in transgenic, knockout, or chemically- or hormonally-treated animals. A wide variety of excellent specific antibodies are available through commercial vendors or from academic and government research laboratories for the detection of testicular germ and somatic cell types in both PFA-fixed cryosections (Fig. 1) as well as paraffin-embedded sections of the mammalian testis (see Fig. 2). We favor using cryosections in most cases, because in our experience they have better epitope retention.

To perform co-immunostaining, primary antibodies must be chosen that are generated in distinct host species. These primary antibodies are

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