Staging Seminiferous Tubules in Unfixed Mouse Testis Sections Using Hoechst and SCP3

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December 2025

Abstract

Spermatogenesis within mammals occurs in the seminiferous epithelium of the testes. When viewing seminiferous tubule cross-sections, the developmental stages of germ cells within the epithelium are not easily distinguishable. The traditional method for staging is performed using Peanut Agglutinin (PNA) lectin staining, which stains acrosomes of developing Spermatids (Wakayama et al., 2022). Since acrosomes are internalized organelles, they are not accessible at the surface. Therefore, this method of staging requires tissue permeabilization, which may not be desirable depending on experimental conditions. Permeabilization is typically a result of tissue fixation, which wipes away the cell membrane and the enzymes within it. Some experiments such as enzyme activity staining require unfixed, and unpermeabilized tissue. Therefore, we propose a new staging method that uses Synaptonemal Complex Protein 3 (SCP3), a protein complex used in meiosis that facilitates genetic recombination and stains the spermatocytes, along with Hoechst, a nuclear stain that penetrates DNA. These stains will enable visualization of cell organization within the epithelium and allow for identification of distinct stages within spermatids meiotic progression without tissue permeabilization.

Keywords: spermatogenesis, seminiferous tubules, permeabilization

Introduction

Germ Cell Development

Spermatogenesis is the process in which germ cells develop into mature Spermatozoa. This occurs in seminiferous epithelium inside of tubules within testes. In mice specifically, spermatogenesis is a continuous cycle with 12 stages in mice. Each stage has different cell types in different stages of development.

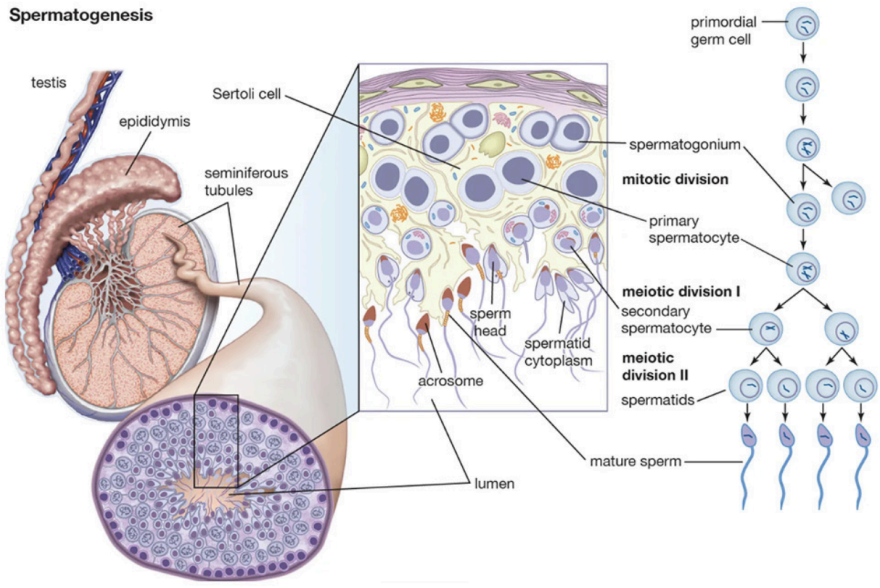

Figure 1: Germ Cell Development (Medline Academics, 2023)

Developing spermatozoa originate from the spermatogonia. This is the stem cell population within seminiferous tubules, found along the basal lamina, which undergo asymmetric division, leaving one cell to remain a spermatogonium, and the other to differentiate into a primary spermatocyte. Primary spermatocytes are the largest germ cells within seminiferous tubules, and the only cells that are actively going through meiosis. After their first meiotic division, they become secondary spermatocytes. Secondary spermatocytes then undergo a second meiotic division, differentiating into spermatids. Within the epithelium, there are two morphological forms of spermatids. In earlier development, there are round

spermatids that have yet to elongate but begin to grow flagellum. These round spermatids elongate and undergo extensive remodeling. Once fully elongated, the cells are considered mature spermatozoa, capable of participating in fertilization.

Staging

Staging is the the process of identifying and labeling specific stages in spermatogenesis by examining the cell types and tissue organization within tubule cross sections. Staging is important because it allows us to track the development of germ cells.

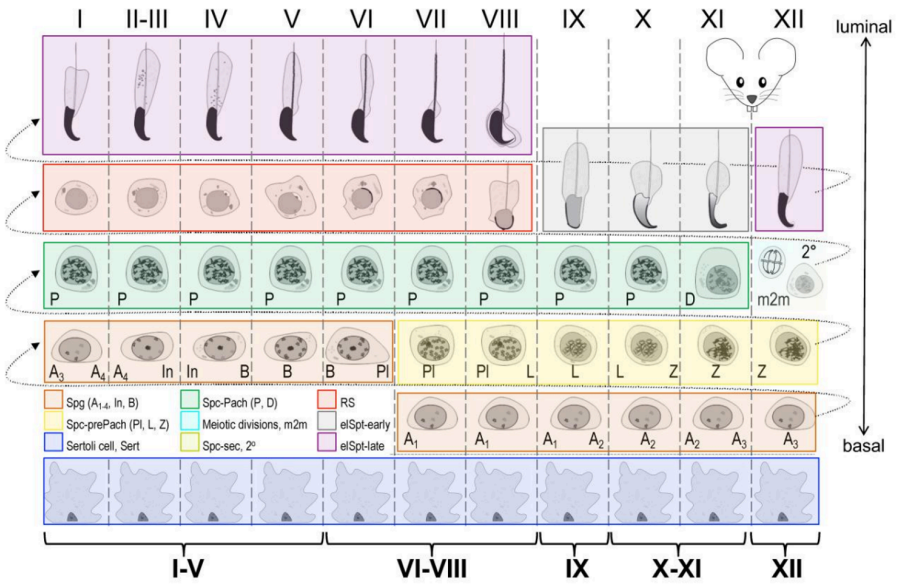

Figure 2: Germ Cell Development within Stages (Meikar et al., 2022)

This figure shows the cell types that are visible in each stage of spermatogenesis through columns. However, the actual development of germ cells is depicted by the arrows. This means that although each column represents a static snapshot of a seminiferous tubule cross-section at a specific stage, the germ cells themselves progress through the epithelium and towards the lumen as they mature and advance through the stages sequentially throughout their development.

Staging with Nuclear Morphology and PNA

Staging is primarily accomplished with nuclear staining, and to discern between similar stages, a marker for a subpopulation of cells is used. This marker for a subpopulation is traditionally Peanut Agglutinin (PNA) lectin staining, which stains acrosomes of developing spermatids. However, acrosomes are internalized organelles so they are not accessible at the surface, meaning in order to stain acrosomes with PNA, the tissue must be permeabilized.

Permeabilization

Permeability is the capacity of tissues to allow substances to move through them. In order to stain acrosomes with PNA, the tissue must be permeabilized, typically accomplished during fixation.

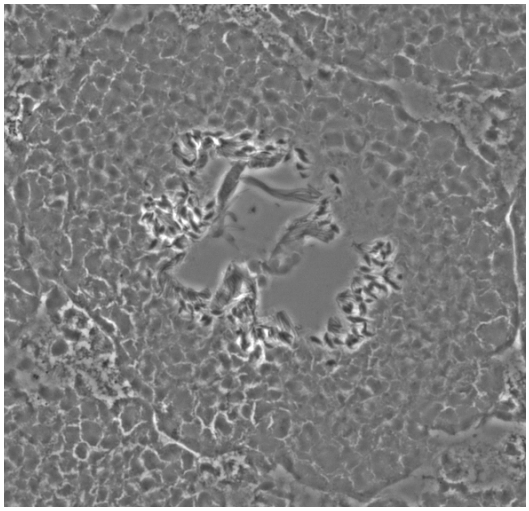

Figure 3: Permeabilized Tubule

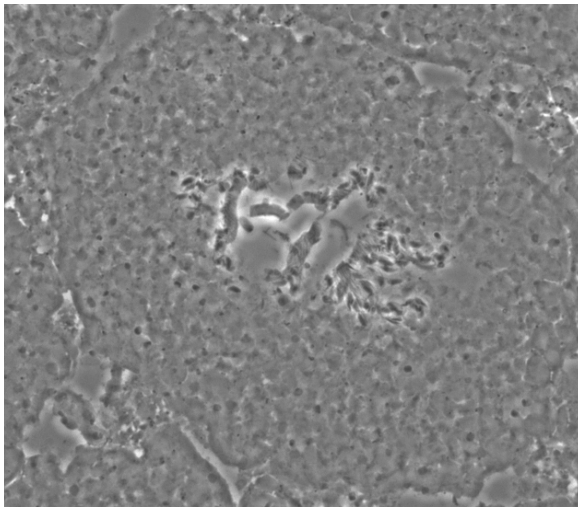

Figure 4: Unpermeabilized Tubule

In the permeabilized tissue parts of the plasma membrane have been washed away, leaving gaps in the tissue. This disrupted cell membrane can be unfavorable depending on the experimental goals. Studies that require unpermeabilized tissue typically aim to maintain the integrity of the tissue by limiting the amount of treatments and washes the tissue undergoes as it

can change the way staining will appear. Additionally, permeabilization can remove membrane-associated proteins.

Hoechst and SCP3

As an alternative method for staging seminiferous tubules, we targeted a different subpopulation of cells rather than acrosomes. Using Synaptinimal Complex Protein 3 (SCP3), a protein complex used in meiosis that facilitates genetic recombination, we were able to stain the spermatocytes of seminiferous tubules. Combined with the nuclear stain Hoechst, SCP3 provided sufficient detail to accurately stage seminiferous tubules without the need for tissue permeabilization.

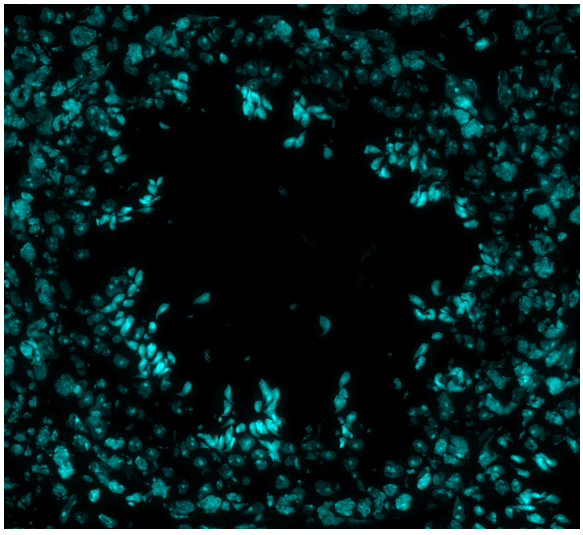

Figure 5: Hoechst

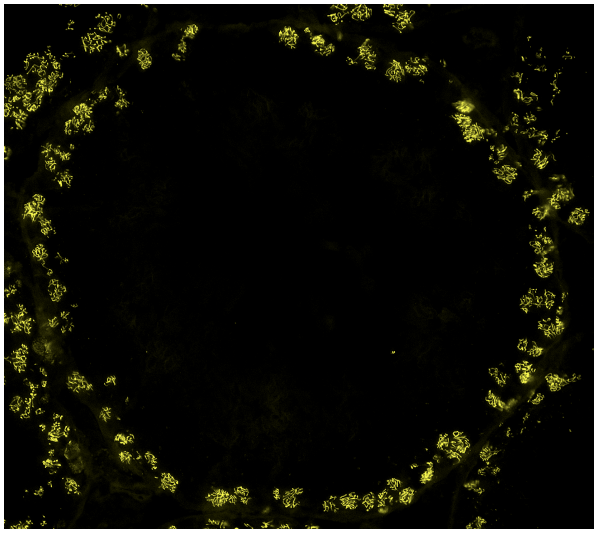

Figure 6: SCP3

Methods

Using a cryostat, 5 µm cross sections were obtained and stored at −80 °C. The following protocol was used to stain these sections with Hoechst and SCP3:

Staining buffer: 0.2M Tris-HCl pH 7.2 with 5% w/v PVA

(PVA = polyvinyl alcohol)

1. Prepare 200 uL staining solution per slide containing 5 ug/mL Hoechst, 5 ug/mL SCP3 488, and 5 ug/mL phalloidin 555
2. Defrost slides and draw a circle around tissue on the back of slide
3. Apply the staining solution to slides for 1 hour at room temperature in a light-protected container
4. Aspirate off staining solution
5. Perform 2 washes for 3 minutes each with staining buffer
6. Coverslip slides and let mountant cure for 1 hour at room temperature before imaging

Once cover slips were fully cured, microscopy images were captured at 40× magnification and imported into Fiji (ImageJ), where Hoechst and SCP3 images were stacked and colors were assigned to their respective channels, with Hoechst being cyan, and SCP3 being yellow.

Results

Because individual stages of spermatogenesis are not easily distinguishable, we categorized them into 3 broader groups: stages 1-5, stages 6-8, and stages 9-12. The following table shows the staging criteria for each group.

Stages	Description
1-5	Presence of smaller spermatocytes, round spermatids, and condensed elongated spermatids in bundles
6-8	Presence of larger spermatocytes, round spermatids and elongated Spermatids begin to align or are aligned at the luminal edge

9-12	Absence of round spermatids. Presence of less condensed elongated spermatids, and two SCP3+ cell types.

Table 1: Staging Criteria

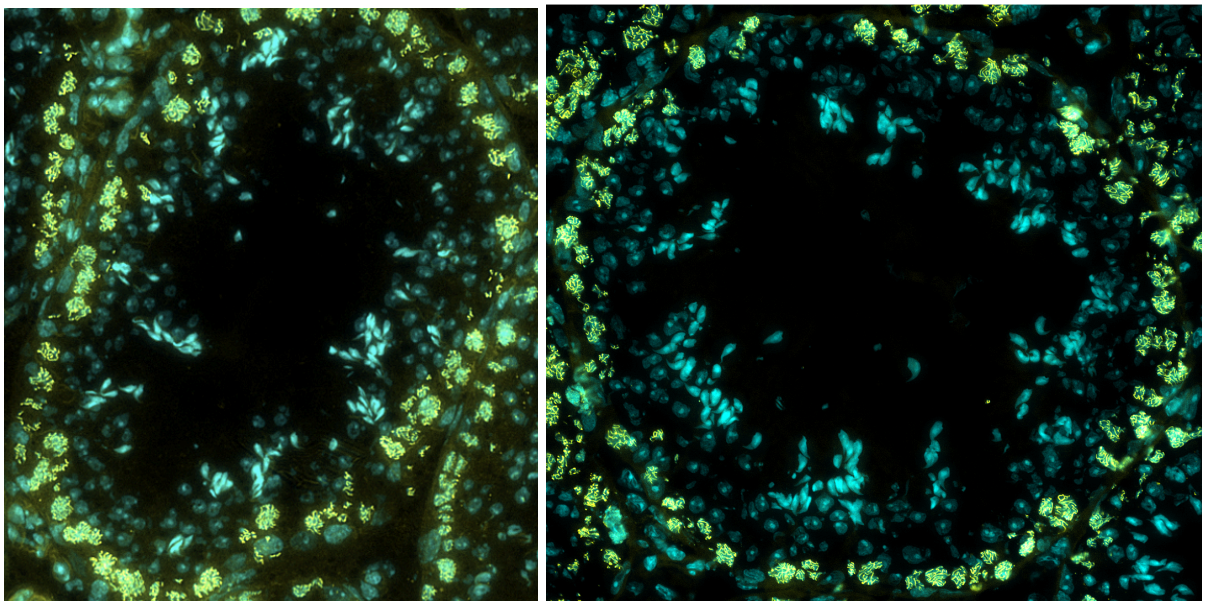

Figure 7: Stages 1-5 Examples

In these images there are distinct condensed bundles of elongated spermatids, presences of round spermatids, and smaller spermatocytes.

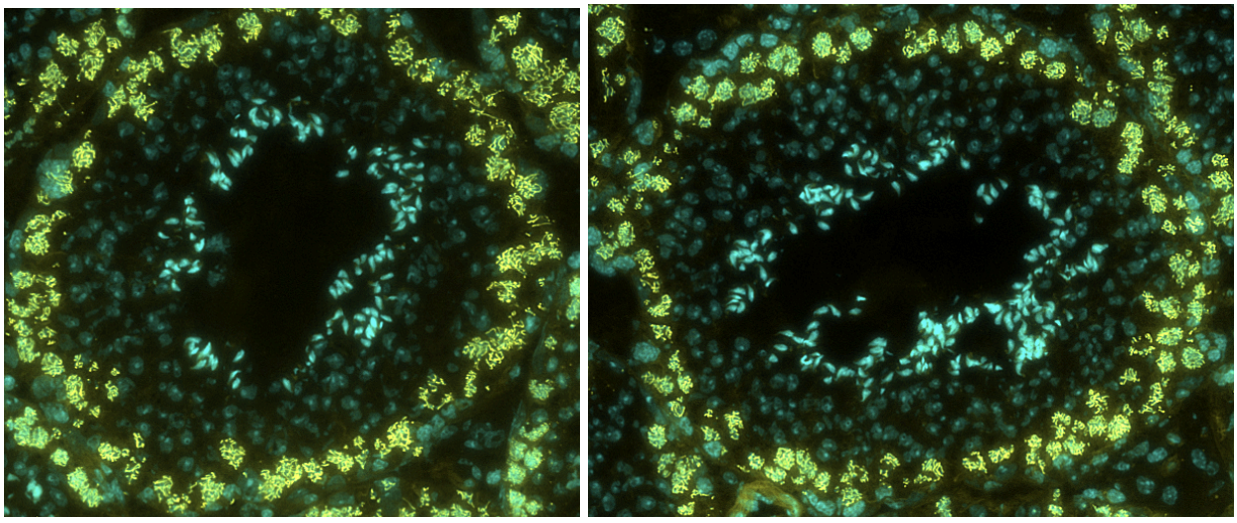

Figure 8: Stages 6-8 Examples

These images show condensed elongated spermatids aligned along the luminal edge, no longer in bundles, presence of round spermatids, and large spermatocytes.

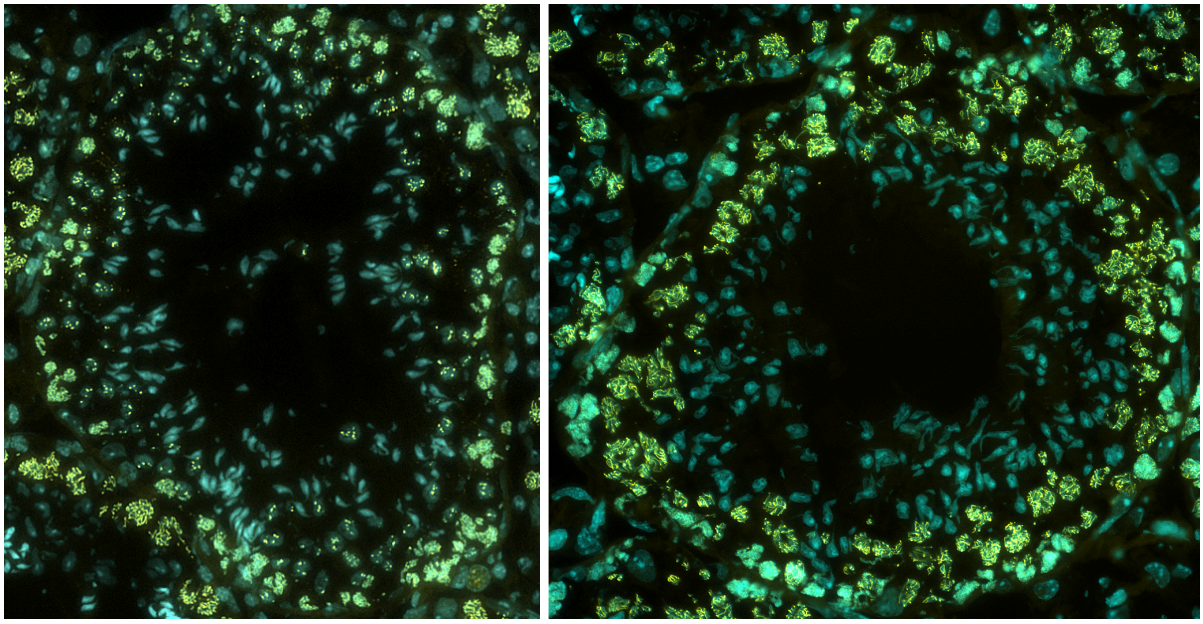

Figure 9: Stages 9-12 Examples

These images show less condensed elongated spermatids, large spermatocytes, no presence of round spermatids, and 2 SCP3+ cell types.

Discussion/Conclusion

Staging tubules allows us to track the development of male germ cells, which is essential for studying the seminiferous epithelium. Because some experiments require minimal handling of tissue, traditional staging methods that rely on permeabilization are not ideal. Using SCP3 along with the nuclear stain Hoechst, we can label and stage a different subpopulation of cells (spermatocytes) without permeabilizing the tissue.

By targeting spermatocytes, this alternative approach shifts away from the traditional method of using PNA lectin to target acrosomes, and relies on the meiotic cell population and nuclear morphology. By preserving the integrity of the cell membrane, this approach is suitable

for applications in which tissue fixation/permeabilization may affect experimental results.

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