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Title: Transillumination-Assisted Dissection of Specific Stages of the Mouse Seminiferous Epithelial Cycle for Downstream Immunostaining Analyses

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **Y, Zeiss Stemi SV 6 which would be much preferred, or if not possible then Olympus SZX9.**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **N**

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

    ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **46**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Juho-Antti Mäkelä**: This method facilitates the identification and dissection of stage-specific seminiferous tubule segments, allowing the study of very specific steps in spermatogenesis **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Noora Kotaja**: Specific stages of the seminiferous epithelial cycle can be recognized in fresh, non-fixed tubules, allowing selection of only the stages of interest for downstream analysis **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Sheyla Cisneros-Montalvo**: It requires some practice to learn to recognize the stages on the basis of their transillumination patterns, but this protocol should help ensure your success **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Introduction of Demonstrator on Camera

1.4. **Noora Kotaja**: Demonstrating the procedure will be Opeyemi Olotu, a PhD candidate from my laboratory **[1][2]**.

1.4.1. INTERVIEW: Author saying the above

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera

Ethics Title Card

1.5. Procedures involving animal subjects have been approved by the National Animal Experiment Board (ELLA) and Regional State Administrative Agency for Southern Finland.

Protocol

2. Testis Dissection and Preparation

2.1. Begin by placing an at least 8-week-old, adult, male mouse in the supine position **[1-TXT]** and disinfecting the ventral abdomen with 70% ethanol **[2]**.

2.1.1. WIDE: Talent placing mouse into position *Videographer: More Talent than mouse in shot* **TEXT: Euthanasia: CO_2 asphyxiation**

2.1.2. Talent spraying/wiping abdomen, with ethanol container visible in frame

2.2. Use sterile scissors to make a V-shaped incision in the abdominalpelvic region **[1]** and use sterile forceps to pull on the epididymal fat pad to locate the testes **[2]**.

2.2.1. Incision being made

2.2.2. Fat pad being pulled/testes being located

2.3. Use scissors to dissect the testes **[1]**, placing the tissues in a sterile 100-millimeter Petri dish with PBS as they are harvested **[2]**.

2.3.1. Testes being dissected

2.3.2. Talent placing the testes into dish

2.4. Use fine-tipped scissors to cut a slit in the tunica albuginea, the thick fibrous sheet encapsulating the testis **[1]** and use forceps to tear the tunica open **[2]** and to force the tubules out **[3-TXT]**.

2.4.1. Slit being cut

2.4.2. Tunica being opened

2.4.3. Tubules being pressed/forced **TEXT: Discard tunica without damaging tubules**

2.5. Place the decapsulated seminiferous tubules into a new Petri dish **[1]** and add enough sterile PBS to cover the bottom of the dish **[2]**.

2.5.1. Talent placing tubules into dish

2.5.2. Talent adding PBS to dish, with PBS container visible in frame

2.6. Then use the forceps to gently pull the tubules apart without damaging the tissues **[1]**.

2.6.1. Tubules being pulled apart *Videographer: Important step*

3. Transillumination-Assisted Microdissection

3.1. For dissection of the tubules, tape the dish onto a dissecting microscope stage **[1]** and turn on the transillumination **[2]**.

3.1.1. WIDE: Talent taping dish

3.1.2. Talent turning on lower light Author NOTE: 2 alternative shots of this: showing the light turning on below the dish or showing the talent turning the light on from the on/off switch

3.2. Use fine forceps to carefully move **[added]** the bundles of tubules to get acquainted with the light absorption and scatter patterns of the different stages **[1]**.

Added shot: An undisturbed testis is being shown through the microscope with lower magnification. This should be shown first

3.2.1. SCOPE: Tubules being moved

3.3. Then use forceps with a hooked tip to lift a tubule of interest **[1]** and use microdissection scissors to cut a segment of an appropriate length **[2]**.

3.3.1. SCOPE: Tubule being lifted and tubule segments being cut NOTE: Use final take *Videographer: Important step*

4. Squash Preparation and Stage Verification

4.1. After cutting the stage-specific tubule segments, use a pipette to collect a segment of interest in a 10-microliters volume of PBS **[1]** and place the segment onto a microscope slide **[2]**.

4.1.1. Talent collecting segment *Videographer: Important step*

4.1.2. Talent adding segment to slide *Videographer: Important step*

4.2. Carefully press a 20- x 20-millimeter cover glass onto the tubule without squashing the cells too much **[1]** and place the slide onto the stage of a phase-contrast microscope **[2]**.

4.2.1. Talent placing cover glass onto tubule *Videographer: Important step*

4.2.2. Talent placing slide onto stage

4.3. Place a filter paper onto the edge of the cover glass to facilitate the spreading of the cells into a monolayer **[1]** and use the 40x objective to verify the cell stages **[2-TXT]**.

4.3.1. Filter paper being placed at edge/cells forming monolayer *Videographer: Important step*

4.3.2. SCOPE: Shot of cells *Videographer: Important step* **TEXT: See text for cell staging information**

Added shot: EXTRA SHOT with higher magnification to show the formed monolayer of cells

Author NOTE: IF USED TOGETHER

use 4.3.2. shot 6 together with EXTRA SHOT 1

OR

use 4.3.2. shot 8 together with EXTRA SHOT 2 or 3

4.4. Once the cells have formed a round monolayer from both ends of the tubule, use forceps to dip the slide into a container of liquid nitrogen for 10 seconds **[1-TXT]**.

4.4.1. Talent dipping slide into LN2 **TEXT: Alternative: Freeze slide on dry ice plate**

4.5. Use a scalpel to flip the cover glass off of the cells **[1]** and immediately place the slide in 90% ethanol for 2-5 minutes **[2]**.

4.5.1. Cover glass being flipped off

4.5.2. Talent placing slide into ethanol.

4.6. Then air-dry and store the slide at room temperature for up to several days **[1-TXT]**.

4.6.1. Talent placing slide at room temperature **TEXT: Store long-term at -80 °C or use for immunostaining**

5. Whole-Mount Seminiferous Tubule Immunostaining

5.1. For whole-mount immunostaining of the seminiferous tubules, use a pipette to transfer segments of interest into a 15-milliliter conical tube of ice-cold PBS on ice **[1]**. After allowing the segments to sediment, carefully remove the PBS **[2]**.

5.1.1. WIDE: Talent adding tubules to tube and placing on ice to sediment

5.1.2. Talent removing PBS

5.2. Add 10 milliliters of fresh ice-cold PBS **[1]** and mix by inversion **[2]**.

5.2.1. Talent adding PBS to tube, with PBS container visible in frame

5.2.2. Talent inverting tube

5.3. Allow the tubules to settle for a few minutes **[1]** before removing the supernatant again as demonstrated **[2]**.

5.3.1. Talent placing tube on ice

5.3.2. Supernatant being removed

5.4. When all of the supernatant has been removed, fix the tubules in 5 milliliters of paraformaldehyde for 5 hours at 20-30 revolutions per minute at 4 degrees Celsius **[1-TXT]**.

5.4.1. Talent adding PFA and placing tube onto rotator, with PFA container visible in frame **TEXT: Fix at 4 degrees Celsius**

5.5. At the end of the incubation, allow the tubules to settle for a few minutes **[1]** before washing the sample three times in fresh PBS at 4 degrees Celsius for 10 minutes per wash with rotation **[2]**.

5.5.1. Talent placing tube on ice

5.5.2. Talent removing PFA and adding PBS to tube, with rotator and PBS container visible in frame

5.6. After the last wash, use a 1-milliliter pipette to transfer 10-20 fixed tubule segments into a 2-milliliter, round-bottom tube **[1]**.

5.6.1. Talent adding segments to tube

5.7. When the segments have settled, replace the supernatant with 1 milliliter of 2% BSA (B-S-A) and 10% FBS (F-B-S) in PBSX (P-B-S-X) for 1-hour incubation at room temperature and 20-30 revolutions per minute **[1-TXT]**.

5.7.1. Talent placing tube onto rotator, with 2% BSA + 10% FBS in PBSX container visible in frame **TEXT: BSA: bovine serum albumin; FBS: fetal bovine serum; PBSX: 0.3% Triton X-100 in PBS**

5.8. At the end of the blocking incubation, wash the samples with 1 milliliter of PBSX **[1]** before labeling the tubules with 250 microliters of primary antibody diluted in 1% BSA in PBSX for 2 hours at room temperature at 20-30 revolutions per minute **[2-TXT]**.

5.8.1. Talent adding PBSX to tube, with PBSX container visible in frame
5.8.2. Talent adding antibody to tube, with antibody containers visible in frame **TEXT: See text for Ab suggestion and preparation details**

5.9. At the end of the incubation, wash the tubules three times in PBSX as demonstrated **[1]** before labeling the samples with 250 microliters of the appropriate secondary antibodies for 1 hour protected from light at room temperature and 20-30 revolutions per minute **[2]**.

5.9.1. Tube rotating on rotator, with PBSX container visible in frame as possible
5.9.2. Talent adding antibody to tube, with antibody containers visible in frame

5.10. At the end of the incubation, wash the tubules three times **[1]** before decanting the tubules onto a microscope slide in a small volume of supernatant **[2]**.

5.10.1. Talent adding PBSX to tube, with PBSX container visible in frame *Videographer: Important step*
5.10.2. Shot of tubules in small volume of PBSX, then tubules being decanted onto slide NOTE: This might be in 2 separate shots *Videographer: Important step*

5.11. Then drain the excess buffer **[2]** and use gel-loading tips to arrange the tubules into linear strips **[1]** before mounting the slide with a coverslip **[3]**.

5.11.1. Tubules being arranged NOTE: switch the order of 5.11.1 and 5.11.2. *Videographer: Important step*
5.11.2. Buffer being removed *Videographer: Important step*
5.11.3. Coverslip being placed onto mounting medium on slide *Videographer: Important step*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.6., 3.2., 3.3., 4.1., 4.2., 4.3., 5.10., 5.11.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
3.2. The most difficult aspect is to learn to identify the differences in light absorption/scatter pattern between the stages of epithelial cycle. To ensure success one has to find proper lighting conditions but it also takes some dedication to learn to see the differences.

Results

6. Results: Representative Mouse Seminiferous Epithelial Cycle Staging

6.1. Under transillumination **[1]**, stages 7-8 appear homogenously dark, because they contain a high number of fully condensed elongating spermatids that are aligned at the apical surface of the epithelium **[2]**.

6.1.1. LAB MEDIA: Figure 5A
6.1.2. LAB MEDIA: Figure 5A *Video Editor: please emphasize dark zone sections of image*

6.2. After mature spermatozoa are released into the lumen during spermiation **[1]**, the tubule appears very pale at stages 9-10 due to the absence of condensed elongating spermatids within the epithelium **[2]**.

6.2.1. LAB MEDIA: Figure 5A *Video Editor: please emphasize asterisks*
6.2.2. LAB MEDIA: Figure 5A *Video Editor: please emphasize pale zone sections*

6.3. This pale zone is followed by the weak spot zone **[1]**, the spotty appearance of which originates from the organization of elongating spermatids with condensed chromatin in bundles **[2]**.

6.3.1. LAB MEDIA: Figure 5A *Video Editor: please emphasize weak spot section*
6.3.2. LAB MEDIA: Figure 5A

6.4. These bundles become very prominent in the subsequent strong spot zone **[1]**.

6.4.1. LAB MEDIA: Figure 5A *Video Editor: please emphasize strong spot section*

6.5. Spermatid bundles migrate towards Sertoli cell nuclei that are located close to the basal lamina **[1]**, which is reflected as a striped appearance of stage two to five tubules when transilluminated **[2]**.

6.5.1. LAB MEDIA: Figure 5B
6.5.2. LAB MEDIA: Figure 5B *Video Editor: please emphasize II-V strong spot image*

6.6. The bundles finally disperse at stage six and condensing elongating spermatids move close to the lumen to be released from the epithelium at stage eight **[1]**.

6.6.1. LAB MEDIA: Figure 5B *Video Editor: please emphasize VII-VIII dark zone image*

6.7. The exact stage of the tubule segment can be accurately verified by phase-contrast microscopy of the squash preparations **[1]**.

6.7.1. LAB MEDIA: Figure 2 Squash prep image row

6.8. Immunostaining of staged squash preparations can be used to study the expression and localization of proteins of interest in the seminiferous epithelium **[1]**.

6.8.1. LAB MEDIA: Supplementary Figure 3

6.9. Seminiferous tubule whole-mount staining **[1]** is typically used to study the cell types that are in contact with the basement membrane of the seminiferous epithelium, either on the tubular **[2]** or interstitial side **[3]**.

6.9.1. LAB MEDIA: Figure 6
6.9.2. LAB MEDIA: Figure 6 *Video Editor: please emphasize Figures 6A and 6B*
6.9.3. LAB MEDIA: Figure 6 *Video Editor: please emphasize Figure 6C*

6.10. The method can also be used to study cells or structures that are located deeper within the epithelium **[1]**, such as the blood-testis barrier **[2]** or postmeiotic germ cells **[3]**.

6.10.1. LAB MEDIA: Supplementary Figure 4
6.10.2. LAB MEDIA: Supplementary Figure 4 *Video Editor: please emphasize Figure S4B*
6.10.3. LAB MEDIA: Supplementary Figure 4 *Video Editor: please emphasize Figure S4C*

Conclusion

7. Conclusion Interview Statements

7.1. **Juho-Antti Mäkelä**: In addition to immunostaining, we can use staged tubule segments for many other downstream analyses, including biochemical RNA and protein assays, flow cytometry, and *ex vivo* tubule culture **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

7.2. **Noora Kotaja**: This method allows a highly detailed analysis of spermatogenesis and therefore helps to answer very specific questions about the critical processes required for the production of sperm and male fertility **[1]**.

7.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera