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Title: Serial Block-Face Scanning Electron Microscopy (SBF-SEM) of Biological Tissue Samples

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Author Questionnaire

1. **Microscopy**: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **Y, Meiji EMZ-5. 2: MOTIC SMZ-140**

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.

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

Protocol Length
Number of Shots: **37**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Justin Courson**: Serial block-face scanning electron microscopy provides an unprecedented view of three-dimensional tissue ultrastructures and can answer a host of questions previously impossible, or exceedingly difficult, to pursue **[1]**.

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

REQUIRED:

1.2. **Alan Burns**: This technique allows the rapid and reproducible production of three-dimensional data sets with minimal tissue charging and artifacts and allows the automated capture of thousands of images daily **[1]**.

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

OPTIONAL:

1.3. **Justin Courson**: Standard transmission electron microscopy provides excellent cellular ultrastructure. However, these images lack three-dimensional context and can be difficult to interpret. SBF-SEM provides three-dimensional context, allowing the interpretation of complex processes **[1]**.

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

OPTIONAL:

1.4. **Alan Burns**: While this protocol is reliable and reproducible, certain steps require precision and are critically important for the success of this methodology. This video will demonstrate these critical steps in detail **[1]**.

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

Protocol

2. Tissue Processing

2.1. Begin by sequentially staining the tissue with osmium ferrocyanide **[1]**, thiocarbohydrazide **[2]**, and osmium tetroxide **[3-TXT]**. Wash the tissue three times between each staining **[1a, 2a, or 3a]**. NOTE: Additional shots 2.1.1a, 2.2.2a and 2.3.3a are added. VO narration was modified to accommodate the shots. I would just show one of the added shots at the end.

2.1.1. WIDE: Talent removing tissue from osmium ferrocyanide, with osmium ferrocyanide container visible in frame

2.1.1a Added: Washing three times

2.1.2. Tissue being placed into thiocarbohydrazide, with thiocarbohydrazide container visible in frame

2.1.2a Added: Washing three times

2.1.3. Tissue being placed into osmium tetroxide, with osmium tetroxide container visible in frame **TEXT: See text for full staining and all solution preparation details**

2.1.3a Added: Washing three times

2.2. After osmium tetroxide incubation, treat the tissue with 1% aqueous uranyl acetate **[1]** at 4 degrees Celsius overnight **[1a]**.

2.2.1. WIDE: Talent placing tissue into uranyl acetate, with UA container visible in frame

2.2.1a Added shot: Talent placing tissue into 4 degrees Celsius fridge

2.3. The next morning, dissolving 0.066 grams of lead nitrate in 10 milliliters of 0.03-molar aspartic acid solution **[1]** and use 1-Normal potassium hydroxide to adjust the pH of the Walton's lead aspartate solution to 5.5 **[2]**.

2.3.1. Talent dissolving lead nitrate to acid, with nitrate and acid containers visible in frame

2.3.2. Talent adding KOH to solution, with KOH container visible in frame, and testing pH.

2.4. After warming the solution in oven [a], wash the tissue five times for 3 minutes per wash in room temperature double distilled water **[1]** before transferring the tissue into 60-degree Celsius-warmed Walton's lead aspartate solution [2] for 30 minutes at 60 degrees Celsius **[2a]**.

2.4a Added shot: Talent warming solution in lab oven

2.4.1. Talent washing tissue

2.4.2. Talent placing tissue into solution, with solution container visible in frame

2.4.2a Added: Talent places tissue into 60 degree Celsius oven

2.5. At the end of the incubation, wash the tissue **[5a]** and dehydrate it in an ascending ice-cold acetone series **[1-TXT]** followed by a 10-minute incubation in room temperature 100% acetone **[2]**.

2.5a Added: Washing the tissue

2.5.1. Talent placing tissue in 30% acetone, with acetone series visible in frame **TEXT: i.e., 30%, 50%, 70%, 95% x2, 100% x3**

2.5.2. Talent placing tissue into 100% acetone, with acetone container visible in frame

2.6. Then, embed tissue in a 1 is to 3 ratio of hard mixed resin and acetone for 4 hours **[1]** on rotating platform **[1a]**, followed by an 8-hour or overnight incubation in a 1:1 ratio of resin to acetone solution **[2]** on the rotating platform **[2a]**, then an overnight incubation in a 3 is to 1 ratio of resin to acetone solution **[3]** on the rotating platform **[3a]**.

2.6.1. Talent placing tissue into 1:3 solution, with acetone and resin containers visible in frame *Videographer: Important/difficult step*

2.6.1a Added: Tissue is placed on rotating platform.

2.6.2. Talent placing tissue into 1:1 solution, with acetone and resin containers visible in frame *Videographer: Important/difficult step*

2.6.2a Added: Tissue is placed on rotating platform.

2.6.3. Talent placing tissue into 3:1 solution, with acetone and resin containers visible in frame *Videographer: Important/difficult step*

2.6.3a Added: Tissue is places on rotating platform.

2.7. The next morning, incubate the tissue in fresh 100% resin for one 4-8-hour **[1]**, one overnight, and one 4-hour incubation at room temperature on rotating platform **[1a]**.

2.7.1. Talent placing tissue into resin, with resin container visible in frame

2.7.1a Added: Tissue is places on rotating platform.

2.8. The next morning, use a wooden stick to mix a small amount of resin with carbon black powder until the resin is saturated with the powder but is still fluid and does not become grainy **[1]**. The resin should resemble thick ink and be able to slowly drip without visible clumps **[2]**.

2.8.1. Resin being mixed, with resin and carbon black containers visible in frame

2.8.2. Shot of black ink-looking resin dripping from stick. NOTE: This one shot is mis-stated as 2.7.1 by the videographer

2.9. Place the tissue sample in a silicone rubber mold **[1]** and capture an image for later reference of the sample orientation within the resin block **[2]**.

2.9.1. Tissue being oriented

2.9.2. Talent taking picture

2.10. When the sample is in place, cover the tissue in the carbon black saturated resin at the tip of the silicone mold with label indicating the experimental and tissue details in the mold at the opposite end of the resin **[1]**.

2.10.1. Resin being added to mold

~~2.10.2. Label being placed~~

2.11. Then place the mold into a 65-degree Celsius oven at an incline for approximately 1 hour **[1]**.

2.11.1. Talent placing mold into oven

2.12. When the carbon black-infused resin has sufficiently cured, remove the mold from the oven **[1]** and, beginning with an empty well, fill the remainder of the mold with clear resin, taking care that the label remains visible **[2-TXT]**.

2.12.1. Talent removing mold from oven

2.12.2. Mold being filled with clear resin **TEXT: If carbon black resin begins to bleed into clear resin, cure sample for an additional 15 min**

2.13. When the sample has been covered with clear resin, place the silicone mold back into the 65-degree Celsius oven not on an incline for 48 hours **[1]**, then remove the mold **[2]**.

2.13.1. Talent placing mold into oven

2.13.2. Added: Talent removing mold from oven

3. Block Preparation

3.1. To prepare the block for imaging, place the resin-embedded specimen onto a microtome chuck with the tapered end sticking approximately 5-6 millimeters out of the chuck **[1]**.

3.1.1. WIDE: Talent placing specimen onto chuck NOTE: Single shot with 3.2.1

3.2. Lock the specimen in place with the set screw **[1]** and place the specimen under a heat lamp **[2]**.

3.2.1. Talent locking specimen in place NOTE: Shot with 3.1.1 by the videographer

3.2.2. Talent placing specimen under heat lamp NOTE: This is labelled as 3.2.1 for take 1 by Videographer

3.3. After several minutes, place the chuck into a stereomicroscope holder **[1]** and use a new double-edged razor blade to make thin sections parallel to the block face until the tissue is visible **[2]**, which will be less reflective and granular compared to the portions of the resin that are devoid of tissue **[3-TXT]**.

3.3.1. Talent placing chuck into holder *Videographer: Important step*

3.3.2. SCOPE: Sections being made *Videographer: Important step*

3.3.3. SCOPE: Shot of less reflective and granular section of resin *Videographer: Important step* **TEXT: Use sample image for tissue location reference**

3.4. Secure an aluminum specimen pin in the trimming pin holder **[1]** and make several deep, crisscrossing scratches in the face of the pin to provide a larger surface area for the glue used to hold the specimen in place **[2]**.

3.4.1. SCOPE: Talent securing pin in trimming pin holder *Videographer: Important step*

3.4.2. SCOPE: Pin face being scratched *Videographer: Important step*

3.5. Place the specimen under heat lamp **[0]** and push the razor 1-2 millimeters straight down into the resin block **[1]** before making a second, perpendicular cut of equal depth into the block **[2]** to trim away the excess resin from the tissue sample to that the block is approximately 3 millimeters in diameter by 2-3 millimeters in height **[3]**.

3.5.0 *Reuse 3.2.2*.

3.5.1. SCOPE: Razor being pressed into block *Videographer: Important step*

3.5.2. SCOPE: Razor being pressed into block *Videographer: Important step*

3.5.3. Added SCOPE: Excess resin being trimmed.

3.6. After this initial trimming, warm the block under the heat lamp again **[1]** and use a new double-edged razor blade to cut off the top of the resin block roughly 1 millimeter below the trimmed portion in a single smooth cut **[2]**.

3.6.1. Talent placing block under lamp

3.6.2. SCOPE: Top of block being removed

3.7. Place the trimming pin holder containing the cut aluminum pin in the stereomicroscope receptacle **[1]** and apply a thin layer of cyanoacrylate glue to the pin face such that it completely covers the pin without forming a visible meniscus **[2]**.

3.7.1. SCOPE: Talent placing holder into receptacle NOTE: Single shot till 3.8.1

3.7.2. SCOPE: Glue being applied NOTE: Single shot from 3.7.1 to 3.8.1

3.8. Use forceps to press the trimmed piece of tissue block onto the center of the specimen pin face for several seconds and allow the glue to set for several minutes **[1]**.

3.8.1. SCOPE: Tissue being pressed into pin face/glue setting NOTE: Single shot from 3.7.1 to 3.8.1

3.9. When the glue has thoroughly dried, locate the tissue on the raised portion of the resin block and use a double-edged razor to trim the raised portion of the resin containing the tissue sample to an area no larger than 1-square millimeter **[1]**.

3.9.1. SCOPE: Shot of tissue, then raised portion being trimmed *Videographer: Important step*

3.10. Slowly and carefully remove as much excess resin as possible, leaving the block slightly longer in one dimension **[1]** and use a final, metal file to angle the excess resin in the area outside of the raised portion containing the tissue sample down toward the edge of the pin **[2]**.

3.10.1. SCOPE: Resin being removed *Videographer: Important/difficult step* NOTE: Single shot for 3.10.1 and 3.10.2

3.10.2. SCOPE: Resin being angled *Videographer: Important/difficult step* NOTE: Single shot for 3.10.1 and 3.10.2

3.11. Remove resin particles and dust from the prepared sample before applying a thin coat of silver paint **[2-TXT]** and gold sputtering to the entire sample block surface **[3]**.

~~3.11.1. SCOPE: Particles/dust being removed~~

3.11.2. SCOPE: Silver paint being applied **TEXT: Dry paint at RT O/N**

3.11.3. SCOPE: Shot of gold sputtered block surface

3.12. After coating, trim any excess silver paint from the block face surfaces **[1]** and place the mounted and trimmed block in the bulbous end of a custom-made transfer pipette tube **[12x]** with the appropriate label attached **[2]**. NOTE: One shot 3.12x is added, VO narration is rearranged to accommodate the shot.

3.12.1. SCOPE: Paint being trimmed *Videographer: Important step* NOTE: Probably combined with 3.11.2

3.12x Added: Storage tube being made from transfer pipette.

3.12.2. Block being placed into tube and label being attached *Videographer: Important step* NOTE: Probably combined with 3.11.3

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.6., 3.3.-3.5., 3.9., 3.12.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
2.6. It is important not to rush this step and to ensure COMPLETE mixing of carbon black and resin, and to have a clear idea of the desired consistency.
3.10. This step can be tricky to do, and if done incorrectly can damage or destroy the tissue block. It is important to maintain a steady hand, work slowly, and maintain a clear view of the block during this step.

Results

4. Results: Representative Serial Block-Face Scanning Electron Microscopy (SBF-SEM) Imaging

4.1. Using SBF-SEM (S-B-F-S-E-M) imaging **[1]**, a network of elastin-free microfibril bundles have been identified within the adult mouse cornea **[2]**.

4.1.1. LAB MEDIA: Figure 5
4.1.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize white fibrils in Figure 5A*

4.2. This network is organized in distinct layers **[1]**, with the fibers closely associated with keratocytes, even lying within shallow invaginations on the keratocyte surface **[2]**.

4.2.1. LAB MEDIA: Figure 5 *Video Editor: please emphasize fibrils in Figure 5B*
4.2.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize arrows in Figure 5A/fibrils within green and orange/yellow grooves in Figure 5A*

4.3. Application of this protocol **[1]** has led to the discovery of a previously unknown population of central corneal nerves **[2]** that fuse with basal epithelial cells at the stromal-epithelial border **[3]**.

4.3.1. LAB MEDIA: Figure 6
4.3.2. LAB MEDIA: Figure 6 *Video Editor: please emphasize purple nerves*
4.3.3. LAB MEDIA: Figure 6 *Video Editor: please emphasize green cell layer*

4.4. SBF-SEM imaging of the central cornea **[1]** reveals the presence of limbal vasculature **[2]**, nerve bundles **[3]**, and associated cells **[4]** that can be manually segmented for 3D reconstruction **[5]**.

4.4.1. LAB MEDIA: Figures 7A and 7B
4.4.2. LAB MEDIA: Figures 7A-7C *Video Editor: please emphasize red vessel in Figure 7C*
4.4.3. LAB MEDIA: Figures 7A-7C *Video Editor: please emphasize blue nerve bundle in Figure 7C*
4.4.4. LAB MEDIA: Figures 7A-7C *Video Editor: please emphasize yellow and magenta cells in Figure 7C*

4.5. Some pitfalls of this imaging procedure include **[1]** using a too long pixel dwell time, resulting in a wavy and distorted image **[2]**, small discharges of electrons from the

block-face, leading to rapid contrast changes and lines **[3]**, knife scratches on the block face due to a damaged knife or debris accumulation on the edge of the knife **[4]** …

4.5.1. LAB MEDIA: Figure 10
4.5.2. LAB MEDIA: Figure 10 *Video Editor: please emphasize Figure 10A*
4.5.3. LAB MEDIA: Figure 10 *Video Editor: please emphasize white arrow in Figure 10B*
4.5.4. LAB MEDIA: Figure 10 *Video Editor: please emphasize black arrows in Figure 10C*

4.6. … artefacts from an extended electron beam focus on the block face while the sample is still in the imaging chamber **[1]**, improper tissue fixation, leading to the separation of cellular structures and connective tissue **[2]**, and image skipping as a result of a large amount of charging within the tissue or resin block **[3]**.

4.6.1. LAB MEDIA: Figure 10 *Video Editor: please emphasize black arrow in Figure 10D*
4.6.2. LAB MEDIA: Figure 10 *Video Editor: please emphasize black arrow in Figure 10E*
4.6.3. LAB MEDIA: Figure 10 *Video Editor: please emphasize black arrows in Figure 10F*

Conclusion

5. Conclusion Interview Statements

5.1. **Justin Courson**: When cutting the tissue-block, it is important to have a good understanding of where the tissue lies within the block so as to not accidentally trim any important sample regions **[1]**.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.3., 3.5.)

5.2. **Alan Burns**: If higher-resolution images of specific structures of interest, cellular interactions, or rare events are desired, imaging can be halted and sections can be cut from the block-face for transmission electron microscopy imaging **[1]**.

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

5.3. **Justin Courson**: SBF-SEM allows a wholly unique view of biological tissues. Using this protocol, we have been able to rapidly acquire quality datasets and to expand the breadth of tissues we can explore **[1]**.

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