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Title: Imaging of Podocytic Proteins Nephrin, Actin, and Podocin with Expansion Microscopy

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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 something similar? **NO**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**
- 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 videographer steps away (≥ 6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, 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? **No**

Current Protocol Length

Number of Steps: 19

Number of Shots: 46

Introduction

1. Introductory Interview Statements

REQUIRED:

- 1.1. **Eva Königshausen**: This expansion microscopy protocol enables the investigator to visualize glomerular proteins with nanoscale resolution on a conventional microscope.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.
- 1.2. **Theresa Schmitz**: The main advantage of expansion microscopy is that it is accessible to a large research community.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Preparation for Expansion Microscopy

- 2.1. Begin by making spacers for the gelation chamber [1]. Cut number 1 and number 1.5 glass cover slips into 5-millimeter stripes using a diamond knife [2-TEXT]. Place a glass slide into a staining chamber [3] and position the number 1.5 cover slip stripes on the glass slide so that they form a 2.5-centimeter square [4]. *Videographer: This step is important!*
 - 2.1.1. WIDE: Establishing shot of talent at the lab bench.
 - 2.1.2. Talent cutting a glass coverslip into stripes. **TEXT: 4 per glass slide**
 - 2.1.3. Talent places slide into a staining chamber.
 - 2.1.4. Talent positioning the stripes onto the slide into a square.
- 2.2. Pipette a droplet of double-distilled water into the corners of the square to adhere the glass cover slip stripes to each other and to the glass slide. Wait for approximately 20 minutes so that the number 1.5 cover slips are stably attached [1]. *Videographer: This step is important!*
 - 2.2.1. Talent pipetting water onto the corners of the square.
- 2.3. Pipet a droplet of water on each number 1.5 cover slip stripe [2]. Place four number 1 glass cover slip stripes onto the number 1.5 cover slips [1]. *Videographer: This step is important!*
 - 2.3.1. Talent placing the #1.0 cover slip stripes on the #1.5 cover slip. **NOTE: 2.3.2. should come before 2.3.1.**
 - 2.3.2. Talent pipetting water on a cover slip stripe.
- 2.4. For the gelation chamber lid, wrap a cover glass with paraffin film, avoiding any folds or dirt on the film [1].
 - 2.4.1. Talent wrapping a cover glass with paraffin film.

3. Anchoring and polymerization (gelation)

- 3.1. For anchoring treatment, remove PBS from the immune-stained cells [1] and add 250 microliters of anchoring buffer per well directly onto the glass cover slip. Incubate the plate for 3 hours at room temperature [2]. Keep the substrate in the dark using a box [3]. *Videographer: This step is difficult!*
 - 3.1.1. Talent removing PBS.
 - 3.1.2. Talent adding anchoring buffer to a well. **NOTE: May be added shot**
 - 3.1.3. Talent putting the substrate in a box.

- 3.2. After the incubation, remove the anchoring buffer [1] and wash the cover slip once with 1.5 milliliters of PBS per well [2].
 - 3.2.1. Talent removing the anchoring buffer from a few wells.
 - 3.2.2. Talent washing the coverslip with PBS.
- 3.3. To stain actin fibers, thaw an ExM compatible phalloidin-solution and incubate it for 45 minutes at room temperature [1]. Meanwhile, dissolve sodium acrylate in double-distilled water using a stirring device [2] and prepare the monomer solution on ice [3].
 - 3.3.1. Talent taking phalloidin out of the freezer.
 - 3.3.2. Talent dissolving sodium acrylate in water.
 - 3.3.3. Monomer solution on ice.
- 3.4. Remove phalloidin from the cells and wash them with 1.5 milliliters of PBS twice at room temperature [1]. Leave 1.5 milliliters of PBS within the well after the last wash [2]. Use forceps and a cannula to lift the cover glass slip from the 6-well plate and place it into the gelling chamber [3].
 - 3.4.1. Talent removing phalloidin and adding PBS to the cells.
 - 3.4.2. Wells with PBS.
 - 3.4.3. Talent lifting the cover glass and placing it in the gelling chamber.
- 3.5. Add APS to the gelling solution [1-TXT] and vortex briefly [2]. Pipette 200 microliters of gelling solution on the sample [3] and cautiously close the chamber, avoiding air bubbles within the gel [4]. Add water to the staining chamber [6] and incubate the staining chamber for at least 1 hour at 37 degrees Celsius to polymerize the gel [5].
 - 3.5.1. Talent adding APS to the gelling solution. **TEXT: APS – ammoniumperoxidsulfate**
 - 3.5.2. Talent vortexing the solution.
 - 3.5.3. Talent pipetting gelling solution on the sample.
 - 3.5.4. Talent closing the gelation chamber.
 - 3.5.5. Talent putting the chamber in the incubator.
 - 3.5.6. Talent adds water to the staining chamber. **NOTE: Move 3.5.6 after 3.5.4.**

4. Homogenization (digestion)

- 4.1. Take the gelation chamber out of the incubator [1]. To open the gelling chamber lid, introduce a razor blade between the lid and the spacer and remove the lid cautiously [2]. Remove the spacers with the razor blade [3] and cut off all extra gel [4].
Videographer: This step is important!

- 4.1.1. Talent taking the chamber out of the incubator.
- 4.1.2. Talent opening the gelation chamber.
- 4.1.3. Talent removing the spacers.
- 4.1.4. Talent cutting the extra gel.
- 4.2. **Use a paintbrush to detach the edges of the gel [0].** Put the slide with the gel and cover glass into a dish filled with PBS [1], then remove the detached cover glass from the gel by shaking it gently [2]. Put the slide below the gel to attach the gel to the slide [3]. *Videographer: This step is important!*
 - 4.2.0 **Added shot: Talent using a paintbrush to detach the edges of the gel.**
 - 4.2.1. Talent putting the slide in a dish with PBS.
 - 4.2.2. Talent shaking the dish.
 - 4.2.3. Talent putting the slide below the gel.
- 4.3. With the gel on the slide, divide the gel into small pieces using the razor blade [1]. Gently push one piece of gel into a well of a 6-well plate with a glass bottom [2] and enfold it with a paintbrush [3]. Use the paintbrush to keep the gel moisturized with a small amount of PBS [4]. *Videographer: This step is important!*
 - 4.3.1. Talent cutting the gel into small pieces.
 - 4.3.2. Talent pushing a piece of gel into the well of a plate.
 - 4.3.3. Talent enfolding the gel with a paintbrush.
 - 4.3.4. Talent wetting the gel.
- 4.4. Using an inverted microscope, take overview images with low numerical aperture for pre-expansion images [1].
 - 4.4.1. Talent imaging the gel.
- 4.5. Dilute Proteinase K to 4 units per milliliter in digestion buffer to make the digestion solution [1-TXT]. Add 500 microliters of the digestion solution to each well [2] and immerse the gel within the solution [3]. Let it digest overnight at room temperature with the lid closed to keep the samples in the dark [4]. *Videographer: This step is difficult!*
 - 4.5.1. Talent adding proteinase K to the digestion buffer. **TEXT: Digestion buffer without Proteinase K can be stored at 4 °C for 1–2 weeks**
 - 4.5.2. Talent adding digestion solution to a few wells.
 - 4.5.3. Talent immersing the gel in the solution.
 - 4.5.4. Talent closing the lid.

5. Expansion and imaging

- 5.1. Remove the digestion solution with a pipette and discard it [1]. Add 1 milliliter of double-distilled water and incubate the immersed gel for 10 minutes at room temperature [2].
 - 5.1.1. Talent removing the digestion solution.
 - 5.1.2. Talent adding water to the gel.
- 5.2. Remove the water [1] and add 1 milliliter of fresh double-distilled water. Continue exchanging water every 10 minutes until a plateau of expansion is reached [2], causing the gel to become optically clear [3-TXT].
 - 5.2.1. Talent removing the water.
 - 5.2.2. Added shot: Talent replacing with fresh water.
 - 5.2.3. Expanded gel. **TEXT: Up to 4.5-fold expansion is achievable**
- 5.3. Remove the water from the gel [0] and immediately start microscopy. Using an inverted microscope, use an air objective at low magnification to find cells in the pre-expansion state [1].
 - 5.3.0 Added shot: Talent removing water. Expanded gel. **NOTE: This one can also be used instead of 5.2.3.**
 - 5.3.1. Talent looking for cells at the microscope.
- 5.4. Switch to 40x and 63x objectives for better resolution. Excite with the wavelength of interest and take the image via the camera [1].
 - 5.4.1. Talent imaging the cells.

Results

6. Results: Podocin co-localizes with nephrin and actin

- 6.1. This ExM (*pronounce 'ex-M'*) protocol enables expansion of up to four-fold. To determine the expansion factor, it is essential to image cells before and after expansion [1-TXT].
 - 6.1.1. LAB MEDIA: Figure 3. *Video Editor: Label A "Before" and B "After".*
- 6.2. Insufficient anchoring and homogenization may lead to distortions and ruptures of cells [1]. Representative examples of ruptured cells are shown here [2].
 - 6.2.1. LAB MEDIA: Figure 4.
 - 6.2.2. LAB MEDIA: Figure 4. *Video Editor: Emphasize where the arrows are pointing.*
- 6.3. This method can be used to investigate the co-localization of F-actin and actin adaptor proteins, such as podocin and nephrin [1]. Podocin is depicted in green, actin in blue, and nephrin in red [2]. White areas indicate co-localization [3].
 - 6.3.1. LAB MEDIA: Figure 5. *Video Editor: Emphasize the podocin and nephrin images.*
 - 6.3.2. LAB MEDIA: Figure 5.
 - 6.3.3. LAB MEDIA: Figure 5. *Video Editor: Emphasize the white areas in the merge images.*

Conclusion

7. Conclusion Interview Statements

7.1. **Lorenz Sellin:** The fluorescent signal intensity and abundance are key for successful ExM. Efficient anchoring of the fluorescent dyes via AcX is of critical importance for this protocol. In our experience, solubilized AcX is good for up to 3-4 months.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.1.*

