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TITLE:

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

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KEYWORDS:

expansion microscopy, nephrin, actin, podocin, super resolution, proExM

SUMMARY:

The presented method enables visualization of fluorescently labeled cellular proteins with expansion microscopy leading to a resolution of 70 nm on a conventional microscope.

ABSTRACT:

Disruption of the glomerular filter composed of the glomerular endothelium, glomerular basement membrane and podocytes, results in albuminuria. Podocyte foot processes contain actin bundles that bind to cytoskeletal adaptor proteins such as podocin. Those adaptor proteins, such as podocin, link the backbone of the glomerular slit diaphragm, such as nephrin, to the actin cytoskeleton. Studying the localization and function of these and other podocytic proteins is essential for the understanding of the glomerular filter's role in health and disease. The presented protocol enables the user to visualize actin, podocin, and nephrin in cells with super resolution imaging on a conventional microscope. First, cells are stained with a conventional immunofluorescence technique. All proteins within the sample are then covalently anchored to a swellable hydrogel. Through digestion with proteinase K, structural proteins are cleaved allowing isotropical swelling of the gel in the last step. Dialysis of the sample in water results in a 4–4.5-fold expansion of the sample and the sample can be imaged via a conventional fluorescence microscope, rendering a potential resolution of 70 nm.

INTRODUCTION:

Albuminuria is a surrogate parameter of cardiovascular risk and results from disruption of the glomerular filter[1]. The glomerular filter is composed of the fenestrated endothelium, the

glomerular basement membrane and the slit diaphragm formed by podocytes. Primary and secondary foot processes of podocytes wrap around the capillary wall of the glomerulum[2]. The delicate structure of foot processes is maintained by cortical actin bundles which also serve as anchors for multiple slit diaphragm proteins and other adaptor proteins[2]. The slit diaphragm's backbone protein is called nephrin and interacts in a homophilic manner with nephrin molecules of opposing podocytes. Via diverse adaptor proteins, nephrin is linked to the actin cytoskeleton[2,3]. Mutations in the nephrin-encoding gene NPHS1 lead to nephrotic syndrome of the Finnish type[4].

One of nephrin's interacting proteins is podocin, a hairpin-like protein of the stomatin family[3]. Podocin recruits nephrin to lipid rafts and links it to the actin cytoskeleton[5]. Podocin is encoded by the NPHS2 gene. Mutations in NPHS2 lead to steroid-resistant nephrotic syndrome[6].

To visualize and co-localize actin adaptor proteins, immunofluorescence techniques may be used. Unfortunately, the diffraction barrier of the light limits the resolution of conventional fluorescence microscopes to 200–350 nm[7]. Novel microscopy techniques, e.g., stimulated emission depletion (STED)[8], photo-activated localization microscopy (PALM)[9], stochastic optical reconstruction microscopy (STORM or dSTORM) or ground state deletion microscopy followed by individual molecule return (GSDIM)[9–11], enable a resolution up to approximately 10 nm. However, these super resolution techniques require highly expensive microscopes, well-trained personnel and are therefore not available in many laboratories.

Expansion microscopy (ExM) is a novel and simple technique that enables super resolution imaging with conventional microscopes and is potentially available to a large research community[12]. In protein retention expansion microscopy (proExM), the sample of interest (cells or tissue) is fixed and stained with fluorophores[13]. Proteins within the sample are then covalently anchored by a small molecule (6-((Acryloyl)amino)hexanoic acid, succinimidyl ester, AcX) into a swellable hydrogel[13]. Through enzymatic digestion with proteinase K (ProK), proteins and fluorophores maintain their relative position within the gel after expansion[13]. After swelling of the gel, the sample expands up to 4.5-fold (90-fold volumetric expansion) leading to an effective lateral resolution of approximately 60–70 nm (300 nm/4.5). Modifications of this technique can even allow for a 10-fold expansion (1,000-fold volumetric expansion), rendering a resolution of 20–30 nm on conventional microscopes[14–16].

Glomerular structures of mouse and human kidneys have been visualized via ExM[17]. Within this paper, we present a detailed proExM protocol to visualize super resolution images of F-actin and the actin-adapter protein podocin within cells using a conventional fluorescence microscope.

PROTOCOL:

1. Splitting and seeding of cells

1.1. Warm up sterile Dulbecco's Modified Eagle's Medium (DMEM) including 10% fetal calf serum (FCS), sterile Phosphate buffered saline (PBS) and sterile trypsin to 37 °C. Activate the clean

bench.

1.2. Prepare a 6-well plate by adding one sterile glass cover slip (10 mm) to each well using sterile forceps.

1.3. Put a 10 cm cell culture dish with Cos7 cells under the clean bench. Under the clean bench, aspirate the cells' medium using a vacuum device.

1.4. Hold the angulated cell culture dish in one hand and add 10 mL of sterile PBS to the side of the cell culture dish to avoid rinsing off cells. Put the cell culture dish down so that PBS rinses the complete cell culture dish.

1.5. Remove the PBS and add 1 mL of trypsin to the middle of the cell culture dish and incubate for 5 min at 37 °C.

1.6. Stop the trypsinization reaction by adding 10 mL of DMEM including 10% FCS and pipette the cell solution up and down with a pipettor to manually separate the cells.

1.7. Analyze the cell number per milliliter using a counting chamber.

1.8. Seed 68,000 cells in 2 mL medium per well onto the glass cover slips in the 6-well plate. Distribute the cells within the 6-well plate by cautiously shaking the plate horizontally and vertically.

NOTE: Cells should lie scattered.

1.9. Incubate the cells overnight in a 37 °C incubator with 5% CO_2.

2. Transfection of cells

2.1. Prepare two sterile 1.5 mL tubes (one for diluted DNA (A) and one for diluted reagent for cationic lipid transfection (B)). Pipette 0.75 µg of nephrin and 0.75 µg of podocin cDNA expression 3 plasmid per well into one 1.5 mL tube and dilute it into 100 µL of reduced serum medium per well. Add 3 µL of cationic lipid transfection reagent per well to the second 1.5 mL tube and dilute with 100 µL of reduced serum medium. Incubate both reactions for 5 min at room temperature.

2.2. Combine both reactions (A and B) to a DNA-lipid complex and incubate for 20 min at room temperature. Cautiously add 200 µL of the DNA-lipid complex to each well.

2.3. Incubate the transfected cells at 37 °C with 5% CO_2 for 48 h without changing the medium.

3. Immunolabeling of cellular structures

3.1. Prepare a fixing solution (4% (w/v) paraformaldehyde in PBS, 1 mL/well), the

permeabilization solution (0.5% (w/v) Triton X-100 in PBS, 1 mL/well), and a blocking solution (5% (v/v) bovine serum albumin in PBS, 1 mL/well).

3.2. Remove the medium with a vacuum device. Add 2 mL of PBS to each well to remove extra medium. Aspirate the PBS completely.

NOTE: To avoid washing away cells with PBS, make sure to pipette PBS not onto the glass cover slip directly.

3.3. Fix cells with 4% (w/v) paraformaldehyde (PFA) dissolved in PBS for 10 min at room temperature.

NOTE: Alternatively, fix cells in 3% (v/v) glyoxal-ethanol[18].

3.4. Discard PFA and wash cells twice in PBS (2 mL each) by avoiding direct pipetting onto the glass cover slips. Permeabilize the fixed cells with Triton X-100 0.5% (w/v) in PBS for 10 min at room temperature.

3.5. Remove the permeabilization solution and wash twice with PBS as indicated in step 3.2.

3.6. Block the cells by adding 1 mL of 5% (v/v) BSA in PBS for 1 h at room temperature. Incubate the cells with 200 µL of the primary antibody (anti-podocin antibody 1:200 in 1% (v/v) BSA in PBS) overnight at 4 °C.

NOTE: Alternatively, incubate the primary antibody for 1 h at room temperature.

3.7. Remove the primary antibody and wash three times with PBS as in step 3.2. Add the secondary antibody (goat anti-rabbit Alexa 488 1:1000 in 1% (v/v) BSA in PBS) for 1 h at room temperature. Keep the cells in the dark using a box.

3.8. Discard the secondary antibody and wash with PBS three times.

3.9. Remove PBS and incubate the cells with 200 µL of anti-nephrin antibody 1:100 in 1% (v/v) BSA in PBS for 1 h at room temperature. Wash with PBS three times. Keep the cells in the dark using a box.

3.10. Remove PBS and incubate with the secondary antibody donkey anti-guinea pig 633, 1:200, for 1 h at room temperature. Wash with PBS three times. Keep the cells in the dark using a box.

4. Expansion microscopy

4.1. Preparation

4.1.1. To form the spacers for the gelation chamber, cut glass cover slips #1.0 and #1.5 into 5

mm stripes (four each per glass slide) using a diamond knife. Position the #1.5 mm cover slip stripes so that they form a square of 2.5 cm length on a glass slide and place them into a staining chamber (**Figure 2 A1–C1**). Pipette a droplet of ddH_2O into each corner of the square to adhere the glass cover slip stripes to each other and to the glass slide (**Figure 2 A2–C2**).

NOTE: Avoid complete drying of ddH_2O as the adhesion force will get lost. If needed, apply more droplets of ddH_2O. Wait for approximately 20 min so that #1.5 mm cover slips are stably attached before starting with step 5.1.2.

4.1.2. Place four #1.0 glass cover slip stripes per cover slip onto the #1.5 mm cover slips. Adhere the stripes by pipetting a droplet on each #1.5 mm cover slip stripe (**Figure 2 A3–C3**).

NOTE: The gel's thickness should be at least 0.15 mm to facilitate handling in the progress of the protocol. However, to avoid excessive gel on top of the cells, keep the spacers height close to the cell substrate. By using #1.5 and #1.0 cover slip stripes as spacers, the spacers height will be approximately 0.3 mm. The cells adhere to the cover glass (height 0.12 mm). Therefore, the gel will be thick enough to handle but excessive gel on top of the cells is avoided using #1.0 and #1.5 cover glass stripes as spacers.

4.1.3. For the gelation chamber lid, wrap a cover glass (#1.5) with paraffin film. Avoid any folds or dirt on the paraffin film (**Figure 2 A5–C5**).

4.2. Anchoring and polymerization (gelation)

4.2.1. Prepare the anchoring buffer (see **Table 1**). For anchoring treatment, replace PBS with 250 µL of anchoring buffer per well directly onto the glass cover slip and incubate for 3 h at room temperature. Keep substrate in the dark using a box.

NOTE: Alternatively, incubate overnight at room temperature. Prepare fresh anchoring buffer for every experiment and wait for 10–15 min until it is dissolved properly. 6-((Acryloyl)amino)hexanoic acid, succinimidyl ester (AcX) should be replaced every 4–5 months to ensure adequate anchoring.

4.2.2. Remove the anchoring buffer and wash once with 1.5 mL PBS per well.

4.2.3. To stain actin fibres, thaw an ExM compatible phalloidin-solution and incubate the phalloidin (5 µL of phalloidin diluted in 195 µL of 1% (v/v) BSA in PBS/well) for 45 min at room temperature. Keep samples in the dark using a box.

4.2.4. In the meantime, dissolve sodium acrylate in ddH_2O using a stirring device. On ice, prepare the monomer solution (see **Table 1**).

NOTE: Dissolved sodium acrylate should be a clear and colorless solution. If the solution is yellow, replace with new sodium acrylate.

4.2.5. Prepare the gelling solution on ice (see **Table 1**). Pipette ammoniumperoxidsulfate (APS) into the gelling solution right before the gelling solution is applied to the gelling chamber.

4.2.6. Remove phalloidin from the cells and wash with 1.5 mL of PBS twice at room temperature. Leave 1.5 mL PBS within the well to facilitate removal of the sample on the glass cover slip.

4.2.7. Place the cells on the cover glass into the gelling chamber using forceps and a cannula to lift the cover glass slip from the 6-well plate.

NOTE: The cells should be on the top of the glass cover slip. The glass cover slip should not touch the spacers.

4.2.8. Add APS to the gelling solution and vortex shortly. Pipette 200 µL of gelling solution on the sample (**Figure 2 A4–C4**). Cautiously close the chamber by avoiding air bubbles within the gel (F**igure 2 A5–C5**).

4.2.9. Incubate the gelling chamber for at least 1 h at 37 °C to polymerize the gel in the wet staining chamber.

4.3. Homogenization (digestion)

4.3.1. Take the gelation chamber out of the incubator. To open the gelling chamber lid, introduce a razor blade between the lid and the spacer. Remove the lid cautiously. Remove the spacers with the razor blade and eliminate all extra gel by cutting it with the razor blade.

4.3.2. Put the slide with the gel and cover glass into a dish filled with PBS. By shaking gently, remove the detached cover glass from the gel. To remove the gel from the dish easily, put the slide below the gel to attach the gel to the slide.

4.3.3. With the gel on the slide, divide the gel in small pieces (quarter of the gel is divided into two to three pieces) using the razor blade. Gently push one piece of gel into a well of a 6-well plate with glass bottom and enfold it by a paintbrush. Keep the gel moisturized with a little amount of PBS using the paintbrush to avoid dehydration of the gel.

NOTE: The cells face downwards.

4.3.4. Using an inverted microscope, take overview images with low numerical aperture to determine the expansion factor after expansion.

4.3.5. Prepare the digestion buffer. Dilute Proteinase K to 4 U/mL in digestion buffer to receive the digestion solution.

NOTE: Digestion buffer without Proteinase K can be stored at 4 °C for 1–2 weeks.

4.3.6. Add 500 µL of the digestion solution to each well and immerse the gel within the solution. Let it digest overnight at room temperature and close the lid keeping the samples in the dark.

NOTE: Alternatively, let it digest at 37 °C for 1 h.

4.4. Expansion

4.4.1. Remove the digestion solution with a pipette and discard it. Add 1 mL of ddH_2O. Incubate the immersed gel for 10 min at room temperature.

4.4.2. Remove the water and add 1 mL of fresh ddH_2O. Wait for 10 min and continue exchanging water every 10 min until a plateau of expansion is reached.

NOTE: Sample expansion up to 4.5-fold is achievable. The gel becomes optically clear.

5. Imaging

5.1. Remove the water from the gel and directly start microscopy. Using an inverted microscope, primarily use an air objective (low magnification) to find imaged cells in the pre-expansion state (step 4.3.4).

5.2. Switch to a 40x (oil/water) and 63x objective for better resolution. Excite with the wavelength of interest and take the image via the camera.

6. Validation

6.1. Take an overview image of the sample. Find and match the same structures within the sample that were imaged in step 4.4.2. Use the channel with the best signal-to-noise ratio for validation (**Figure 3 A–B**).

NOTE: Adjust the imaging parameters to achieve similar brightness as in the image acquired in the non-expanded state (step 4.4.2).

6.2. Overlay pre- and post-expansion images by rotating and shifting them with ImageJ. Use the distance measuring tool in ImageJ to measure the distances between clearly identifiable structures. Measure at least 10 different structures.

NOTE: Alternatively, use a Python script to measure distances as described in[14].

6.3. Calculate the expansion factor by dividing post-/pre-expansion measurements.

6.4. To determine distortions, take images with a higher numerical aperture (**Figure 4 A–B**). Overlay these images and analyze them with ImageJ or as described in[15].

NOTE: Determination of distortions should be performed routinely but not necessarily on every sample.

REPRESENTATIVE RESULTS:

The concept and timing of this proExM protocol is depicted in **Figure 1**. On day 5, transfected cells are fixed and stained with fluorescent antibodies targeting the protein of interest (**Figure 1A,B**). On day 6, treatment with AcX leads to formation of amine groups on all proteins (including fluorophores) (**Figure 1A,B**)[12]. Upon polymerization of the hydrogel, these amine groups bind covalently to the hydrogel (day 6). After polymerization of the gel, homogenization (digestion) is performed with proteinase K resulting in the destruction of structural proteins of the cell (day 6, **Figure 1A,B**). Fluorescently labeled antibodies remain mostly preserved after digestion. Due to the disruption of structural proteins, water dialysis of the hydrogel results in isotropic expansion of the cell within the hydrogel on day 7 (**Figure 1A,B**). Imaging of the sample is performed with a conventional fluorescence microscope (**Figure 1A**). Data validation to determine the expansion factor and to exclude distortions should be performed (**Figure 1A**).

To perform expansion of the cell isotropically, the gelation step is essential. **Figure 2** shows the lateral and top view of a gelation chamber. Glass cover slips build the spacers of the gelation chamber (**Figure 2 A1–3/C1–3**). The cover glass with the fixed and stained cells is positioned with the cells upward onto a glass slide (**Figure 2 A4–C4**). The lid of the gelling chamber is wrapped with parafilm and is closed bubble-free (**Figure 2 A5–C5**).

This ExM protocol enables expansion of up to four-fold. To determine the expansion factor, it is essential to image cells before and after expansion (**Figure 3 A + B**). Insufficient anchoring and homogenization may lead to distortions and ruptures of cells. **Figure 4 A + B** shows representative examples of ruptured cells in different magnification images.

This method can be used to investigate the co-localization of F-actin and actin adaptor proteins, e.g., podocin and nephrin (**Figure 5**). Podocin is depicted in green while actin is labeled in blue (**Figure 5**). Nephrin is marked in green. White areas indicate co-localization.

FIGURE AND TABLE LEGENDS:

Figure 1: Concept and timing of this ExM protocol. (**A**) In the "protocol" column, each step of the protocol is outlined. (**A + B**) After seeding and transfecting cells, immunofluorescent labeling is performed (Immunolabeling). (**A + B**) The small molecule AcX (red dot) binds to all proteins and anchors them to the hydrogel (Anchoring). (**A + B**) Via polymerization all proteins including fluorophores are covalently bound via AcX to the hydrogel (Polymerization). (**A + B**) Homogenization leads to digestion of structural matrix proteins. (**A + B**) Expansion is achieved by dialysis in water. (**A**) Imaging and validation of imaging finalizes the experiment. (**A**) The entire protocol requires 7 days (column "day") with many incubation steps (total time per day column "total time"), but actual bench time is much less as indicated in the respective column "bench time". Modified from[14].

Figure 2: Building the gelation chamber. Side view (**A1**) and top view (**B1 + C1**) of a glass slide with four #1.5 cover stripes. By adding a droplet of water between the glass slide and the cover slip stripes, the stripes will adhere to the glass slide (**side view A2**, **top view B2, C2**). Droplets of water on the #1.5 cover stripes lead to adhesion of #1.0 cover stripes laid on top of the #1.5 cover stripes (**side view A3**, **top view B3, C3**). The sample on the cover slip is placed in the middle of the rectangle using forceps. The gel is pipetted on top (**side view A4**, **top view B4, C4**). (**A5**) Side view and top view (**B5** and **C5**) of the assembled gelation chamber including the closed lid which is built from a cover slip wrapped in parafilm.

Figure 3: Cells before and after expansion. (**A**) Cells before expansion stained for actin. The box indicates in which area the expanded cells in **Figure 3B** lie. (**B**) Cells after expansion stained for actin in red and podocin in green. Podocin co-localizes with actin in the cell periphery. Scale bar = 5 µm, expansion factor = 2.

Figure 4: Distortions and ruptures of cells. (**A + B**) Representative microscopic images of cos7 cells immuno-stained for actin (red). Cells were fixed, stained, anchored, digested and expanded. (**A**) Ruptures of cells. Arrows indicated ruptured areas. Scale bar = 5 µm, expansion factor = 4. (**B**) Ruptures and distortion of cells. White arrows indicated ruptured areas. Scale bar = 5 µm, expansion factor = 4.

Figure 5: Podocin co-localizes with nephrin and actin. Cos7 cells immunofluorescently labeled for podocin, actin, and nephrin. (**A**) Cos7 cells stained for podocin (green), actin (blue), and nephrin (red) with ExM. Podocin co-localizes with actin and nephrin. Scale bar = 200 nm, expansion factor = 4. (**B**) Magnification of the indicated area in (**A**), Scale bar = 40 nm.

Table 1: Solutions for ExM.

DISCUSSION:

The presented method enables the investigator to visualize cellular proteins, e.g., podocin, nephrin, and cytoskeletal components, e.g., F-actin. Within this protocol, transfected cos7 cells are used as a model to study interaction of slit diaphragm proteins with F-actin. Unfortunately, immortalized podocyte cell lines do not express sufficient endogenous amounts of slit diaphragm proteins[19].

With this method, cellular proteins can be visualized with nanoscale resolution using a conventional fluorescence microscope. The most critical steps within the protocol are: 1) sufficient anchoring of protein amine groups to the hydrogel with AcX, 2) adequate polymerization of the hydrogel, 3) optimal timing for digestion and 4) selection of compatible fluorophores.

Anchoring of cellular proteins to the hydrogel is essential for this method in order to preserve the protein's position within the hydrogel during expansion. AcX is a small molecule that binds to amine groups of proteins within cells and tissues. AcX creates a carbon-carbon double band

with proteins, enabling incorporation of the proteins into the hydrogel in the polymerization step[20]. AcX also integrates antibodies so that labeling with immunofluorescence antibodies can be performed before AcX treatment. Insufficient anchoring may lead to ruptures and distortions of cells. Due to modification of amine groups by fixatives, one needs to optimize the fixative or the time of fixation. In addition, insufficient storage or non-optimized anchoring conditions may result in ruptures and distortions. Based on our experience, AcX loses its optimal effect when used for more than 3–4 months.

Polymerization of the gel is temperature dependent. We, therefore, recommend keeping the polymerization solutions on ice before pipetting it into the gelation chamber. In addition, the handling time of the gelation step should be kept short (less than 5 min) in order to avoid premature gel formation. Thorough mixing of the polymerization solution prevents uneven polymerization. Air bubbles will affect the expansion process when touching the sample and can be prevented by adding more polymerization solution.

After incorporation of the cellular proteins within the hydrogel, the mechanical homogenization step (or digestion) is needed to ensure expansion. Different methods, e.g., heat and detergent or enzymatic digestion, exist and need to be customized to the investigated sample[12,14,20]. Within this protocol, the protease Proteinase K is used for enzymatic digestion. Proteinase K is applied at a dosage sufficient to destroy structural proteins whilst preserving most other proteins including fluorescent antibodies[12]. If digestion is incomplete, the sample expansion is insufficient. In addition, the sample can tear during the expansion process (**Figure 3**). If an inadequate sample expansion has occurred, water replacement is recommended. Alternatively, the time for the enzymatic digestion can be adjusted or a new aliquot of the Proteinase K opened.

If the sample is over-digested, fluorescence signals will be diminished. In this case, the digestion time should be reduced. In ExM in general, the fluorescence signal intensity per unit of volume is reduced due to the volumetric expansion of the sample[14]. Therefore, longer exposure times during imaging need to be considered.

It is essential to select ExM compatible fluorophores. Cyanine dyes are degraded during the polymerization step[13]. Fluorescence proteins based on bacteriophytochromes are also largely destroyed[13]. However, most GFP-like proteins will be preserved[13]. In addition, streptavidin can also be applied pre-expansion, labeling post-translational modifications such as S-nitrolysation via a small molecule tag[13].

Phalloidin, a small labeling molecule to target the actin cytoskeleton, is not compatible with ExM[21]. To overcome insufficient anchoring of phalloidin, trivalent anchoring (TRITON) has been introduced[21]. This approach offers simultaneous targeting, labeling and grafting of biomolecules[21].

This method can be modified to stain RNA molecules (ExFish)[22]. In iterative expansion microscopy (iExM) or X10 microscopy, the resolution of 60–70 nm can be extended to approximately 25 nm by applying a second swellable gel within the first expanded hydrogel or conducting a single

expansion step using a different hydrogel[15,16]. Ultrastructure expansion microscopy (U-ExM) enables super resolution of proteins preserving their attribution to an ultrastructural element (e.g., mitochrondria, microtubules)[23]. A combination of ExFish (RNA and DNA) and proExM methods have previously been performed as well[22,24]. The presented protocol uses transfected cos7 cells as a model to investigate slit diaphragm proteins. We expect that other resident cultured kidney cells, e.g., HEK293T cells, can be similarly used for this protocol. Depending on the cell line, adjustments may need to be made for the different culturing and transfection conditions.

ExM enhances resolution of immuno-stained samples by about 4-fold reaching a lateral spatial resolution of 70 nm[13]. Compared to other super-resolution techniques, ExM is performed on a conventional fluorescence microscope[13,14]. Therefore, no expensive equipment or specifically trained personnel is necessary to conduct the ExM method[14]. Even though not all fluorophores are compatible with ExM, there are generally many available antibodies with optimized fluorophores with photo-physical properties needed for super resolution microscopy[14]. The main disadvantage of this method is that ExM is incompatible with live samples[12,14].

In the future, improving the hydrogel's chemical composition may lead to even higher spatial resolution[12]. The combination of different protocols may also enable visualization of proteins, RNA, DNA, or lipids in complexes within the same sample with such high resolution[12].

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

A

Day	Protocol	Total Time	Bench Time
1	Seeding of cells	>12 h (o/n)	30 min
2-4	Transfection	6-8 h + 48 h	1 h
5	Immunolabeling	16-18 h (o/n)	2-3 h
6	Actin Staining and Anchoring	5-6 h	1.5 h
6	Polymerization	2 h	1-1.5 h
6	Homogenization	> 12 h (o/n)	15 min
7	Expansion	1-2 h	30-45 min
7	Imaging	2-3 h	2-3 h
7	Validation	30-60 min	30-60 min

B

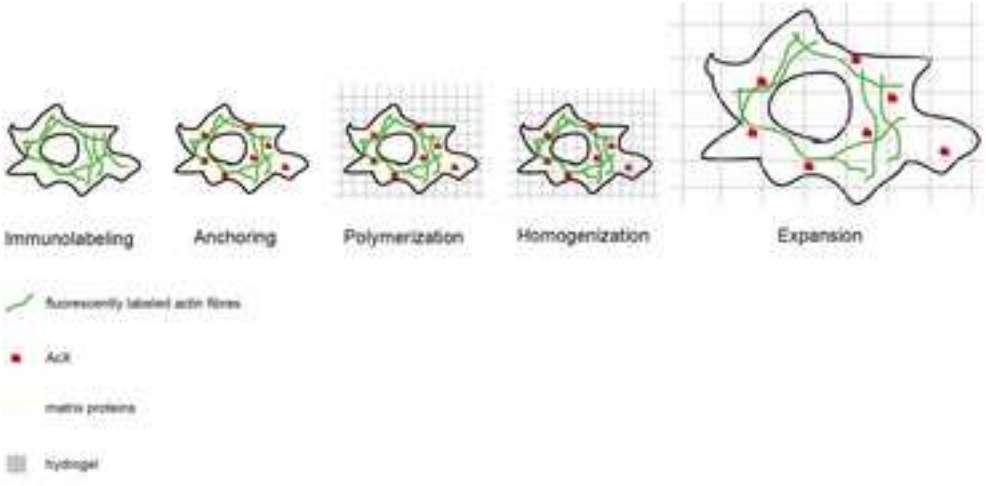

Figure 2

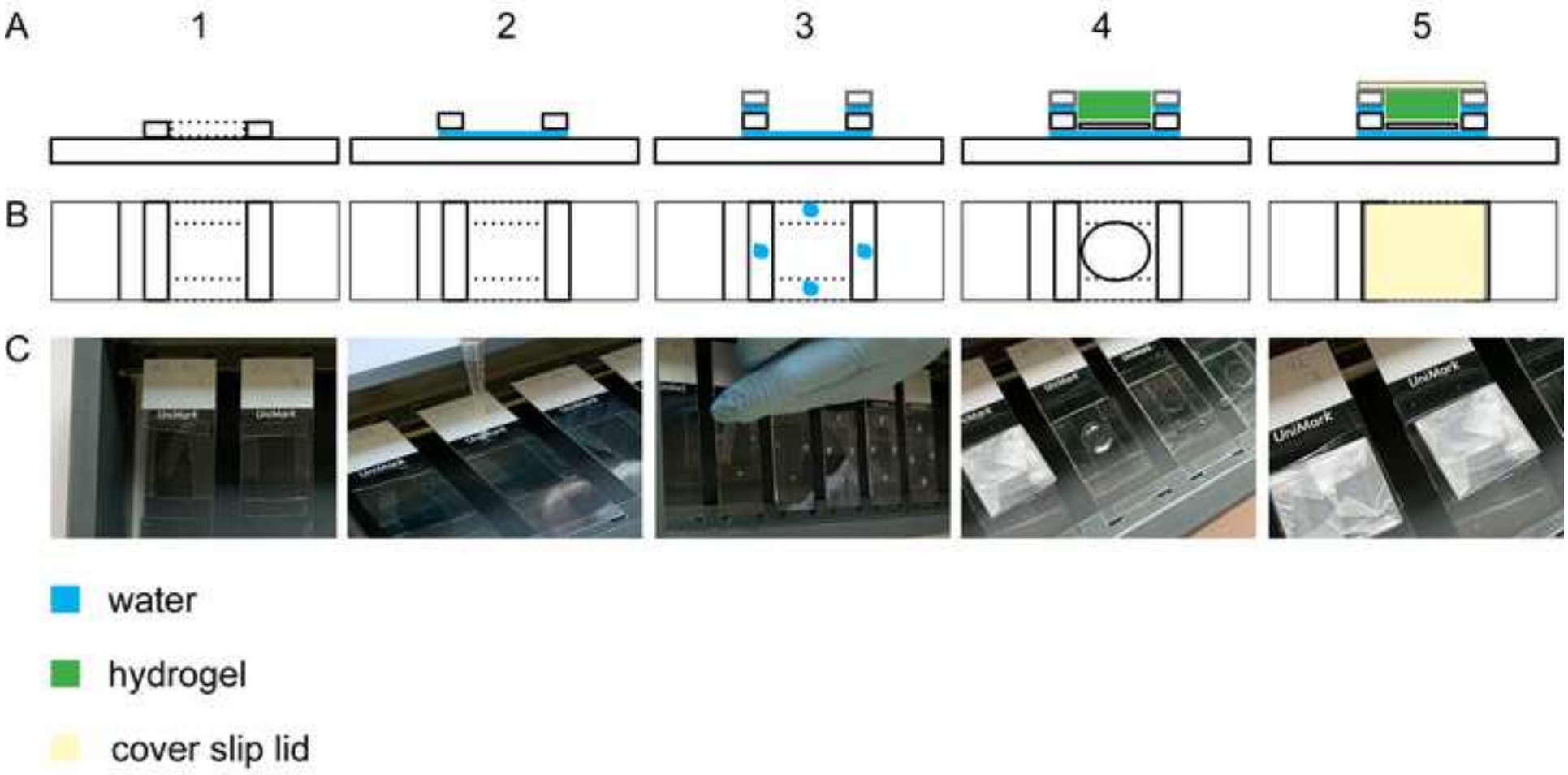

Figure 3

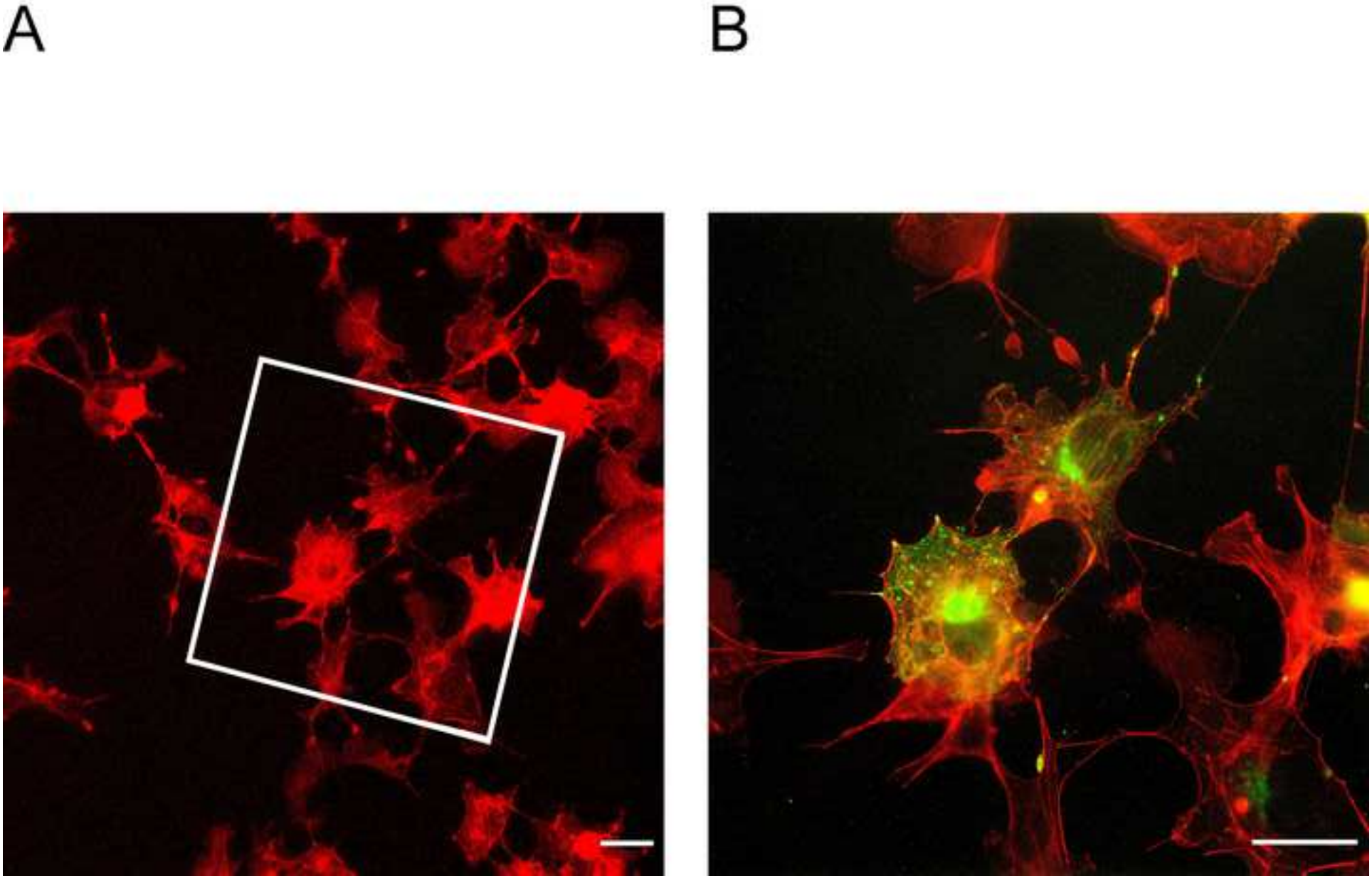

Figure 4

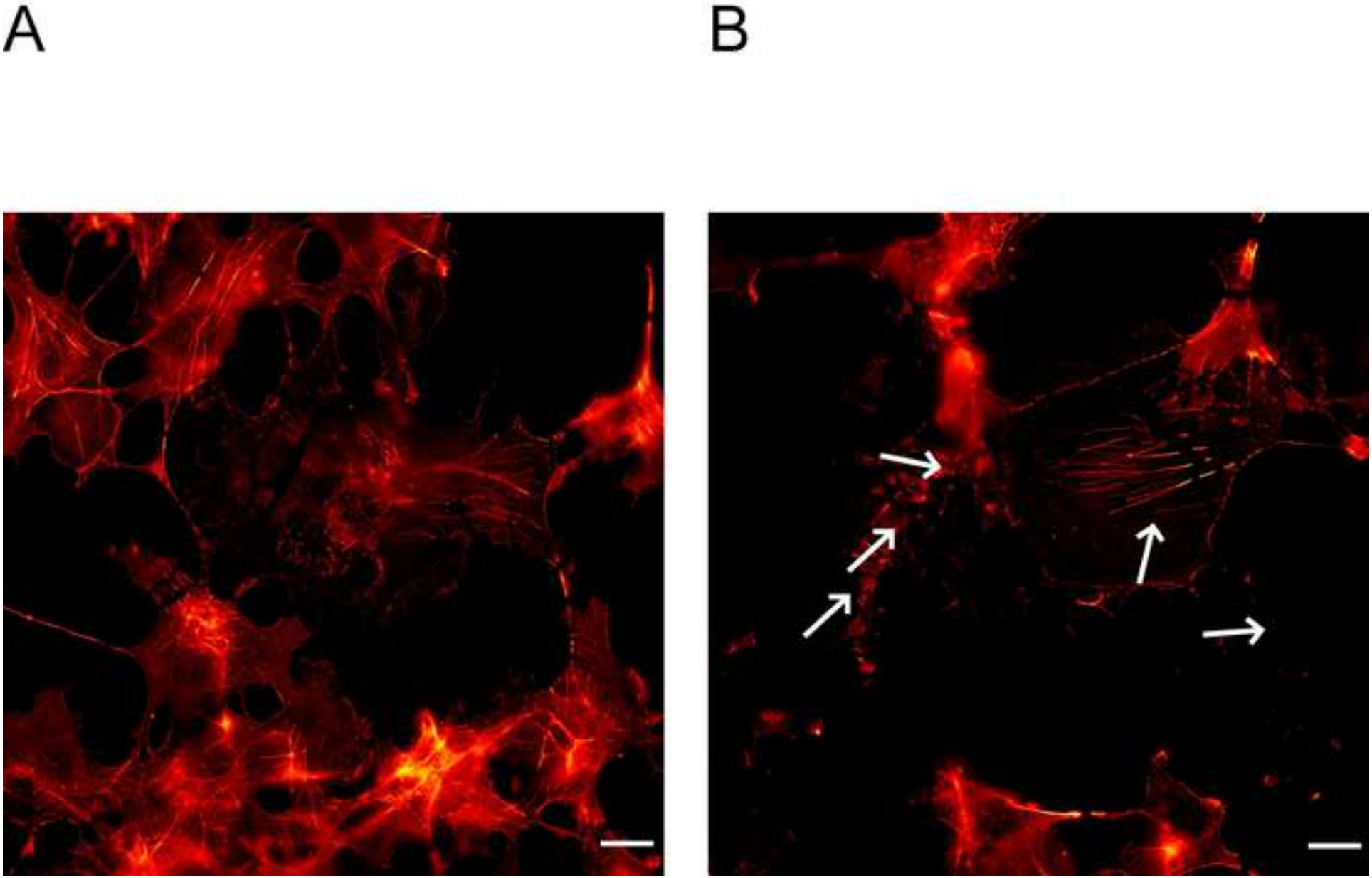

Figure 5

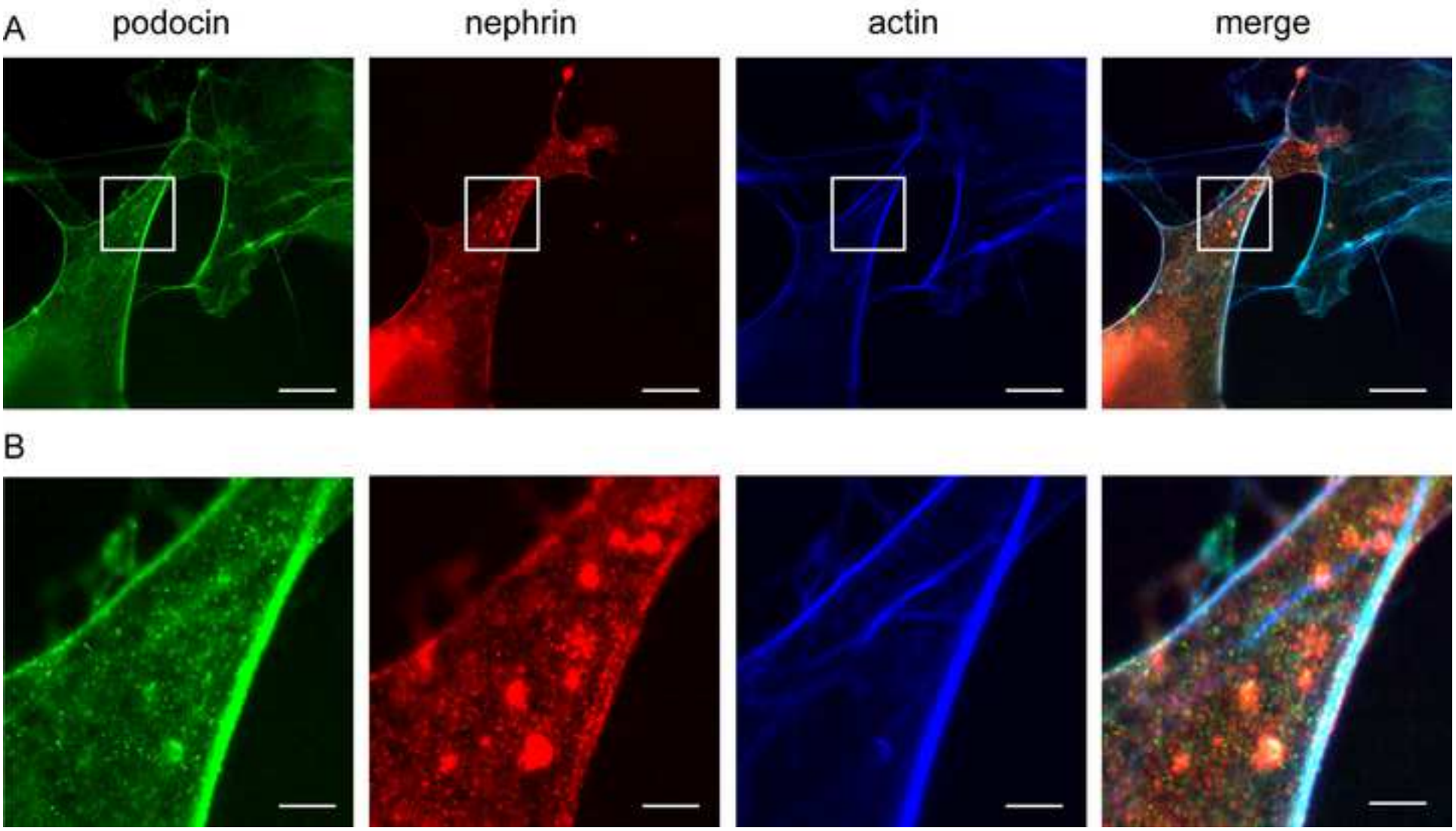

Table 1

Anchoring buffer	final concentration
NaHCO3	150 mM
Acryloyl-X, SE (AcX)	0.1 mg/ml

Monomer solution	Stock solution concentration g/100 ml
sodium acrylate	38
acrylamide	50
N,N'-Methylenebisacrylamide	2
sodium chloride	29.2
PBS	10x
water	
total	

Gelling solution	Stock solution concentration
monomer solution	NA
APS	10%
TEMED	>99%
water	NA
total	

Digestion solution	final concentration
Tris Cl, pH 8.0	1 M
EDTA pH 8.0	0.5 M
Triton X-100	0.005%
Guanidin HCL	8 M
water	
proteinase K	4 U/ml

amount (ml)
2.25
0.5
0.75
4
1
0.75
9.4

amount (µl)
190
4
4
2
200

final concentration (g/100 ml)
8.6
2.5
0.15
11.7
1x

final concentration (mg/ml)
NA
0.1
0.1
NA

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_Revised edt LS.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
6-((Acryloyl)amino)hexanoic acid, succinimidyl ester, Acryloyl-X, SE	invitrogen	A-20770	store up to 4 months
6-Well glass bottom plates	Cellvis	P06-1.5H-N	
Acrylamide >99%	Sigma-Aldrich	A3553-100G	
APS	Sigma-Aldrich	A3678-25G	
Deckgläser (cover glasses)	Engelbrecht	K12432	24x32 mm #1.0
Diamont cutter	VWR	201-0392	for cutting the cover slips
Guanidine HCl	Sigma-Aldrich	G3272-100G	8 M Stock can be kept at RT
Marten hair paintbrush	Leon Hardy	3 (770)	
Menzel Deckgläser (cover glasses)	Thermo Fischer	15654786	24x24 mm #1.5
N,N`-Methylenbisacrylamide	Sigma-Aldrich	M7256-25G	
Objektträger UniMark	Marienfeld	703010	
Proteinase K	New England Biolabs	P8107S	
Sodium Acrylate	Sigma-Aldrich	408220	check purity
Sodium Bicarbonate	Sigma-Aldrich	S5761	
Staining chamber			produced at the university's workshop
TEMED	ROTH	2367.1	
Antibodies			
Actin-ExM 546	chrometra	non-available	1:40
Anti Podocin produced in rabbit	Sigma	P-0372-200UL	1:200
Donkey anti guinea-pig CF633	Sigma	SAB4600129-50UL	1:200
Goat anti rabbit 488	Life Technologies	A11034	1:1000
Guinea pig anti nephrin	Origene	BP5030	1:100
Software			
FIJI			
Visiview			
microscope			
AXIO Observer Z1	Zeiss	non-available	

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Dr. Eva Königshausen	0211 81 17726		26.10.2020

Rebuttle document manuscript JoVE62079

Dear Mr Nguyen,

Thank you for the in-depth review of the manuscript. In our means your comments were very helpful to improve the manuscript substantially. Please find below the point by point reply:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thank you very much for this comment. We have proofread the manuscript to ensure that there are no spelling or grammar issues.

2. 1.8: Do you mean 68,000 cells? Please replace the period in the decimal with a comma.

Response: We have replaced the period with a comma.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: lipofectamine, OptiMEM, Acryloyl-X-SE, CF 633, LabelX, ExFish,

Response: Thank you for pointing this out. We have replaced commercial language with generic terms instead. ExFish is the abbreviation for a method in which expansion microscopy of FiSH labeled cells or tissues is performed. We did therefore not replace ExFish.

4. What does Actin-ExM contain?

Response: According to the manufacturer, "Actin-ExM is a unique phalloidin derivative that enables single-step staining of F-Actin for fluorescence microscopy. Available with a choice of fluorophores, compatible with gelation. Actin ExM can be incorporated into established ExM methods by simply treating the sample with Actin ExM for thirty minutes, after immunolabelling and immediately before gelation".

5. Please do not abbreviate journal names in the reference list.

Response: Thank you, we have changed abbreviated journal names to full journal names in the reference list.

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Response: Thank you for your comment. All figures have been created by the authors of this manuscript. We therefore do not need to obtain copyright permission. We would however like to reference reviews as indicated as our figures resemble figures in those reviews.

7. Please sort the Materials Table alphabetically by the name of the material.

Response: The Materials Table has been sorted alphabetically.

Reviewer #1
Minor concerns
Would be good to describe if the specific amounts and ratios of reagents as described in the technique need to be optimized for every cell types, or if the methodology will generally work for all the typical cell types that can be cultured from the kidney.

Response: Thank you for this excellent point. We have added a comment within the discussion, that this protocol can be transferred to other resident cell types *e.g. HEK293T* cells. Adjustments in the protocol need to be made for culturing and transfection conditions.

Reviewer#2
Major concerns:
1. The manuscript describes the importance of looking at podocyte specific structural molecules such as podocin, nephrin, and F-actin. However the use Cos7 cells. Why did the authors not use podocytes? This needs to be justified

Response: Thank you for pointing this out. We have used transfected Cos7 cells as a model because immortalized podocyte cell lines do not express slit diaphragm proteins at all or in very low amounts (Rinschen et al., Am J Physiol Cell Physiol 2016). We have added a comment in the discussion section to explain this rationale.

2. There is a section for transfection. It is unclear what the purpose of this step is, as it is not described what they are transfecting. And why?

Response: Thank you for this comment. We have added the transfected DNA (nephrin and podocin) to section 2.1 (transfection of cells). The purpose of the transfection is to incorporate the slit diaphragm proteins nephrin and podocin into Cos7 cells which do not endogenously express them.

3. The description of the ExM in section 4 was not clear and need to be more descriptive. Why use 1.5 and then #1.0 cover slips spacers? The is no references to figures throughout.

Response: Thank you for this helpful comment. We have added a passage explaining the rationale behind the use of #1.0 and #1.5 cover glass stripes as spacers to section 4.1.2. In addition, we have referenced figures throughout the protocol sections.

Minor Concerns:

The materials section is not well presented. A better description of the solutions used in a table format is needed.

Response: Thank you for pointing this out. We have added a table of solutions used within this protocol in the materials section.

Yours sincerely,

Lorenz Sellin

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Author(s): Eva Königshausen, Ch. Theresa Schmitz, L. Christian Kump and Lorenz Sellin

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