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Nanoscopic Imaging of Human Tissue Sections via Physical and Isotropic Expansion

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Nandita Singh, Ph.D.

JoVE

Dear Nandita,

It gives us great pleasure to submit our protocol paper "Nanoscopic Imaging of Human Tissue Sections via Physical and Isotropic Expansion" to *JoVE*. In this manuscript, we describe a simple and inexpensive protocol for nanoscale imaging of common types of clinical specimens, based on a novel tissue expansion technique, termed Expansion Pathology (ExPath). ExPath is capable of imaging nanoscale structure in tissue sections, such as tertiary podocyte foot processes in kidney, that was previously inaccessible to conventional wide-field or confocal microscopes. We describe how to both execute the protocol in detail and discuss key steps for successful tissue expansion and imaging. We are also happy to assist the *JoVE* team with production of a video to instruct users how to perform ExPath experiments in details.

This paper is not under review anywhere else. For reasons of competition, we request the following individuals not review this paper: Viviana Gradinaru, Joshua Vaughan, and Kwanghun Chung. The following individuals may be qualified reviewers of the paper:

1) Professor Joseph V. Bonventre, M.D. Ph.D., Professor, Chief of the Renal Unit and Director of the Bioengineering Division at Brigham and Women's Hospital (joseph_bonventre@hms.harvard.edu)

2) Professor Adrian Lee, Ph.D., Professor, Department of Pharmacology and Chemical Biology, Director, Women's Cancer Research Center, University of Pittsburgh Cancer Institute, Pittsburgh Foundation Chair in Precision Medicine (leeav@upmc.edu)

3) Professor Bhanu P. Jena Ph.D., George E. Palade University Professor and Distinguished Professor of Physiology at the Wayne State University School of Medicine (bjena@med.wayne.edu)

Most Sincerely,

Yongxin (Leon) Zhao, Ph.D.

TITLE:

Nanoscopic Imaging of Human Tissue Sections via Physical and Isotropic Expansion

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KEYWORDS:

expansion microscopy, fluorescence microscopy, nanoscale imaging, super-resolution imaging, pathology, expansion pathology, immunohistochemistry

SUMMARY:

Nanoscale imaging of clinical tissue samples can improve understanding of disease pathogenesis. Expansion pathology (ExPath) is a version of expansion microscopy (ExM), modified for compatibility with standard clinical tissue samples, to explore the nanoscale configuration of biomolecules using conventional diffraction limited microscopes.

ABSTRACT:

In modern pathology, optical microscopy plays an important role in disease diagnosis by revealing microscopic structures of clinical specimens. However, the fundamental physical diffraction limit prevents interrogation of nanoscale anatomy and subtle pathological changes when using conventional optical imaging approaches. Here, we describe a simple and inexpensive protocol, called expansion pathology (ExPath), for nanoscale optical imaging of common types of clinical primary tissue specimens, including both fixed-frozen or formalin-fixed paraffin embedded (FFPE) tissue sections. This method circumvents the optical diffraction limit by chemically transforming the tissue samples into tissue-hydrogel hybrid and physically expanding them isotropically across multiple scales in pure water. Due to expansion, previously unresolvable molecules are separated and thus can be observed using a conventional optical microscope.

INTRODUCTION:

Investigating the molecular organization of tissues in a three-dimensional (3D) context can provide new understanding of biological functions and disease development. However, these nanoscale environments are beyond the resolution capabilities of conventional diffraction limited microscopes (200−300 nm), where the minimal resolvable distance, d, is defined by $d \propto$

λ/NA. Here λ is the wavelength of light and NA is the numerical aperture (NA) of the imaging system. Recently, direct visualization of fluorescently labeled molecules has been made possible by newly developed super-resolution imaging techniques[1–3], including stimulated emission depletion (STED), photo-activated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), and structured illumination microscopy (SIM). Although these imaging techniques have revolutionized understanding of biological function at the nanoscale, in practice, they often rely on expensive and/or specialized equipment and image processing steps, can have slower acquisition time comparing to conventional optical imaging, require fluorophores with specific characteristics (such as photo-switching capability and/or high photostability). In addition, it remains a challenge to perform 3D super-resolution imaging on tissue specimens.

Expansion microscopy (ExM), first introduced in 2015[4], provides an alternative means of imaging nanoscale features (<70 nm) by physically expanding preserved samples embedded in a swellable polyelectrolyte hydrogel. Here, key biomolecules and/or labels are anchored in situ to a polymer network that can be isotopically expanded after chemical processing. Because the physical expansion increases the total effective resolution, molecules of interest can then be resolved using conventional diffraction-limited imaging systems. Since the publication of the original protocol, where custom synthesized fluorescent labels were anchored to the polymer network[4], new strategies have been used to directly anchor proteins (protein retention ExM, or proExM)[5–9] and RNA[9–12] to the hydrogel, and increase physical magnification through iterative expansion[13] or adapting gel chemistry[8,14,15].

Here we present an adapted version of proExM, called expansion pathology (ExPath)[16], which has been optimized for clinical pathology formats. The protocol converts clinical samples, including formalin-fixed paraffin-embedded (FFPE), hematoxylin and eosin (H&E) stained, and fresh-frozen human tissue specimens mounted on glass slides, into a state compatible with ExM. Proteins are then anchored to the hydrogel and mechanical homogenization is performed (**Figure 1**)[16]. With a 4-fold linear expansion of the samples, multicolor super-resolution (~70 nm) images can be obtained using a conventional confocal microscope having only a ~300 nm resolution and can also be combined with other super-resolution imaging techniques.

PROTOCOL:

1. Preparation of stock reagents and solutions

1.1. Prepare gelling solution components.

NOTE: Solution concentrations are given in g/mL (w/v percent).

1.1.1. Make the following stock solutions: 38% (w/v) sodium acrylate (SA), 50% (w/v) acrylamide (AA), 2% (w/v) N,N'-methylenebisacrylamide (Bis), and 29.2% (w/v) sodium chloride (NaCl). Dissolve the compounds in doubly deionized water (ddH_2O). Use the amounts in **Table 1** as a reference; prepared solutions can be scaled up or down in volume as needed. For example, to

make 10 mL of a 38% (w/v) SA solution, add 1.9 g SA to a graduated 10 mL cylinder and add ddH_2O to a volume of 5 mL.

1.1.2. Prepare 9.4 mL of monomer solution at a 1.06x concentration as shown in **Table 1**.

NOTE: This will result in a 1x concentration after addition of the initiator, accelerator, and inhibitor. The monomer stock can be stored at 4 °C for up to 3 months, or at -20 °C for long-term storage.

1.1.3. Prepare the following stock solutions separately in ddH_2O: 0.5% (w/v) of the inhibitor 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (4HT), which inhibits gelation to enable diffusion of the gelling solution into tissues, 10% (v/v) of the initiator tetramethylethylenediamine (TEMED), which accelerates radical generation by ammonium persulfate (APS), and 10% (w/v) APS which initiates the gelling process.

NOTE: Stock solutions of 4HT and TEMED can be prepared in 1 mL aliquots and stored at -20 °C for at least 6 months. APS has been found to lose efficacy after long-term storage and is best prepared in small quantities (<0.1 mL) immediately before gelling.

1.2. Prepare digestion buffer (50 mM Tris pH 8.0, 25 mM EDTA, 0.5% [w/v] nonionic surfactant, 0.8 M NaCl) by combining 25 mL of 1 M Tris pH 8 (3.03 g of Tris base in 25 mL of ddH_2O), 25 mL of EDTA (0.5 M pH 8), 2.25 g of nonionic surfactant, and 23.38 g of NaCl. Add ddH_2O for a total volume of 500 mL.

NOTE: The solution can be scaled up or down as needed and be stored in at 4 °C. Proteinase K (ProK) will be added immediately before the digestion step.

1.3. Prepare 20 mM sodium citrate solution by combining 2.941 g of sodium citrate tribasic dihydrate with 500 mL of ddH_2O and adjusting pH to 8.0 at room temperature (RT). Scale the volume of stock as needed.

1.4. Prepare a stock solution of 6-((acryloyl)amino)hexanoic acid, succinimidyl ester (acryloyl-X, SE; AcX), the anchoring compound. Dissolve AcX in 500 µL of anhydrous dimethyl sulfoxide (DMSO) for a final concentration of 10 mg/mL.

NOTE: The solution can be stored in a desiccated environment at -20 °C in 20 µL aliquots.

1.5. If not using commercially available buffers for immunostaining, prepare blocking buffer. Use a blocking buffer of 5% (v/v) normal animal serum and 0.1% (w/v) nonionic surfactant in 1x phosphate-buffered saline (PBS) and select the serum based on the host animal of the secondary antibodies. For example, to prepare 500 mL blocking buffer for antibodies raised in goat, combine 25 mL of goat serum, 0.45 g of nonionic surfactant, and 1x PBS to a volume of 500 mL.

2. Preparation of archived and freshly prepared clinical tissue slides for ExPath

2.1. Convert the tissue into an ExPath compatible format. Choose one of the four following steps (2.1.1–2.1.4) based on how the specimen was prepared: FFPE slides, stained FFPE slides, or unfixed or fixed frozen tissue slides in optimum cutting temperature (OCT) solution.

NOTE: These are based on standard recovery steps for pathology samples and are not specific to the ExPath protocol.

2.1.1. FFPE clinical samples

2.1.1.1. Prepare 30 mL of 95% ethanol, 70% ethanol, and 50% ethanol. Measure out 30 mL of xylene, 100% ethanol, and ddH_2O.

2.1.1.2. Place the slide with the sample in a 50 mL conical using forceps and add 15 mL of xylene. Cap the tube and place it horizontally on an orbital shaker at approximately 60 rpm and incubate at RT for 3 min for each solution. Repeat with the remaining 15 mL xylene.

2.1.1.3. Repeat step 2.1.1.2 with 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol, and ddH_2O in place of xylene.

2.1.2. Stained and mounted permanent slides

2.1.2.1. Place the slide in a 100 mm Petri dish and cover with xylene. Carefully remove the coverslip using a razor blade. If the coverslip is not easily removed, return the slide to the xylene until the coverslip loosens.

2.1.2.2. Process using the steps for FFPE samples (steps 2.1.1.1–2.1.1.3).

NOTE: In the case of H&E stained slides, the stains are eliminated during the expansion process.

2.1.3. Unfixed frozen tissue slides in OCT solution

2.1.3.1. Fix the tissue in acetone at -20 °C for 10 min.

2.1.3.2. Wash the samples with 1x PBS solution 3 times for 10 min each at RT.

2.1.4. Previously fixed, frozen clinical tissue slides

2.1.4.1. Incubate the slides for 2 min at RT to melt the OCT solution.

2.1.4.2. Wash the sample with 1x PBS solution 3 times for 5 min each at RT.

2.2. Perform heat treatment for antigen retrieval on all samples after format conversion.

2.2.1. Add 20 mM citrate solution (pH 8 at RT) in a heat resistant container, such as a slide staining jar.

NOTE: There should be enough solution to cover the tissue mounted on the slide (50 mL for a standard slide staining jar).

2.2.2. Heat the citrate solution to 100 °C in the microwave and place the slide in the solution. Immediately transfer the container to an incubation chamber and incubate at 60 °C for 30 min.

NOTE: The protocol can be paused here. Slides can be placed in Petri dishes and covered in 1x PBS and stored at 4 °C.

2.3. Stain the sample using standard immunofluorescence (IF)/immunohistochemistry (IHC) staining protocols.

NOTE: Specific primary and secondary antibody concentrations and staining durations are dependent on the concentrations suggested by the manufacturer or by optimization for the specific experiment.

2.3.1. Use a hydrophobic pen to draw a boundary around the tissue section(s) on the slide to minimize the volume of solution needed to cover the tissue. Place the slide in a dish large enough to fit the slide. For a standard 3-inch slide, use a 100 mm Petri dish.

NOTE: The hydrophobic pen does not interfere with the polymerization of the sample nor the digestion process.

2.3.2. Incubate the tissue with blocking buffer for 1 h at 37 °C, 2 h at RT, or 4 °C overnight to reduce nonspecific binding.

2.3.3. Dilute primary antibodies to the desired concentration in the appropriate amount of prepared blocking buffer (or other preferred staining buffer). Incubate the tissues with the primary antibody solution for at least 3 h at RT or 37 °C, or overnight at 4 °C.

NOTE: Samples should be placed in a humidified container (such as a Petri dish with a damp wipe) to prevent the tissue from drying out. Typically, antibodies have been diluted to 1:100−1:500 in 200−500 µL of buffer, depending on the tissue size and antibody used.

2.3.4. Wash the tissue with prepared blocking buffer (or other preferred washing buffer) 3 times for 10 min at RT.

2.3.5. Dilute secondary antibodies (and 300 nM 4’,6-diamidino-2-phenylindole [DAPI] if desired), in prepared blocking buffer (or other preferred staining buffer) to a concentration of approximately 10 µg/mL. Incubate the tissue in the secondary antibody solution for at least 1 h at RT or 37 °C.

NOTE: Timing may be adjusted depending on the antibodies used and the thickness of the tissue. Secondary antibodies containing cyanine dyes (Cy3, Cy5, Alexa 647) are not compatible with the ExM protocol when applied pre-polymerization. Suggested dyes include Alexa 488 (green), Alexa 546 (orange/red), and Atto 647N or CF633 (far-red). DAPI must be reapplied after expansion, as it is washed away during the expansion process.

2.3.6. Wash the tissue with prepared blocking buffer (or other preferred washing buffer) 3 times for 10 min each at RT.

NOTE: The protocol can be paused here. Slides can be placed in Petri dishes and covered in 1x PBS and stored at 4 °C.

2.3.7. Perform fluorescent imaging using a conventional wide-field microscope, confocal microscope, or other imaging system of choice.

NOTE: This step is required to determine biological length using the expansion factor by comparing pre- and post-expansion images. To facilitate post-expansion imaging, easily identifiable regions of interest should be selected and images at both low and high magnification should be collected.

3. In situ polymerization of specimens

3.1. Incubate the specimen in anchoring solution.

3.1.1. Prepare the anchoring solution (typically 250 µL is enough to cover the tissue section) by diluting the AcX stock solution in 1x PBS to a concentration of 0.03 mg/mL for samples fixed with non-aldehyde fixatives or 0.1 mg/mL for samples fixed with aldehyde fixatives, which have fewer free amines available to react with AcX.

3.1.2. Place the slide in a 100 mm Petri dish and pipette the anchoring solution over the tissue. Incubate for at least 3 h at RT or overnight at 4 °C.

3.2. Incubate the samples in gelling solution.

3.2.1. Prepare at least 100-fold excess volume of gelling solution. Per 200 µL, combine the following, in order: 188 µL of monomer solution, 4 µL of 0.5% 4HT stock solution (1:50 dilution, final concentration: 0.01%), 4 µL of 10% TEMED stock solution (1:50 dilution, final concentration 0.2%), and 4 µL of 10% APS stock solution (1:50 dilution, final concentration 0.2%).

NOTE: Gelling solution should be made immediately before use. The solution should be kept at 4 °C and the APS solution should be added last, to prevent premature gelling.

3.2.2. Remove excess solution from the tissue section and place the slide in a 100 mm Petri dish.

Add fresh, cold gelling solution to the sample and incubate the mixture on the tissue for 30 min at 4 °C, to allow diffusion of solution into the tissue.

3.3. Construct a chamber on the slide around the sample (**Figure 2A**) without disturbing the gelling solution.

3.3.1. Make spacers for the gelling chamber by thinly cutting pieces of cover glass using a diamond knife.

NOTE: To facilitate imaging post expansion, the spacers should be close in thickness to the tissue specimen to reduce the amount of blank gel above the tissue. Number 1.5 glass can be used for standard clinical samples (5−10 µm). Cover glass pieces can be stacked for thicker samples.

3.3.2. Secure the spacers on either side of the tissue using droplets of water (~10 µL).

3.3.3. Carefully place a cover glass lid over the slide, making sure to avoid trapping air bubbles over the tissue (**Figure 2B**).

3.4. Incubate the sample at 37 °C in a humidified environment (such as a closed Petri dish with a damp wipe) for 2 h.

NOTE: The protocol can be paused here. The slide chamber can be stored inside a sealed Petri dish at 4 °C.

4. Sample digestion

4.1. Remove the lid of the gelling chamber by gently sliding a razor blade under the coverslip and slowly lifting the coverslip off the gel surface. Trim the blank gel around the tissue to minimize volume. Cut the gel asymmetrically to track the orientation of the gel after homogenization, since the sample will become transparent.

4.1.1. Dilute ProK by 1:200 in digestion buffer (final concentration 4 U/mL) before use. Prepare enough solution to completely submerge the gel; a single well of a four-well plastic cell culture plate requires at least 3 mL per well.

4.1.2. Incubate the sample in a closed container containing the digestion buffer for 3 h at 60 °C. If the sample does not detach from the slide during digestion, use a razor blade to gently remove the sample.

NOTE: The specimen should be completely submerged in digestion buffer to prevent the sample from drying out and placed in a covered container (small slide box, plastic well, Petri dish, etc.) that can be sealed with film.

5. Sample expansion and imaging

5.1. Use a soft paint brush to transfer the specimen into 1x PBS in into a container compatible with the desired imaging system and large enough to accommodate the fully expanded gel. Make sure that the tissue is placed with the sample-side down if imaging on an inverted system or up if imaging on an upright system to minimize the distance from the imaging objective to the sample. Flip the gel using a soft paint brush if needed.

NOTE: Side-illumination from an LED can be used to make them visible in liquid. A standard 6-well plate can accommodate samples that have a pre-expanded diameter less than 0.6 cm. A glass bottom well plate should be used for imaging on an inverted system.

5.2. Wash the samples in 1x PBS at RT for 10 min. If desired, re-stain the sample with 300 nM DAPI as the digestion process washes away the DAPI stain. Remove PBS and stain with 300 nM DAPI diluted in 1x PBS for 20 min at RT, followed by a 10 min wash with 1x PBS at RT.

NOTE: The samples can be covered with 1x PBS and stored at 4 °C before proceeding to the next step.

5.3. To expand the samples, replace the PBS and wash with an excess volume of ddH_2O (at least 10x the final gel volume) 3−5 times for 10 min each, at RT.

NOTE: After the 3rd or 4th wash, the specimen's expansion should begin to plateau. For storage, to prevent bacterial growth, the ddH_2O can be supplemented with 0.002%−0.01% sodium azide (NaN_3). In this case, the final expansion factor is reversibly reduced by 10%.

5.4. Perform fluorescence imaging using a conventional wide-field microscope, confocal microscope, or other imaging system of choice.

NOTE: To prevent gels from drifting, excess liquid can be removed from the well. Gels can also be immobilized with 1.5−2% low-melt agarose. Prepare 1.5−2%(w/v) low-melt agarose in water in a container 2−4 times the volume of solution. Warm the solution in a 40 °C water bath or in a microwave for 10−20 s to melt the solution. Pipette the melted agarose around the edges of the gel. After allowing the agarose to harden at RT or 4 °C, add water to the sample to prevent dehydration.

REPRESENTATIVE RESULTS:

If the protocol has been successfully carried out (**Figure 1**), samples will appear as a flat and transparent gel after mechanical homogenization (**Figure 3A**) and can expand by a factor of 3−4.5x in water (**Figure 3B**), providing an effective resolution of ~70 nm depending on the final expansion factor and imaging system used[5,16]. **Figure 4** shows example images of a 5 µm thick FFPE kidney sample processed using the ExPath protocol. Full expansion of the gelled samples resulted in a 4.5x expansion factor in water, giving an effective resolution of ~63 nm when imaged using a 0.95 NA objective. The tissue was first pre-processed with xylene to remove paraffin and rehydrated (section 2.1.1), followed by antigen-retrieval with the citrate buffer (section 2.2). The

recovered tissue was then stained with antibodies for alpha-actinin 4 (ACTN4) and vimentin, along with DAPI to visualize nuclear DNA and wheat germ agglutinin (WGA), to label carbohydrates. The specimen was then imaged using a spinning disk confocal microscope (**Figure 4A,B,D**). Tissues were treated following the above protocol and fully expanded in water (**Figure 4C,E**). Similarly, **Figure 5** shows example images of H&E stained normal breast tissue (**Figure 5A**) that was treated with xylene (section 2.1.2) to remove the cover glass and then treated as an FFPE sample (section 2.1.1). During homogenization, the H&E stain is eliminated from the tissue. Post-expansion images of the DAPI stained sample obtained on a spinning disk confocal microscope (**Figure 5B**) compared with pre-expansion images indicate an expansion factor of 5.1, giving an effective resolution of ~43 nm when imaged with an NA 1.15 lens.

Example data for unfixed frozen kidney slices processed using the ExPath protocol can be seen in **Figure 6**. The tissue was first fixed in cold acetone (section 2.1.3) and stained with ACTN4, vimentin, DAPI, and WGA. Comparison of pre-expansion (not shown) and post-expansion images indicate an expansion factor of 4.5. Comparison data from the acetone-fixed frozen kidney samples to that of the FFPE samples (**Figure 4C,E**) shows that there is a decrease in quality of the ACTN4 staining in the expanded FFPE sample, which may be due to a degradation of the antigenicity due to the fixation method[16].

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the expansion pathology (ExPath) workflow. Pre-processing of clinically archived tissue slides is first performed based on the storage format. Samples are then stained using conventional immunostaining protocols and pre-expansion images are obtained. Samples are then treated with acryloyl-X SE (AcX) to anchor proteins to the hydrogel. In situ polymerization is preformed prior to mechanical homogenization using proteinase K (ProK). Samples can then be expanded in ddH_2O prior to imaging.

Figure 2: Gelation chamber for pathology samples. (**A**) Two spacers, such as two pieces of #1.5 cover glass cut with a diamond knife, are placed on either side of the tissue after incubating the sample in gelling solution at 4 °C. The spacers should be thicker than the tissue slices, to prevent compression of the sample. (**B**) A lid, such as a piece of #1.5 cover glass, is used to cover the sample prior to incubation at 37 °C.

Figure 3: Example of sample expansion. A 5 µm thick kidney sample post homogenization, (**A**) pre- and (**B**) post-expansion in ddH_2O is shown. Grid squares are 5 x 5 mm.

Figure 4: Representative results from 5 µm thick FFPE kidney samples. (**A**) Pre-expanded image. (**B**) Magnified pre-expansion image of the outlined region of interest in panel A and (**C**) the corresponding post-expansion image of the same region of interest after the sample was fully expanded in water. (**D**) Magnified image of the outlined region of interest in panel B and (**E**) the corresponding post-expansion image of the same region of interest after the sample was fully expanded in water. Cracking, distortions, and loss of labeled targets can be the result of inadequate anchoring and/or homogenization (**F,G**). All images were obtained using a spinning

disk confocal microscope with a 20x (NA 0.95; water immersion) objective (**A-E,G**) or 10x (NA 0.5) objective (**F**). Blue, DAPI; green, vimentin; red, alpha-actinin 4 (ACTN4); magenta, WGA. Scale bars (yellow scale bars indicate post-expansion images): are 100 µm (**A,F,G**); (**B**) 50 µm, (**C**) 50 µm (physical size post expansion 225 µm; expansion factor 4.5); (**D**) 25 µm; (**E**) 25 µm (physical size post expansion 112.5 µm; expansion factor 4.5).

Figure 5: Representative results from H&E stained normal breast tissue. (**A**) Brightfield image of pre-expanded H&E stained tissue taken with a 40x (0.95 NA) objective. (**B**) Post-expansion DAPI image of the same region of interest after the sample was fully expanded in water. The image was obtained on a spinning disk confocal microscope using a 40x (NA 1.15; water immersion) objective. Scale bars (the yellow scale bar indicates post-expansion images) represent 10 µm in panel A and 2 µm in panel B (physical size post expansion 50.1 µm; expansion factor 5.1).

Figure 6: Representative results of fresh frozen kidney samples after expansion, obtained on a spinning disk confocal microscope with a 60x (NA 1.4; oil immersion) objective. Blue, vimentin; green, alpha-actinin 4 (ACTN4); red, collagen IV; grey, DAPI. Scale bar = 10 µm (physical size post expansion 45 µm; expansion factor 4.5).

Table 1: Components of monomer solution. All concentrations are given in terms of g/mL (w/v) except PBS.

DISCUSSION:

Here, we present the ExPath protocol[16], a variant of proExM[5] that can be applied to the most common types of clinical biopsy samples used in pathology, including FFPE, H&E stained, and fresh-frozen specimens on glass slides. Format conversion, antigen retrieval, and immunostaining of the specimens follow commonly used protocols that are not specific to ExPath. Unlike the original proExM protocol[9], ExPath relies on a higher concentration of EDTA in the digestion buffer, which improves the expansion of formalin-fixed tissues, as demonstrated in the original ExPath study[16]. The protocol has been validated in 5−10 µm thick clinical specimens[16], but could also be applied to thicker tissue samples with some modification. The most critical steps in this protocol are: 1) the timing of the gelation steps; 2) setup of the gelation chamber; 3) parameters for sample homogenization; and 4) handling of the gel.

The most critical parameter for this protocol is the timing of the gelation steps. If the gelling solution prematurely polymerizes, the sample will not be sufficiently anchored to the gel matrix. Inadequate anchoring and premature gelation can cause distortions, limit expansion, and result in the loss of target molecules (**Figure 4F,G**). The initiation of gelation is temperature dependent, therefore it is important to keep the mixed gelling solution at 4 °C before placing it on the target specimen. The initiator, APS, should be freshly prepared and added immediately before applying the gelling solution. APS is not stable at RT and can lose efficacy after undergoing freeze-thaw cycles. Insufficient anchoring can also be the result of reduced reactivity of the anchoring compound, AcX, which can lose activity after long term storage or after contact with water. AcX has been found to retain activity after 6 months of storage in a desiccated environment at -20 °C.

If premature gelation is not the suspected cause of distortion, a new stock solution of AcX can be prepared.

During the setup of the gelation chamber, air bubbles can become trapped under the cover glass lid. If these bubbles are on top of or directly touching the sample, they can cause distortions. They can be moved away from the specimen by adding more gelling solution through the side of the chamber. To help prevent air bubbles, a small drop of gelling solution can be deposited on the lid before placing it over the tissue.

Sample digestion is dependent on time, temperature, as well as tissue properties, such as thickness and tissue type. Inadequate digestion can also cause distortions and result in an expansion factor that is smaller than expected. If incomplete digestion is suspected, the digestion time and/or ProK concentration can be increased, particularly in the case of thicker tissues. ProK can also lose activity over time. To preserve its activity, ProK should be stored at -20 °C and can be aliquoted into smaller volumes to avoid free-thaw cycles.

Care should be taken when handling homogenized samples, particularly when fully expanded. If the specimen is hard to locate when submerged in liquid, illuminating the container from different angles can scatter incident light to allow visualization of the gel. Fully expanded gels are fragile and can break during handling. Use of soft brushes and plastic spatulas is recommended to transfer the expanded gels.

The ExPath protocol provides a cost-effective alternative to current super-resolution imaging and electron microscopy techniques to interrogate nanoscale structures in clinical biopsy specimens. Although ProK provides even expansion of the sample after homogenization, the loss of proteins prevents interrogation of other targets of interest post-expansion. However, the protocol can be easily performed in any regular wet lab. Most importantly, imaging of nanoscale features can be carried out on conventional wide-field or confocal microscopes that are commonly found in biology laboratories and imaging core facilities.

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DISCLOSURES:
YZ and OB are two of the inventors who have filed for and obtained patent protection on a subset of the technologies described here (US patents US20190064037A1, WO2018157074A1, and WO2018157048A1).

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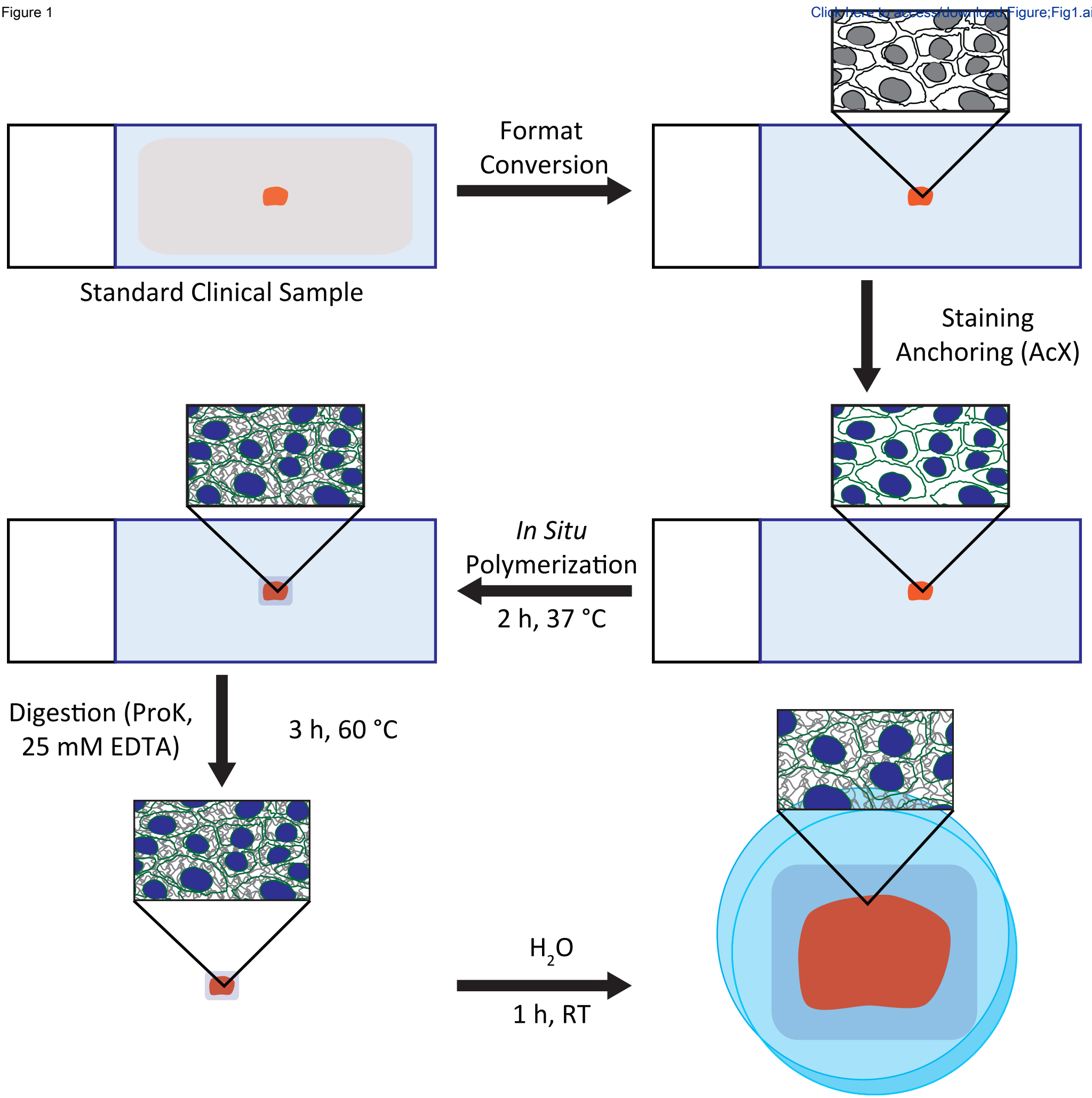
Figure 1



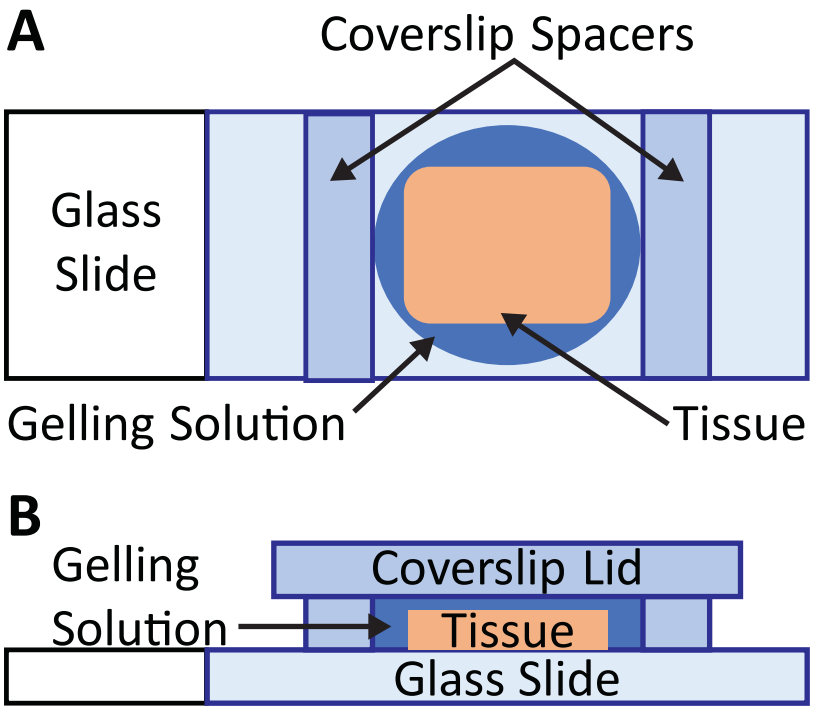


Figure 3

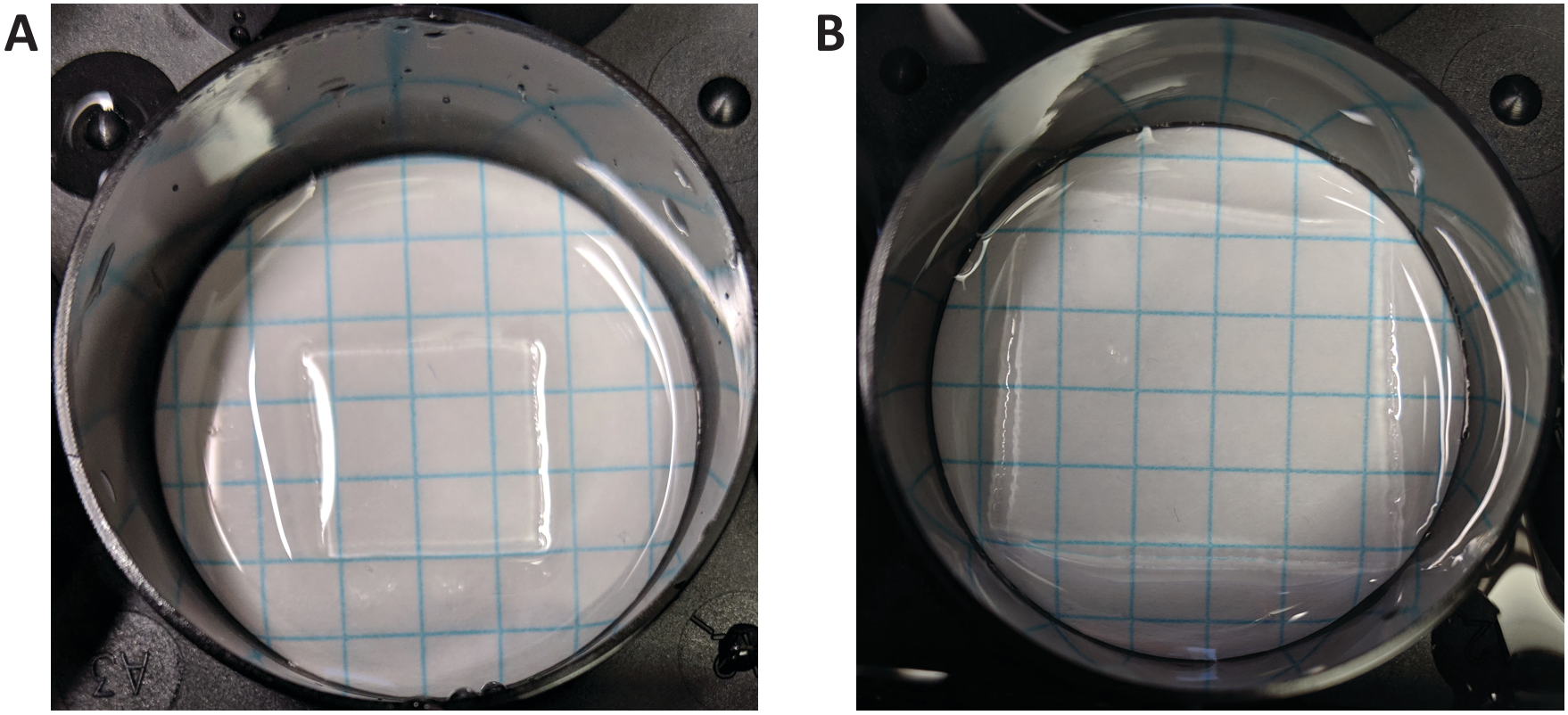


Figure 4

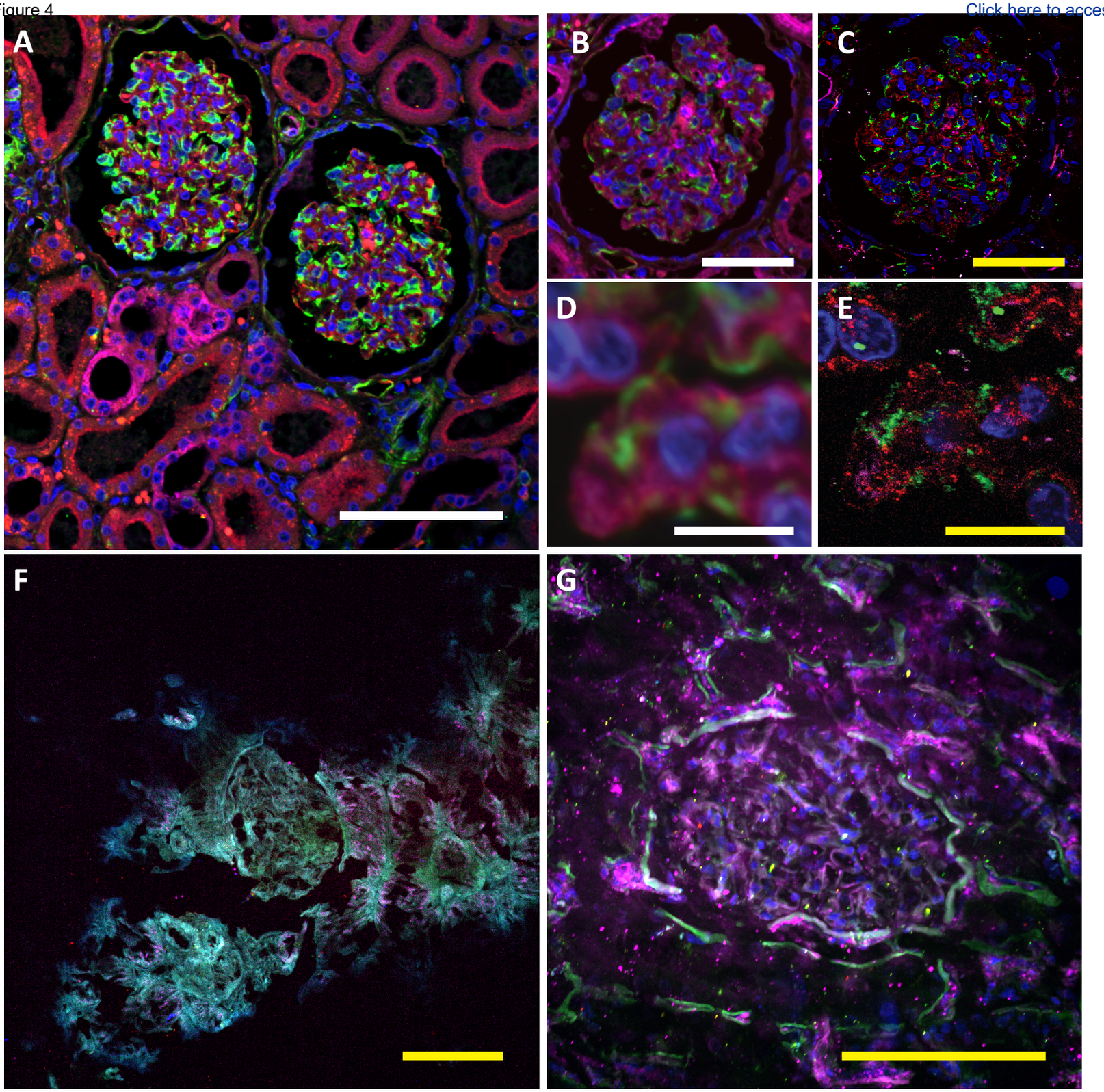


Figure 5

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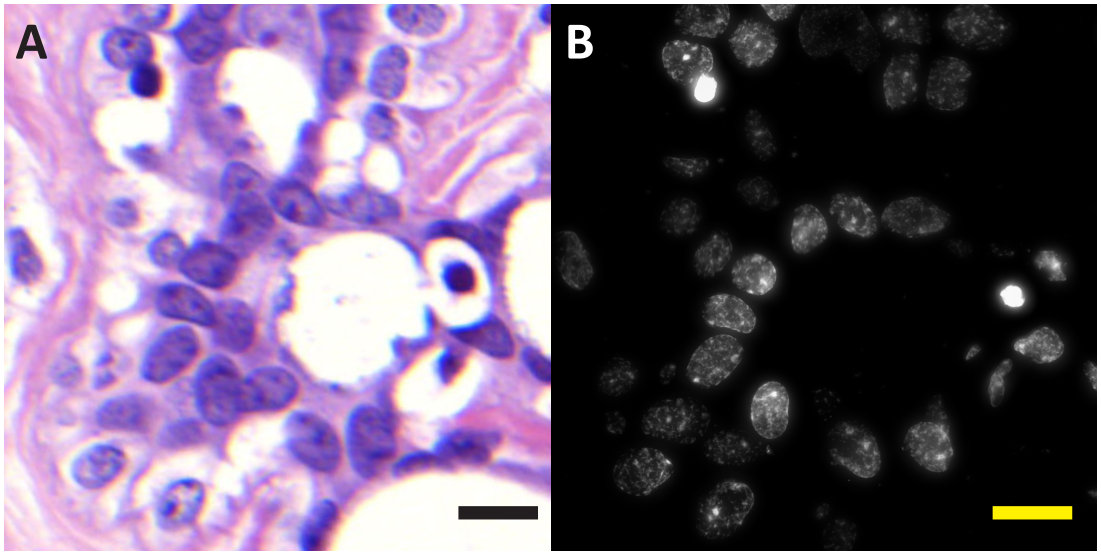

Figure 6

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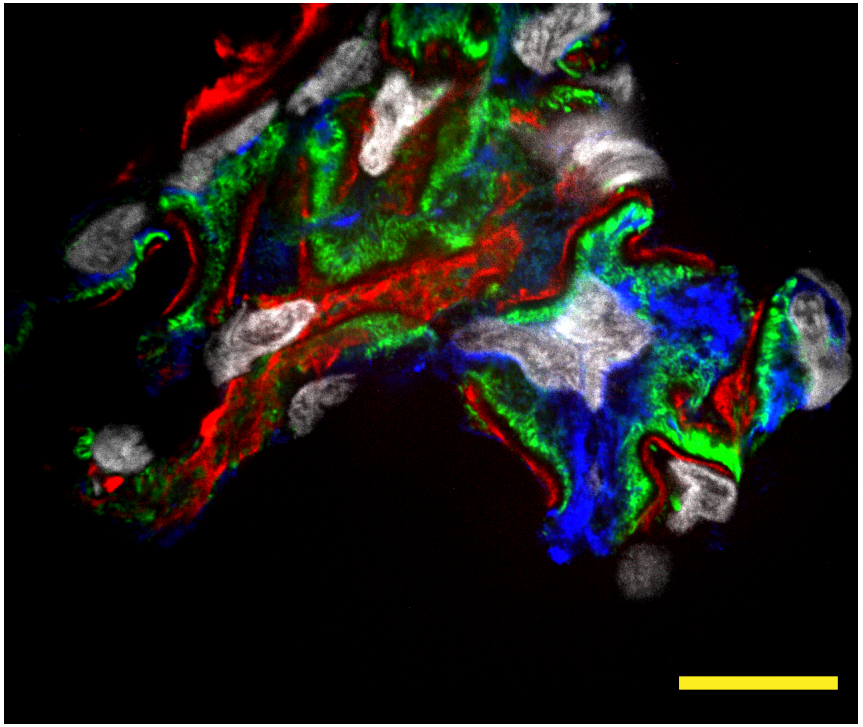

Component	**Stock concentration**	**Stock volume (mL)**	**Final concentration**
Sodium acrylate	0.380 g/mL	2.25	0.086 g/mL
Acrylamide	0.500 g/mL	0.50	0.025 g/mL
N,N'-Methylenebisacrylamide	0.020 g/mL	0.50	0.001 g/mL
Sodium chloride	0.292 g/mL	4.00	0.117 g/mL
PBS	10x	1.00	1x
Water		1.15	
Total Volume		9.40	

Final amount per 10 mL

0.86 g
0.25 g
0.10 g
1.17 g
1x

Table of Materials

Click here to access/download;Table of Materials;60195_ExPath Materials_Revision_v1.xlsx

Name of Reagent/ Equipment	Company	Catalog Number
4-hydroxy-TEMPO (4HT)	Sigma Aldrich	176141
6-well glass-bottom plate (#1.5 coverglass)	Cellvis	P06-1.5H-N
Acetone	Fischer Scientifc	A18-500
Acrylamide	Sigma Aldrich	A8887
Acryloyl-X, SE (AcX)	Invitrogen	A20770
Agarose	Fischer Scientifc	BP160-100
Ammonium persulfate (APS)	Sigma Aldrich	A3678
Anti-ACTN4 antibody produced in rabbit	Sigma Aldrich	HPA001873
Anti-Collagen IV antibody produced in mouse	Santa Cruz Biotech	sc-59814
Anti-Vimentin antibody produced in chicken	Abcam	ab24525
Aqua Hold II hydrophobic pen	Scientific Device	980402
Breast Common Disease Tissue Array	Abcam	ab178113
DAPI (1 mg/mL)	Thermo Scientific	62248
Diamond knife No. 88 CM	General Tools	31116
Ethanol	Pharmco	111000200
Ethylenediaminetetraaceticacid (EDTA) 0.5 M	VWR	BDH7830-1
FFPE Kidney Sample	USBiomax	HuFPT072
Forceps		
Goat Anti-Chicken IgY (H+L), Highly Cross-Adsorbed CF488A	Biotium	20020
Goat Anti-Chicken IgY (H+L), Highly Cross-Adsorbed CF633	Biotium	20121
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Alexa Fluor 546	Invitrogen	A11010
MAXbind Staining Medium	Active Motif	15253
MAXblock Blocking Medium	Active Motif	15252
MAXwash Washing Medium	Active Motif	15254
Micro cover Glass #1 (24x60mm)	VWR	48393 106
Micro cover Glass #1.5 (24x60mm)	VWR	48393 251
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma Aldrich	T9281
N,N′-Methylenebisacrylamide	Sigma Aldrich	M7279
Normal goat serum	Jackson Immunoresearch	005-000-121
Nunclon 4-Well x 5 mL MultiDish Cell Culture Dish	Thermo Fisher	167063
Nunclon 6-Well Cell Culture Dish	Thermo Fisher	140675
Nunc 15mL Conical	Thermo Fisher	339651

Nunc 50mL Conical	Thermo Fisher	339653
Orbital Shaker		
Paint brush		
pH Meter		
Phosphate Buffered Saline (PBS), 10x Solution	Fischer Scientifc	BP399-1
Plastic Petri Dish (100 mm)	Fischer Scientifc	FB0875713
Proteinase K (Molecular Biology Grade)	Thermo Scientific	EO0491
Razor blade	Fischer Scientifc	12640
Safelock Microcentrifuge Tubes 1.5 mL	Thermo Fisher	3457
Safelock Microcentrifuge Tubes 2.0 mL	Thermo Fisher	3459
Sodium acrylate	Sigma Aldrich	408220
Sodium chloride	Sigma Aldrich	S6191
Sodium citrate tribasic dihydrate	Sigma Aldrich	C8532-1KG
Tris Base	Fischer Scientifc	BP152-1
Triton X-100	Sigma Aldrich	T8787
Wheat germ agglutinin labeled with CF640R	Biotium	29026
Xylenes	Sigma Aldrich	214736

Comments/Description

Inhibitor

Initiatior

Nuclear stain

Can be substituted with non-commercial staning buffer of choice.
Can be substituted with non-commercial blocking buffer of choice.
Can be substituted with non-commercial washing buffer of choice.

Accelerator

For preparing blocking buffer. Dependent on animal host of secondary antibodies.
Multi-well plastic culture dish

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Author(s):	Aleksandra Klimas, Octavian Bucur, Brigdet Njeri, Yongxin Zhao

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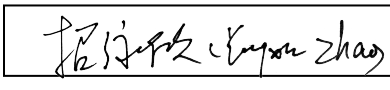
Signature:	
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We thank the editor and reviewers for their comments and suggestions, as well as the opportunity to respond. Accordingly, we have addressed concerns raised by the reviewers and revised the manuscript with addition of new figures (Figures 5 and 6) and example data as per their suggestions. Please see our responses below in blue.

Editorial comments:

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The text has been modified accordingly.

4. Please do not number the Table of Materials in the manuscript.

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The text has been modified accordingly and the Table of Materials has been updated.

6. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

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The text has been modified accordingly.

8. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing)

to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

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Reviewer #1:

Manuscript Summary:

Excellent Manuscript

Major Concerns:

None

Minor Concerns:

None

We thank the reviewer for the kind review.

Reviewer #2:

Manuscript Summary:

The manuscript by Klimas et al. reports on a method to analyze pathology samples (Expansion Pathology), prepared and archived using common methods in the pathology lab, with improved spatial resolution compared to traditional sample preparation methods. The method, in brief, is a physical one for expanding the sample, thus creating larger structures to image using a traditional optical microscope.

Major Concerns:

The method is very similar to the authors' reported work in their 2016 Nature Biotechnology article, however, in many respects the already published method in Nature Biotechnology is clearer and easier to follow than the outline provided in this submission. This also appears to be a protocol that may have considerable variability across labs or users. As a result, several points for clarification should be considered.

We thank the reviewer for the comments, which give us the opportunity to further clarify the ExPath technology and better inform the readers on the applicability of the protocol.

1. The description of the super resolution imaging techniques on page 2 is misleading and should be corrected. The authors group all these imaging technologies into one to generate a list of negative qualities, which only a subset of these qualities may apply for a given technique or experiment. Thus the selection of one of these common super resolution techniques versus the expansion technology technique is much more nuanced than the authors present. For example, STORM can be performed on nearly any microscope and many more "traditional" fluorophores have been used for STORM in recent years.

We agree with the reviewer that every imaging technology comes with their pros and cons, and we are also aware of the recent, rapid advancement of other super resolution imaging techniques. Accordingly, the text has been modified to reflect the nuance of the different techniques (lines 50-54) to better aid readers in choosing the most suitable approach for answering their specific scientific questions. While STORM has become increasingly accessible to the community, it still requires modification of a conventional fluorescent microscope. Due to the principle of STORM, it is essential to choose fluorophores with robust photo-switching capability to generate high-quality STORM images. It is worth noting that ExPath is essentially a method of sample preparation and is orthogonal to other imaging techniques. Because it does not rely on modification of the optical system, it can also be used synergistically with other super resolution techniques, including STORM, to gain additional resolution.

2. The limitations of the Expansion Pathology technique should be made clearer. For example, is there a limit to the size or thickness of sample that may be suitable for this technique? Perhaps sample age is another consideration? Are there limits to the type of tissue?

As presented, the ExPath protocol is designed to work with standard clinical tissue sections in varied formats (FFPE, H&E stained, and fresh frozen specimens) and has been validated in 5-10 µm-thick clinical tissue sections over a broad range of tissue types. Thicker tissues require longer proteinase K digestion and the digestion time is highly dependent on tissue types and thickness. We encourage users to explore various digestion temperature and time on new tissue type that is not previously reported for ExPath imaging. Accordingly, we have modified the text to clarify this (lines 411-412).

3. While the authors report an effective resolution of ca. 70 nm in their published work, this is not acceptable and misleading. The effective resolution will depend on the numerical aperture of the objective and the wavelength of light they are using (as described by Abbe). The authors report using a 10x and 20x objective (critically, they don't report the numerical aperture). A 70-nm resolution could correlate to using 400-nm light and a 0.6 numerical aperture objective, but the authors need to make it clear that this is highly dependent on the imaging system (the parameters stated above). A more general equation for the spatial resolution that may be achieved (e.g., that based on the Abbe equation) is warranted. Using a high-quality 100x objective, one may reasonably achieve 140 nm resolution (and easily tile many images for a larger field of view) without any of the reported time-consuming sample processing.

We agree with the reviewer that the total resolution is indeed dependent on the specifics of the imaging system used. The text has been modified to emphasize that ExPath improves the effective resolution by the factor of expansion and the role of the NA of the chosen optics has been emphasized (lines 44-46, 72-71). Effective resolutions are now given in terms of the NA of the objective, assuming use with 532 nm light, per convention.

4. The reference to the use of DAPI in steps 2.4.5 and 2.7.4 is confusing. Are both these optional? Why is the second use in step 2.7.4 required?

Use of DAPI is optional, per the desire of the user. DAPI must be re-stained after expansion as the DAPI dye is washed away during the proteinase K digestion at 60 C or higher.

Minor Concerns:

5. Some of the language the authors use is too informal, which may generate confusion. For example, bottom of page 2 "brought up."

The text has been modified for clarity.

6. The notation 4x is used for both indicating the number of times to perform a step and a concentration. Consistent use this notation would provide better clarity (also 4-fold is sometimes also used in addition to 4x).

We thank the reviewer for the suggestion. The text has been modified for clarity.

7. The Figure legend 4C & E requires editing for clarity, same region highlighted in A....

We thank the reviewer for bringing this to our attention and the legend has been modified accordingly.

8. While the focus on the manuscript is not the imaging step, the imaging details are sparse. For example, perhaps it needs to be clear that the location of image collected pre-expansion will need to be again identified post-expansion and how this might be done, etc.

We thank the reviewer for the suggestion and we have emphasized the purpose of obtaining pre-expansion images (step 2.3.7).

Other:

Required equipment is not listed.

Additional equipment has been added to the required equipment list.

Step 2.1.1. what shaker speed/range is required.

This has been added in the text.

Step 2.2, should this refer to all samples, since each step above referred to different sample preparation methods?

This is needed for all samples and has been clarified in the text.

There is a typo in step 2.2.1; there appears to be a numbering issue between 2.3 and 2.4, and again at 2.6.1.

With the given layout, AcX is not defined on first usage.

This has been fixed in the text.

Reviewer #3:

Manuscript Summary:

This manuscript describes the ExPath protocol, a previously published adaptation of expansion microscopy specifically geared towards the processing of pathology specimens. In particular, ExPath describes how to achieve expansion of samples obtained from the various fixation and preparation methods commonly employed in clinical practice to obtain and store pathology samples. The ExPath protocol achieves this by combining previously established methods for processing such samples for immunohistochemistry with the previously established proExM protocol (currently the most widely used approach to expansion microscopy). The result is a protocol that achieves up to 4.5-fold expansion of clinical samples, for a resolution of up to 70 nm.

While this protocol is generally straightforward and should be rather easy to implement for most clinical or biology laboratories from the original publication (Zhao & Bucur et al., Nature Biotechnology, 2017), a concise and visual protocol will surely be useful to many scientists nonetheless. The protocol presented here generally satisfies these requirements - it is generally easy to follow, concise, and clear. I have no major concerns precluding publication in principle, but the one major concern I have should be addressed, as without this it is almost impossible for the reader to evaluate the usefulness of the technique for all applications the protocol claims to cover. There are several minor concerns that should be addressed in full before publication as well. These minor concerns mainly pertain to ambiguous language, to missing explanations for some recommendations given, and to potentially too abbreviated guidelines for scientists without prior experience with expansion microscopy to follow successfully.

If these issues can be addressed in full (as I have no doubt they can), this manuscript should be highly considered for publication.

We thank the reviewer for the comments, and we have added additional example data and clarification in the text to better assist the reader in using the ExPath protocol.

Major Concerns:

1) The authors strive to provide a protocol for differently prepared types of clinical pathology samples (steps 2.1.1.-2.1.4.). Yet, they only show one example, for the preparation type described in step 2.1.1. (Figure 4). I highly recommend to show an example for each of the four sample preparation approaches this protocol encompasses (steps 2.1.1.-2.1.4.). Otherwise, it will be impossible for the reader to evaluate the potential usefulness of this protocol for their application. In short, every general case the protocol claims to cover should be demonstrated with at least one example.

We thank the reviewer for this suggestion and additional examples have been included in the text (Representative Results Section) and Figure 5 and 6 have been added. The text has also been changed to emphasize the format conversion of the samples is not necessarily specific to ExPath and can be used on any of the standard formats used in clinical biopsy samples.

Minor Concerns:

1) As in the original expansion microscopy publication and many follow-up manuscripts, concentrations for the gel components for the monomer solution are given in "g/100 ml" (Table 2; page 2, lines 79-83, step 1.1.1.). This is an unusual unit, and it can cause confusion during conversions to calculate the amounts required for preparing monomer solution. I suggest to convert all amounts given in this way to the more intuitive "g/ml". I further suggest to provide not only the amounts of stock solution necessary to prepare the monomer solution in Table 2, but also the amounts of each component in weight for preparing the monomer solution without stock solutions (as given on page 2, lines 79-83, step 1.1.1.).

The units have been changed to g/mL to reflect that w/v% is used (Step 1) Example volumes are also provided, and the fact stock solution volumes can be scaled up or down as needed is also emphasized.

2) The manuscript notes that APS solution should typically be prepared "immediately before preparing the gelling solution" (page 3, lines 95-96, step 1.1.2.). This differs from the recommendation for the preparation of the other solutions used in step 1.1.2., namely monomer solution, TEMED, and TEMPO. It would be useful if the authors could clarify why APS is prepared with this timing by them, and what they understand by "typically" - is this an absolute requirement, is it just their idiosyncratic practice, will this influence the outcome of the protocol? Whatever the reasoning for preparing the APS solution with this timing, the authors should make it explicit.

This has been clarified in the text (lines 421-424); APS is not stable in solution at RT or when undergoing freeze-thaw cycles. To obtain a more consistent result, freshly preparing APS is recommended, based on our own experience. In fact, it is also more convenient since preparing fresh APS does not require the time-consuming thawing step.

3) The authors should specify the type and manufacturer of hydrophobic pen they use (page 4, lines 154-156, step 2.4.1.), as the quality of these products varies widely, with potential implications for later steps of the protocol (also see comment 6)).

This has been added to the equipment list.

4) The authors make extensive use of commercial products for blocking, staining, and washing during the immunostaining procedure: MAXblock(TM) Blocking Medium (page 4, lines 158-159, step 2.4.2.), Maxbind(TM) Staining Medium (page 4, lines 161-164, step 2.4.3.), and MAXwash(TM) Washing Medium (page 4, lines 169-170, step 2.4.4.). This can pose a problem to many laboratories, as such commercial solutions are typically much more expensive than typically used self-made solutions for

blocking, staining, and washing during immunohistochemistry. To make the protocol more accessible (with accessibility being one of the stated major advantages of ExPath over other super-resolution methods; page 9, lines 353-359), I recommend to include alternative formulations of these buffers that can be prepared in the lab.

We have added an optional blocking buffer solution (step 1.5; step 2.3) that can be used in place of the commercially available buffers and have emphasized that the reader can also use their buffer of choice if preferred.

5) The authors should state their reasoning for adjusting the concentration of AcX when anchoring samples fixed with aldehyde fixatives or non-aldehyde fixatives (page 5, lines 193-195). It seems counter-intuitive to increase the concentration of AcX for samples fixed with non-aldehyde fixatives (0.3 mg/ml) compared to samples fixed with aldehyde fixatives. AcX reacts with the same functional chemical groups as aldehyde fixatives (primary amines), which suggests that aldehyde fixatives reduce the number of available targets for anchoring. This in turn would suggest that it would be desirable to increase the concentration of AcX in samples fixed with aldehyde fixatives, to better saturate the available remaining primary amines. Maybe the authors also reason that less available targets for AcX means that less AcX is required (assuming that AcX is not supplied in vast excess, which would be my assumption, in both fixation with aldehyde fixatives and non-aldehyde fixatives). In either case, the authors should share their reasoning here. Also, the unit is missing from the concentration for samples fixed with non-aldehyde fixatives (page 5, line 194); this unit is presumably mg/ml.

We have clarified that 0.03 mg/mL AcX is used for non-aldehyde fixed samples and 0.1 mg/mL AcX is used for aldehyde fixed samples due to more available amines in the non-aldehyde fixed samples (step 3.1.1).

6) The authors should provide information and guidance on whether (and if so, how) to remove the hydrophobic pen applied in step 2.4.1. (page 4, lines 154-156) before constructing the gelling chamber in step 2.6.3. (pages 5-6, lines 216-223). Can the pen be left on, does it interfere with the gel in any way, does it need to be removed, and if yes how?

The content of hydrophobic pen does not interfere the polymerization reaction, and it can be left on the slide. This is clarified in the text (step 2.3.1).

7) A common problem in expanding tissue slices is that the slice is embedded not perfectly straight and flat, leading to microscopic waves in the sample that translate to even bigger waves after expansion. This often makes imaging difficult. Can the authors provide guidelines on how to avoid this during embedding of the slices into the gel (pages 5-6, lines 216-223, step 2.6.3.)?

As the protocol aims to conserve the morphology of sample and scale it proportionally to the expansion factor, the protocol unfortunately cannot fix the artifact caused by the sectioning and mounting process prior to the ExPath procedure.

8) The authors should give their reasoning for placing the gel sample-down into the 6-well glass bottom plate after digestion (page 6, lines 248-251, step 2.7.3.). I assume that this is to have the tissue close to the glass bottom for imaging later on, but it would help the novice user to have this stated explicitly.

We thank the reviewer's suggestion. This is indeed to reduce the distance from the sample to the imaging objective. The importance of minimizing this distance has been emphasized in the text (step 5.1 note).

9) The authors state that the expanded gel can be immobilized with 1.5-2% low melt agarose for imaging (page 7, line 275, step 2.8.). The authors should detail how this is done and include low melt agarose in their list of materials (Table 1).

As suggested, a protocol for immobilizing the gel in low melt agarose has been added to the text (step 5.3 note) and the material has been added to the list of materials.

10) The authors state that the gel they employ expands "by a factor f ~4.5x in water [...], providing an effective resolution of ~70 nm" (page 7, lines 278-279). Both statements are accurate, but only for the best possible case. It is quite common for the gel composition employed here to fluctuate in expansion factor between 3.0 and 4.5 - with resolution decreasing in direct correspondence to the expansion factor. This is attested by daily use in many laboratories and documented in the literature. To not overstate the case, the authors should revise their phrase to account for this expected variability.

We thank the reviewer for pointing out the need for clarification. The range of expansion has been modified in the text (lines 333-335) and the reliance of the effective resolution change on this expansion factor has been emphasized throughout the text.

11) The authors claim that increasing the EDTA concentration in the digestion buffer to 25 mM is necessary to achieve complete homogenization of formalin-fixed tissue samples (page 8, lines 320-321). It is unclear why this should be the case, especially considering that calcium ions (removed by chelation with EDTA) are claimed to increase the stability of proteinase K (Truckenbrodt et al., Nature Protocols, 2019). In this light, one might expect that reducing the amount of EDTA in the digestion buffer would improve results, rather than increasing it. The authors should comment on this and explain their reasoning for increasing the concentration of EDTA.

This has been more thoroughly demonstrated in the original ExPath paper (Zhao et al. Nature Biotechnology 2017) and the text has been altered to more explicitly reference the text (lines 409-411).

12) The authors state that making a new stock solution of AcX can address problems with insufficient anchoring resulting in tears or distortions of the sample. It has been suggested in the past that adjusting the composition of the anchoring buffer can also lead to improvements, particularly for tissue slices (MES buffer, suggested by Tillberg/Chen et al., Nature Biotechnology, 2016). The authors should comment on this.

The anchoring buffer presented here has been optimized for the ExPath protocol. Per the reviewer's suggestion, we have added information to the text concerning the storage of AcX (lines 424-427).

13) The authors state that proteinase K can lose activity over time, potentially impacting results (page 8, lines 344-345). The authors should provide guidelines on the optimal storage conditions to preserve proteinase K activity, and on its expected shelf-life.

Storage requirements and expected shelf life of ProK have been added to the text (lines 438-440).

Reviewer #4:

Minor Concerns:

As the authors state in the Discussion,last paragraph: "Although Pro(teinase) K provides even expansion of the sample after homogenization, the loss of proteins prevents interrogation of other targets of interest post-expansion. However, the protocol can be easily performed in any regular wet lab." The technique may not achieve high levels of accuracy because of this but benefits from its simplicity . It seems that the preservation of fluorescence signals for the specific antigens could depend on the anchorage of secondary antibodies and efficacy of ProK digestion. For example, in Figure 4 B and C, there was a clear loss of the red signal (a-ACTN4: podocytes) and some loss of the green signal (Vimentin: mesangial cells?). It is not clear that if the figure 4 C explicitly shows a-ACTN4 positive podocyte food processes. Podocyte foot processes of aTdTomato transgenic animal can be imaged with conventional microscopes.

https://jasn.asnjournals.org/content/23/5/785

The authors may need further investigate varying anchoring and ProK digestion protocols to optimize the staining with various primary and secondary antibodies anchoring to avoid false negative staining.

We thank the reviewer for the comments, and we have added additional example data (Figures 5 and 6; Representative Results Section) and clarified the post-expansion stain quality is dependent on the method the specimen was originally preserved (lines 356-357).

Reviewer #5:

Manuscript Summary:

The authors describe the protocol for Expansion Microscopy in depth and it would be useful for researchers performing this technique to see the detailed video protocol. The protocol is detailed, and covers all aspects of the technique. The written protocol itself is not straight forward to be adopted by non-experts but combined with a video tutorial, it will be more comprehensive and clear. The protocol is sufficient for publication in JoVe and will be useful for non-experts int he field.

We thank the reviewer for the comments, and we have modified the text to address the minor concerns.

Minor Concerns:

Minor suggestions, which the authors can clarify in their final revision.

1. The steps to prepare monomer stock solution in 1.1.1 are not clear. The authors describe the amounts for 100 mL buffer but write a note that final volume of monomer stock solution is 9.4 mL. They can clarify this.

The units have been changed to g/mL to reflect that w/v% is used and clarified necessary volumes of stock solutions (step 1).

2. In the step 2.1.1 it is not clear what does 2x mean? Does it mean 2 times solution?

The text has been modified for clarity and consistency.

3. In the step 2.2.1 what solution is at 60 C?

The text has been modified to clarify citrate solution is used (step 2.2.2).

4. Finally, the four critical steps described in the protocol would be better explained with additional pictures. Hopefully this will improve with clear instructions in the video recording of the article.

We agree with the reviewer that the video recording will hopefully provide better clarification for the critical steps.5. Cite Asano et. al. protocol published in Current Protocols in Cell Biology

The protocol citation has been added.