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Nanoscale imaging of clinical specimens using conventional and rapid-expansion pathology

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In pathology, microscopy is an important tool for the analysis of human tissues, both for the scientific study of disease states and for diagnosis. However, the microscopes commonly used in pathology are limited in resolution by diffraction. Recently, we discovered that it was possible, through a chemical process, to isotropically expand preserved cells and tissues by 4–5× in linear dimension. We call this process expansion microscopy (ExM). ExM enables nanoscale resolution imaging on conventional microscopes. Here we describe protocols for the simple and effective physical expansion of a variety of human tissues and clinical specimens, including paraffin-embedded, fresh frozen and chemically stained human tissues. These protocols require only inexpensive, commercially available reagents and hardware commonly found in a routine pathology laboratory. Our protocols are written for researchers and pathologists experienced in conventional fluorescence microscopy. The conventional protocol, expansion pathology, can be completed in ~1 d with immunostained tissue sections and 2 d with unstained specimens. We also include a new, fast variant, rapid expansion pathology, that can be performed on <5-μm-thick tissue sections, taking <4 h with immunostained tissue sections and <8 h with unstained specimens.

Introduction

Optical microscopy is one of the most important technologies used in biology and medicine. Conventional optical microscopy reveals details of specimens by optically magnifying specimen images through lenses. However, the resolution of such microscopes is restricted by the diffraction limit to ~250 nm or so. The development of super-resolution microscopy (SRM) has enabled smaller structures to be visualized[1–5]. However, such techniques require specialized equipment and extensive training and are difficult to apply to extended objects, such as tissue specimens, due to their slow speed or physical limits. Such techniques can also require custom labels and dyes, which can limit the number of colors that can be detected in a specimen. Perhaps because of these challenges, SRM techniques have not often been applied to human tissues, such as those studied in pathology and other biomedical sciences.

We recently discovered that a dense (i.e., spaced nanometers apart) mesh of swellable polymer hydrogel (sodium polyacrylate) could be synthesized throughout a preserved biological specimen. Through synthesis of this hydrogel, anchoring key biomolecules or labels (e.g., proteins, RNA and DNA) to the polymer, mechanically softening the tissue (i.e., disrupting protein-protein interactions or even entire sets of proteins that are no longer needed) and swelling of the polymer-specimen composite, it was possible to evenly expand a biological specimen by 4–5× in linear dimension (i.e., length, width and height)[6–9]. Embedding of tissues in hydrogels for imaging purposes has a long

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history, going back to the early 1980s[10]. We call the physical magnification concept expansion microscopy (ExM). It is useful because it enables nanoscale resolution imaging on ubiquitous, conventional diffraction-limited microscopes[11]. After physical magnification, molecules within a diffraction-limited region are separated and can therefore be distinguished by ordinary microscopes. Since diffraction-limited microscopes (e.g., widefield, spinning disk confocal and lightsheet microscopes) can image very quickly, ExM greatly facilitates nanoscale resolution imaging of large specimens, such as human tissues.

We designed the polymer composition, polymer architecture, mechanical softening and physical expansion to enable the expansion process to be as even as possible. The process has also been validated to have low distortion (e.g., a few percent over length scales of tens to hundreds of microns) in various species and tissue types[6–9]. ExM can utilize anchoring molecules that link proteins (including expressed fluorescent proteins and applied fluorescently labeled antibodies) to the polymer, resulting in protein retention ExM (proExM for short) protocols[7,12,13], or anchoring molecules that link RNA to the polymer for post-expansion fluorescent in situ hybridization (FISH) imaging (expansion microscopy FISH)[9]. Most methods of expansion increase sample size by ~3× (for expansion microscopy FISH) to ~4.5× (for proExM) in linear dimension, meaning that a 300-nm resolution lens would have a new effective resolution ranging from 300/4.5 to 300/3, or ~ 60–100 nm, depending on the protocol used. In addition, the process of polymer embedding and expansion can be applied twice to the same sample, resulting in ~4.5 × 4.5 (~20×) linear expansion, or a resolution of 300/20, or ~20 nm (the resolution is slightly larger than the theoretical resolution of 15 nm because it is limited by the size of the antibody, when applied before expansion) via a process known as iterative ExM[14].

For non-clinical, non-pathology specimens, detailed protocols have been published elsewhere[15], and reviews giving an overview of the technique have been published that explore the history of the field and relevant concepts[11,16,17]. For clinical and pathology specimens, we recently developed a proExM variant that we call expansion pathology (ExPath for short; Fig. 1a)[8]. ExPath is capable of processing clinical specimens, including formalin-fixed paraffin-embedded (FFPE), hematoxylin and eosin (H&E)-stained and/or fresh frozen and fixed human tissue specimens on glass slides, so that they then are ready for expansion. Using steps optimized and streamlined for clinical and pathology specimens (Fig. 1a), including a strong mechanical digestion step, ExPath enables expansion of a broad range of preserved human tissues, such as, but not limited to, breast, prostate, ovary, lung, colon, kidney, liver, pancreas, skin and lymph node. Using our previously published method (conventional ExPath), here described in protocol form, the whole expansion process takes ~1 d to complete. Taking into account the time for specimen pre-processing and immunostaining, conventional ExPath requires 2 d to complete. We also describe here for the first time a rapid form of ExPath (rExPath), which requires only 4 h to complete for immunostained tissue sections or <8 h to complete if started with unstained specimens. rExPath is derived by optimizing parameters of immunostaining, gelling and proteinase K digestion for minimal processing time, while still enabling low-distortion expansion of tissues. rExPath works only for thin tissue sections (≤5 μm).

Overview of the ExPath procedure

ExPath begins by (Fig. 1b) converting human tissue specimens prepared in various ways into a state appropriate for expansion. Shown are both the steps for conventional ExPath and rExPath. For example, FFPE samples are deparaffinized using a series of immersions in xylene, ethanol and water. Tissues are then placed in 100 °C citrate buffer, so that the temperature of the buffer gradually decreases to 60 °C during 30 min of incubation in a 60 °C incubator, as a suggested antigen retrieval step, followed by standard immunostaining. We encourage users to apply their own antigen retrieval methods and buffers, to achieve the best performance on immunostaining. Indeed, since antigen retrieval and immunostaining are performed before expansion, these steps can, in general, be performed in whatever way is most familiar to the practitioner, since these steps will not be affected by any expansion microscopy procedures, which occur later. To facilitate calculation of the expansion factor, or to provide large-scale low-resolution survey data, the stained tissue can be imaged in the pre-expansion state on a conventional microscope (optional). Then, the specimen is treated with Acryloyl-X, a small molecule that reacts with amines on proteins to attach a small group that can be linked into the polyacrylate gel. The specimen is then immersed in a solution containing the monomer sodium acrylate, which then undergoes free radical polymerization for in situ polymer synthesis, yielding a hydrogel-tissue composite specimen. The gelled specimen is then incubated with

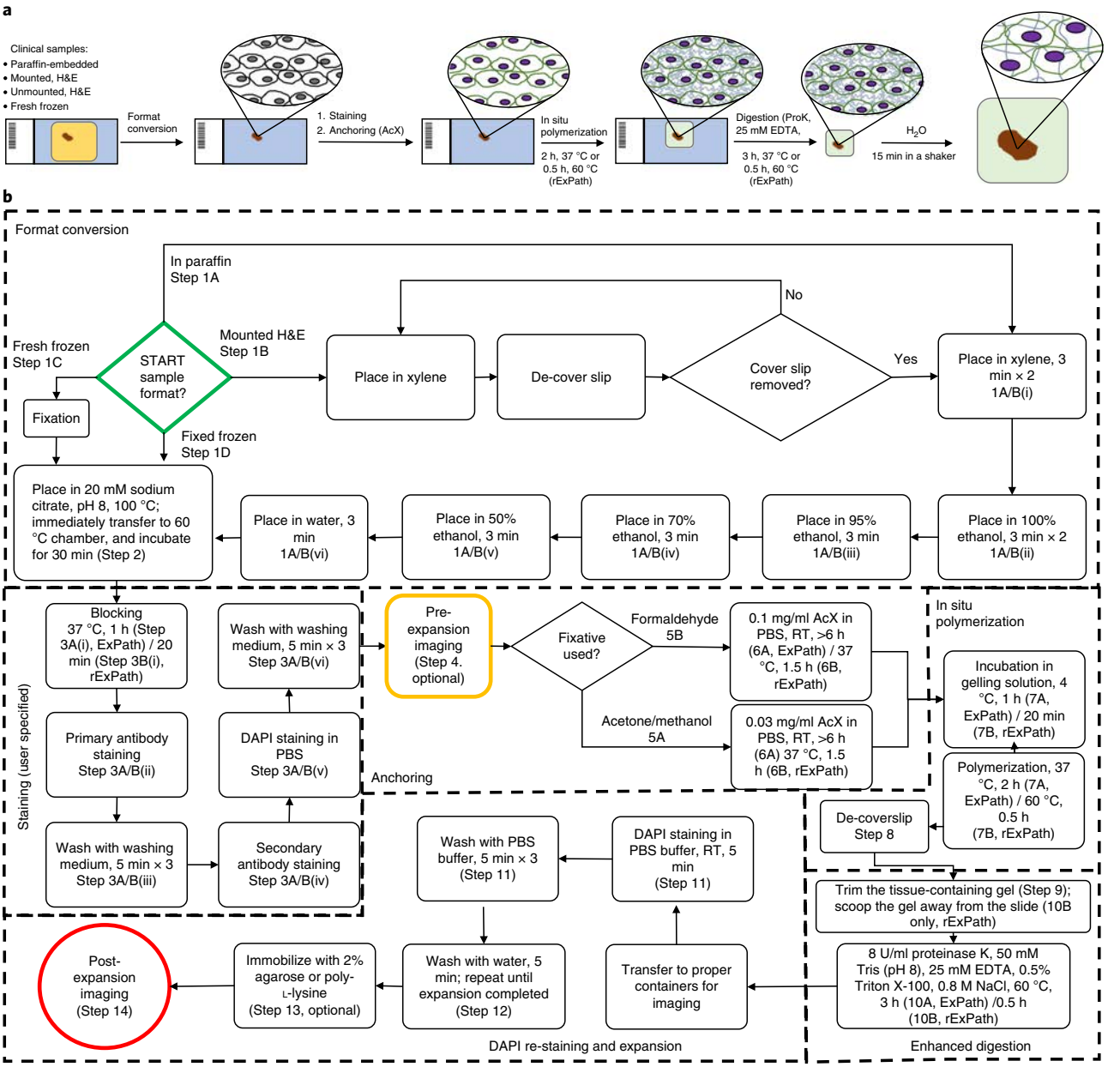

Fig. 1 | Workflows of conventional (ExPath) and rapid (rExPath) expansion pathology. a, Schematic of ExPath/rExPath workflow (details in **b**). ProK, proteinase K. **b**, Detailed outline of ExPath/rExPath workflow. Notation such as 1A/B(vi), refers to steps in the text.

a digestion solution that disrupts the mechanical properties of the specimen so it can be expanded. After mechanical homogenization, the hydrogel-embedded specimen can be expanded by dialysis in water. Osmotic force draws water into the sample, and as the polymer threads expand away from each other, the charged polymer chains repel one another yet further, resulting in large-scale expansion of the tissue-gel hybrid.

Comparison with other approaches

SRM approaches

Due to their complexity, slow speed and/or high cost of equipment, classical SRM methods such as stimulated emission depletion (STED) microscopy[18,19], super-resolution structured illumination microscopy (SIM)[20,21], photoactivated localization microscopy[5], stochastic optical reconstruction microscopy[22] and DNA points accumulation for imaging in nanoscale topography[23], have not found

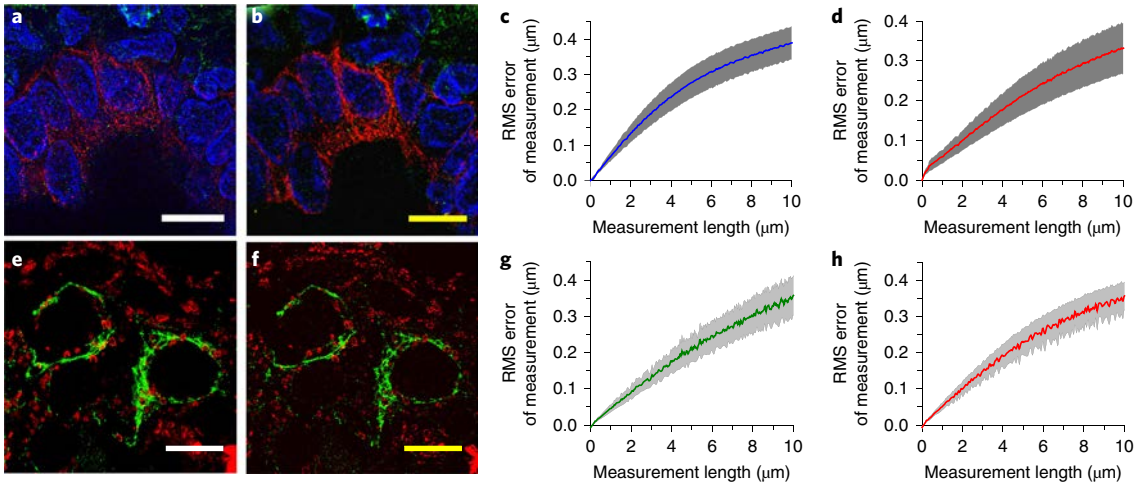

Fig. 2 | Validation of conventional (ExPath) and rapid (rExPath) expansion pathology. a, Super-resolution SIM image of normal human breast tissue. Blue, DAPI; green, anti-vimentin; red, anti-KRT19. **b**, ExPath image of the specimen in **a** acquired with a spinning disk confocal microscope. **c** and **d**, Root-mean square (RMS) length measurement error as a function of measurement length for ExPath versus super-resolution SIM images of human breast tissue (blue solid line, mean of DAPI channel; red solid line, mean of KRT19 channel; shaded area, s.e.m.; $n = 5$ fields of view from specimens from four different patients; average expansion factor: 4.0 (s.d.: 0.2)). Scale bars: 10 μm (**a** and **b**) (scale bars of the color yellow indicate biological scale throughout; physical size after expansion, 43 μm; expansion factor: 4.3). **a** and **b** adapted from ref. [8]. **e**, STED image of normal human breast tissue. Green, anti-vimentin; red, anti–voltage-dependent anion channel (VDAC). **f**, rExPath image of the specimen in **e** acquired with a spinning disk confocal microscope. **g** and **h**, RMS length measurement error as a function of measurement length for rExPath versus STED images of human breast tissue (green solid line, mean of vimentin channel; red solid line, mean of VDAC channel; shaded area, s.e.m.; $n = 3$ fields of view from specimens from three different patients; average expansion factor: 4.8 (s.d.: 1.0)). Scale bars: 10 μm (**e** and **f**) (physical size after expansion, 50 μm; expansion factor: 5.0).

routine usage for the imaging of pathological or clinical specimens. In comparison, ExPath requires only hardware to which typical biomedical laboratories already have access, which can enable large areas or volumes to be rapidly imaged. As with previous methods of ExM, both ExPath and rExPath yield low levels of distortion (a few percent over length scales of interest in pathology) (Fig. 2a–h), when compared to other super-resolution methods, such as SIM and STED. However, unlike some SRM methods, ExPath is not compatible with live imaging, since the physical expansion process is not compatible with the living state.

Tissue-clearing methods

Since ExPath expands hydrogel-tissue composites in water, the final product is mostly water, with original biomolecules or labels greatly diluted, and thus samples are highly transparent (Fig. 3a). ExPath also achieves a reduction in autofluorescence (which can be high in heavily formalin-fixed human tissues) due to removal of unanchored molecules by the expansion process (Fig. 3b–h). Other techniques clear tissues by homogenizing the refractive index (RI) within a specimen; for example, See deep brain (SeeDB), 3D imaging of solvent-cleared organs (3DISCO), benzyl alcohol/benzyl benzoate (BABB) and immunolabeling-enabled imaging of solvent-cleared organs (iDISCO)[24–27] utilize solvent-based dehydration and RI matching, Scale and clear, unobstructed brain/body imaging cocktails and computational analysis (CUBIC)[28,29] are based on hyper-hydration–based clearing and clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue-hydrogel (CLARITY) and passive clarity technique (PACT)/perfusion-assisted agent release in situ (PARS)[30,31] use hydrogel-supported lipid removal and RI matching. In contrast, ExPath homogenizes RI throughout specimens via dilution of the components of the tissue-hydrogel composite in water. After expansion, >99% of the volume of the gel is composed of water, and thus the RI of the sample is nearly equal to that of water (1.33), resulting in transparency. Thus, as a byproduct of the goal of increasing resolution, ExPath achieves the side effect of clearing of human tissues.

Limitations

Physical tissue expansion can be performed only on preserved biological specimens, such as fixed cells, tissues and organs/organisms. As with electron microscopy, many SRM methods, and all

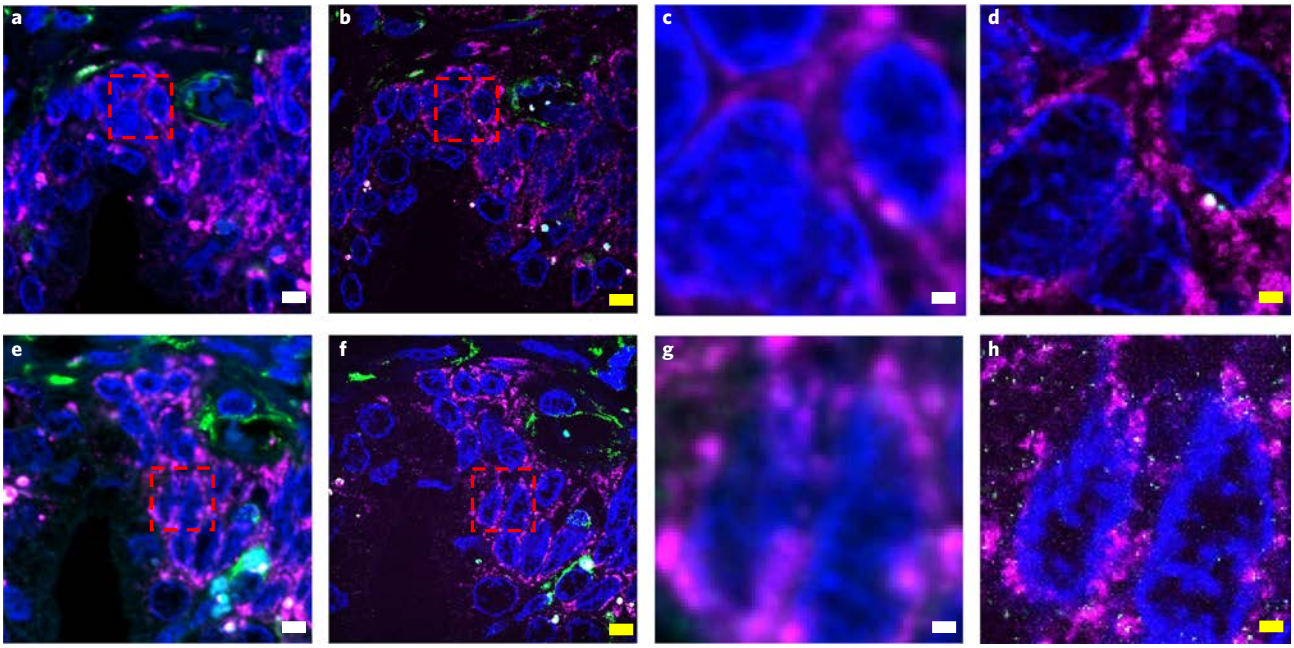

Fig. 3 | Comparison of ExPath and rExPath on adjacent human prostate FFPE tissue sections. a, Pre-expansion image of a normal human prostate FFPE tissue section acquired with a spinning disk confocal microscope. Blue, DAPI; green, vimentin; magenta, VDAC. **b**, ExPath image of the specimen in **a** acquired with the same confocal microscope. Expansion factor: 5.0. **c**,**d**, Fields of view zoomed into the corresponding areas outlined by a dashed red box in **a** and **b**, respectively. **e**, Pre-expansion image of a normal human prostate FFPE tissue section adjacent to that in **a** acquired with a spinning disk confocal microscope with the same staining and imaging parameters. **f**, rExPath image of the specimen in **e** acquired with the same confocal microscope and same imaging parameters. Expansion factor: 5.0. **g**,**h**, Fields of view zoomed into the corresponding areas outlined by a dashed red box in **e** and **f**, respectively. Scale bars (biological scale): 5 μm (**a**, **b**, **e** and **f**); 1 μm (**c**, **d**, **g** and **h**). All the scale bars are yellow in the images of expanded samples (**b**, **d**, **f** and **h**).

clearing methods developed to date, chemical fixation or other forms of preservation are required. Depending on the nature of the fixation step, specific steps of the ExPath/rExPath protocol may vary (Fig. 1b). Current ExPath/rExPath protocols typically aim for the investigation of three to five different protein or DNA targets, limited by the typical three to five different colors that can be distinguished spectrally on a fluorescence microscope. On a practical level, a large specimen, once physically expanded, may need to be trimmed to fit into the designated reservoir for imaging.

The digestion, or mechanical homogenization, step is performed by adding a strong protease, proteinase K, which chops up proteins indiscriminately. Proteinase K is a potent non-specific protease and enables essentially complete mechanical homogenization of the properties of the tissue-gel hybrid, thus enabling full and even expansion during the later water addition step. Because ExPath and rExPath adapt this aggressive proteinase K digestion method, most epitopes in the tissue are destroyed and not available for post-expansion immunostaining. Therefore, pre-expansion immunostaining is necessary, before the anchoring and gelation steps.

Another result of expansion is that imaging time will increase, to accommodate the increased volume of the sample (although expanded samples, being transparent, can be imaged using extremely fast diffraction limited methods, such as light-sheet microscopy[9,32]). For typical experiments, it is advised to image the sample first at low magnification to determine regions of interest, followed by high-magnification imaging of the most relevant regions. Nevertheless, even for typical microscopes common in biology, imaging time for expanded samples can be an order of magnitude, or more, faster than for classical SRM methods.

Experimental design

General considerations and differences between ExPath and rExPath

ExPath/rExPath is a straightforward chemical process. All steps in ExPath/rExPath can be carried out in a standard wet laboratory setting. We recommend having a dedicated microscope with proper excitation and emission filters and objectives, preferably on a vibration isolation table, in a dark room. In general, we recommend first using conventional ExPath, which enables very thorough

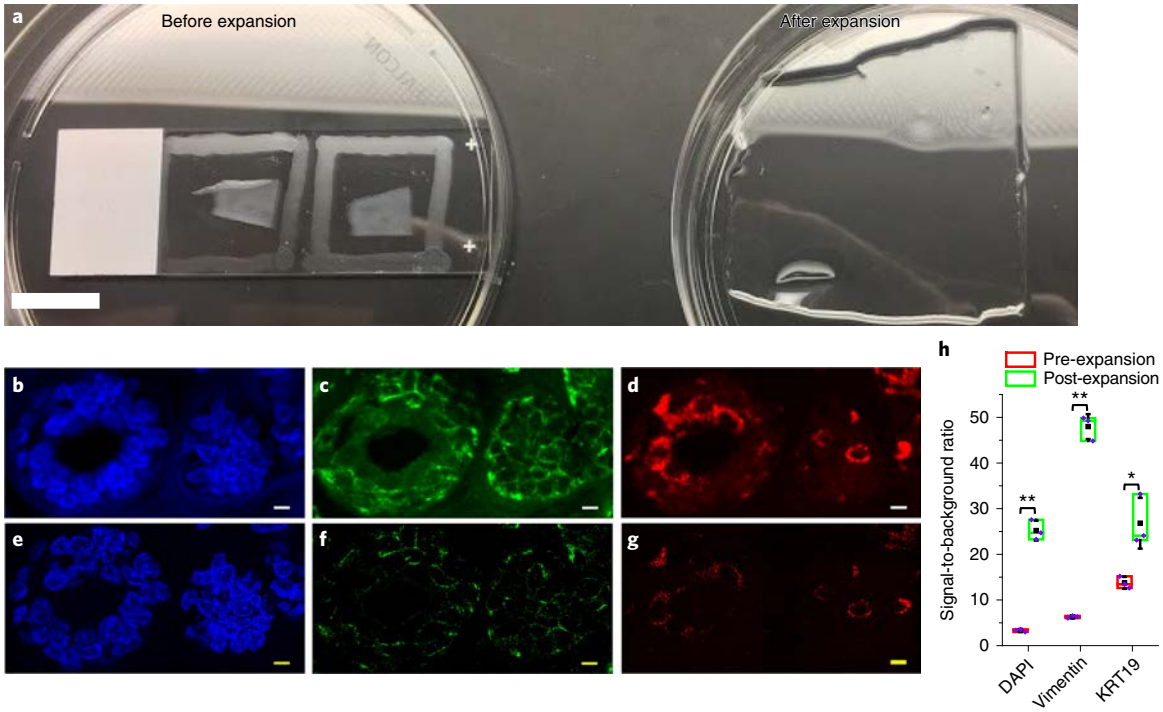

Fig. 4 | ExPath reduction of tissue autofluorescence. a, Photo of human kidney tissue sections before (left) and after (right) expansion with the ExPath protocol. **b**–**g**, Confocal images of normal human breast tissue, labeled with DAPI (blue) and antibodies against vimentin (green) and KRT19 (red), showing pre- (**b**–**d**) and post- (**e**–**g**) expansion data. **h**, Signal-to-background ratio for pre-expansion (red) and post-expansion (green) states of $n = 3$ samples of breast tissue from three patients. Average expansion factor: 4.1 (s.d.: 0.1). **, $P < 0.01$; *, $P < 0.1$; two-tailed paired t-test. The ends of whiskers are defined by the s.d.; the upper and lower boundaries of the box are defined by the maximum and minimum, respectively; the segment in the rectangle indicates the median; and the square symbol indicates the mean. The purple diamond-shaped dots indicate the individual data points. Scale bars (yellow indicates post-expansion image): 15 mm (**a**); 5 μm (**b**–**d**); 5 μm (**e**–**f**) (physical size after expansion, 18 μm; expansion factor: 4.0). **b**–**h** adapted from ref. [8].

proteinase K homogenization, and thus low distortion (Fig. 2a–d), over various sample sizes and thicknesses commonly studied in pathology. For ≤5-μm tissue sections, in time-sensitive applications (such as in a potential clinical setting), rExPath is recommended, since it enables greatly accelerated processing. rExPath is characterized by a low distortion similar to that of ExPath on 5-μm tissue sections (Fig. 2e–h). In addition, a comparison between ExPath and rExPath on 5-μm-thick, adjacent, formalin-fixed prostate sections with identical immunostaining and imaging conditions yielded similar images (Fig. 3), suggesting that decreasing the procedure time in rExPath, including lowering the duration of proteinase K homogenization, does not compromise the quality or resolution of obtained images. In addition, autofluorescence is reduced to the same extent by rExPath as with ExPath. Note that rExPath may not work for thick (e.g., 100-micron-thick) tissue sections. Both ExPath and rExPath can be performed by researchers in all areas of biology and medicine, as long as they have basic operational knowledge of histology, immunostaining and imaging. The availability of a microscope and a computer to analyze the data is also required.

Specimen format and organ type

We have been able to expand fixed human tissues prepared in a wide range of different formats, such as FFPE (ExPath: Fig. 4b–g (breast tissue), Fig. 3a–d (prostate tissue), Fig. 5 (prostate, lung, breast, pancreas, ovary, liver, kidney and colon) and Fig. 6 (lymph node tissue); rExPath: Fig. 2e,f (breast tissue) and Fig. 3e–h (prostate tissue)), H&E-stained (ExPath: Fig. 7a,b (breast tissue); rExPath: Fig. 7e,f (breast tissue)) and frozen human tissue specimens on glass slides (ExPath: Fig. 7c,d (kidney tissue); rExPath: Fig. 7g,h (lung tissue)). We have also been able to expand human tissue specimens from a wide range of organs, including but not limited to breast, prostate, ovary, lung, colon, pancreas, liver and kidney (Fig. 5). We have not yet encountered tissue types that pose problems for ExPath expansion. However, we have not yet tried expansion of human bone or muscle tissue with ExPath/rExPath.

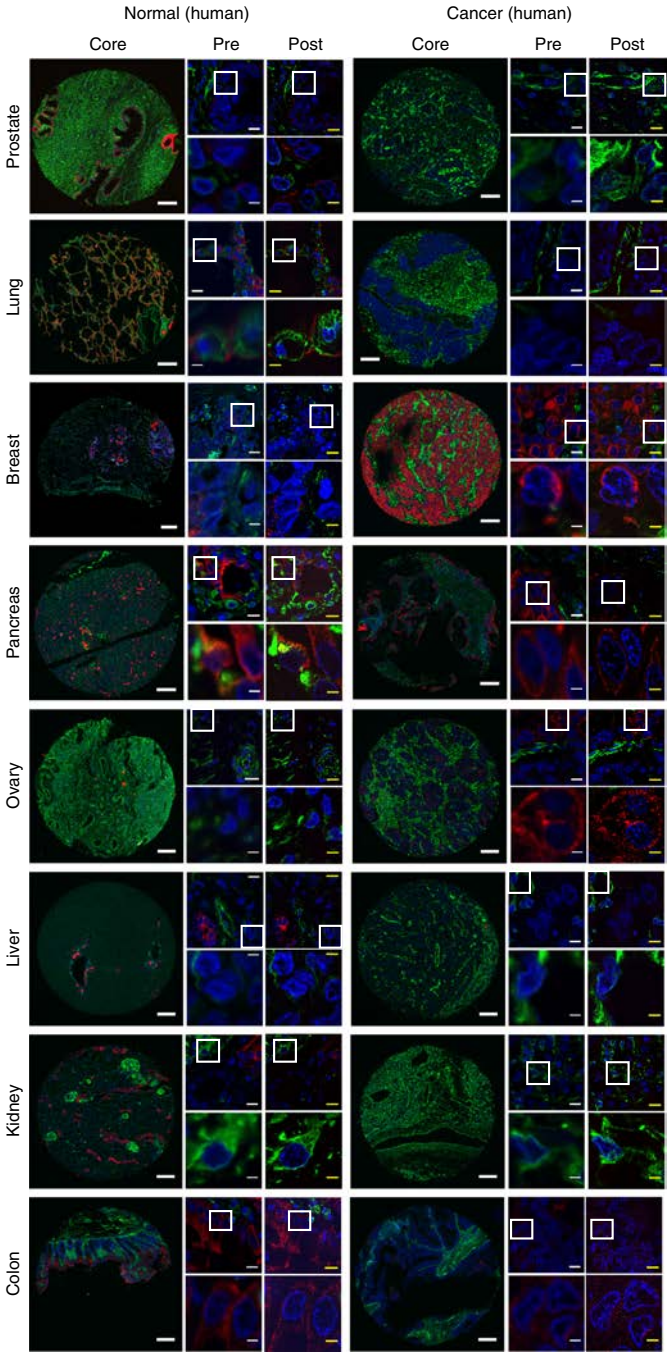

Fig. 5 | ExPath imaging of a wide range of human tissue types. Images of various tissue types for both normal (left images) and cancerous (right images) tissues from human patients. From top to bottom, different rows show different tissue types as labeled (e.g., prostate, lung, breast, etc.)[8]. Within each block of images for a given tissue × disease type, there are five images shown. The leftmost of the five images shows a core from a tissue microarray (scale bar, 200 μm). The middle column within the five images shows two images, the top of which is a small field of view (scale bar, 10 μm), and the bottom of which zooms into the area outlined in the top image by a white box (scale bar, 2.5 μm). The right column within the five images shows the same fields of view as in the middle column but after expansion (yellow scale bars; top images, 10–12.5 μm; bottom images, 2.5–3.1 μm; physical size after expansion, top images, 50 μm; bottom images, 12.5 μm; expansion factors: 4.0–5.0×). Blue, DAPI; green, vimentin; red, KRT19. Adapted from ref. [8] with permission.

Tissue fixation and format conversion

Physical tissue expansion methods work on fixed tissue. Thus, fixation is required for specimens that are not already fixed (e.g., fresh frozen samples). Several fixation methods are available, with formalin

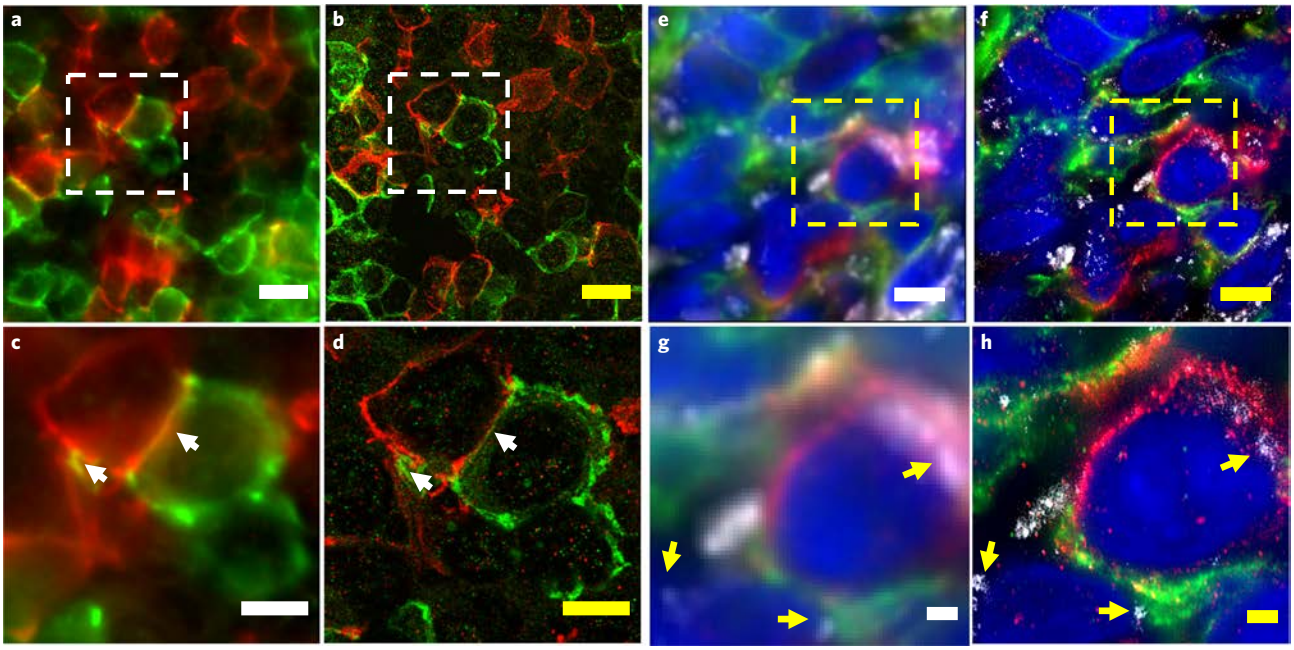

Fig. 6 | Rapid ExPath imaging of lymph node specimens from patients. a, Pre-expansion image of a normal human lymph node specimen acquired with a spinning disk confocal microscope. Green, IgD; red, CD8. **b**, rExPath image of the specimen in **a** acquired with the same confocal microscope. Expansion factor: 4.0. **c** and **d**, Fields of view zoomed into the corresponding areas outlined by a dashed white box in **a** and **b**, respectively. White arrows indicate examples of pre-expansion overlapped IgD and CD8 patterns being resolved after expansion. **e**, Pre-expansion image of a human lymph node specimen with HIV acquired with a wide-field fluorescence microscope. Green, CD8; red, PD-1; gray, p24; blue, DAPI. **f**, rExPath image of the specimen in **e** acquired with the same microscope. Expansion factor: 4.58. **g**,**h**, Corresponding fields of view zoomed into the areas outlined by a dashed yellow box in **e** and **f**, respectively. Yellow arrows indicate examples of p24 being localized with sub-diffraction limit precision. Scale bars (biological scale): 5 μm (**a** and **b**); 1 μm (**c** and **d**); 10 μm (**e** and **f**); 2 μm (**g** and **h**).

being one of the methods of choice in pathology, since it is used for FFPE tissues and can also be applied to fresh frozen tissue. Depending on the format of the specimen, the tissue needs to be deparaffinized and rehydrated (for FFPE unstained tissues), the cover slip needs to be removed and the tissue rehydrated (for H&E or immunofluorescence/immunohistochemistry stained tissue slides) or the tissue needs to be washed with PBS (after fixation for fresh/frozen tissue). Although sodium citrate (i.e., antigen retrieval) is traditionally used for tissues fixed with formalin, we also recommend heat treatment of samples with sodium citrate to improve antigen exposure in samples fixed with acetone (e.g., fresh frozen kidney samples fixed with acetone after sectioning).

Labeling

For fluorescent dye–conjugated antibodies, we strongly advise using oxidation-resistant, photostable fluorescent dyes (Table 1). There are two reasons. First, in situ polymerization will destroy some dyes. Second, after expansion, the labels are diluted, and thus samples require longer exposure times to obtain a good signal-to-noise ratio. ExPath is not compatible with direct imaging of genetically encoded fluorescent proteins, since they are degraded during the aggressive digestion procedures of the ExPath protocol. However, antibodies against such proteins can be used to make them visible in the ExPath context, if desired (e.g., in a transgenic animal model of a pathological state).

In addition to pre-expansion immunostaining, post-expansion DNA FISH is possible with ExPath and rExPath. Due to the dense hydrogel matrix, the large size of traditional bacterial artificial chromosome-based FISH probes precludes their efficient delivery into expanded specimens. Instead, we recommend using as FISH probes single-stranded oligonucleotides with an average size shorter than 150 bases, such as commercially available SureFISH probes or customized oligo probes. Because DNA FISH is performed after the sample is gelled and mechanically homogenized, it does not affect immunostaining performed before gelation. It is thus feasible to co-stain the same tissue section with an antibody against a target protein (before expansion) and with a FISH probe against the corresponding gene in the genome (after expansion).

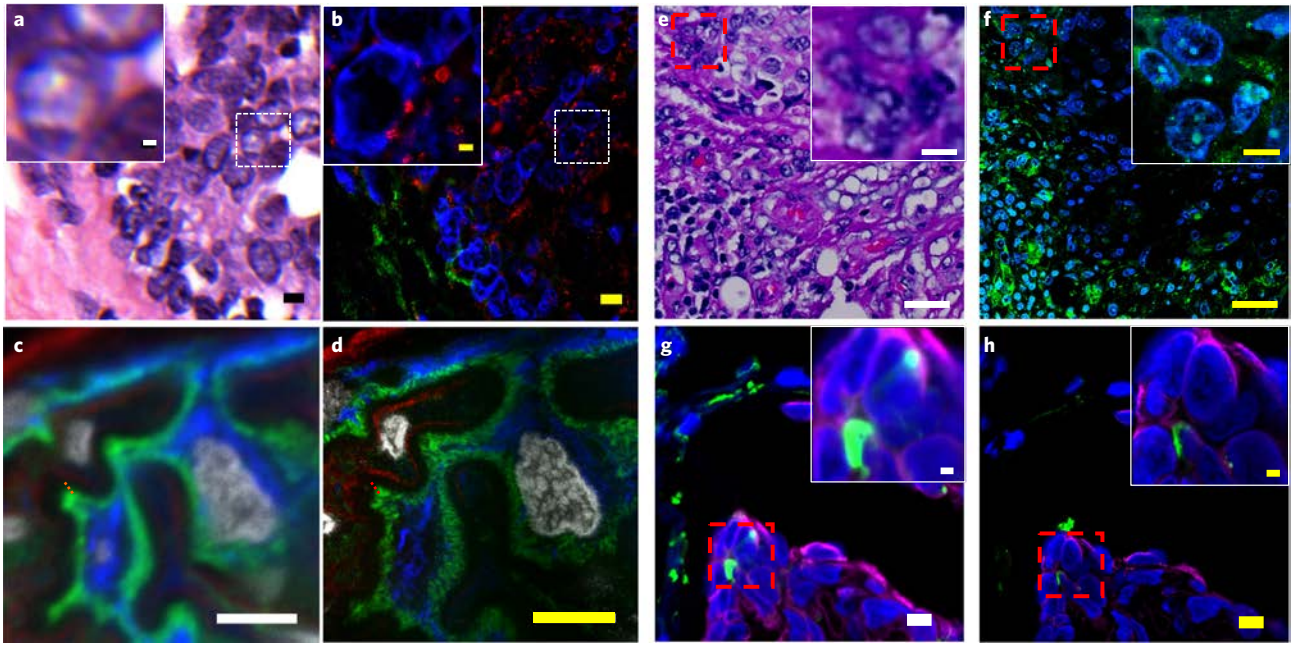

Fig. 7 | ExPath and rExPath imaging of H&E-stained tissue sections and frozen tissue sections. a, H&E-stained human breast specimen with atypical ductal hyperplasia. The inset (upper left) is a magnified view of the area framed by the small dotted square. **b**, ExPath widefield fluorescent image of the specimen in **a** stained with antibodies against Hsp60 (red) and vimentin (green) and with DAPI (blue). **c**, Pre-expansion confocal image of a normal human kidney specimen (fresh frozen, fixed in acetone before processing) showing part of a glomerulus acquired with a spinning disk confocal microscope. Blue, vimentin; green, actinin-4; red, collagen IV; gray, DAPI. **d**, ExPath image of the specimen in **c** using the same microscope. **e**, H&E-stained human breast specimen with atypical ductal hyperplasia. The inset (upper right) is a magnified view of the area framed by the small dotted red square. **f**, rExPath widefield fluorescent image of the specimen in **g** stained with antibodies against vimentin (green) and with DAPI (blue). **g**, Pre-expansion confocal image of a normal human lung specimen (fresh frozen, fixed in acetone before processing) acquired with a spinning disk confocal microscope. The inset (upper right) is a magnified view of the area framed by the small dotted red square. Blue, DAPI; green, vimentin; magenta, pan-cytokeratin. **h**, rExPath image of the specimen in **g** using the same microscope. Scale bars (biological scale): 5 μm, inset 1 μm (**a**); 5 μm, inset 1 μm (**b**) (physical size after expansion, 23 μm; inset, 4.6 μm; expansion factor: 4.6); 5 μm (**c**); 5 μm (**d**) (physical size after expansion, 23.5 μm; expansion factor: 4.7); 20 μm, inset 3.3 μm (**e**); 20 μm, inset 3.3 μm (**f**) (physical size after expansion: 80 μm, inset 13.2 μm; expansion factor: 4.0); 5 μm, inset 1 μm (**g**); 5 μm, inset 1 μm (**h**) (physical size after expansion: 25 μm, inset 5 μm; expansion factor: 5.0). **a**–**d** adapted from ref. [8].

Table 1 | Examples of fluorescent dyes suitable for ExPath

Dye	Excitation maximum, nm	Emission maximum, nm	Brightness after expansion (% of pre-expansion intensity)	Source
CF405M	408	452	51 ± 4	Biotium
Alexa488	495	519	48 ± 2	Life Technologies
Alexa546	556	573	68 ± 3	Life Technologies
Alexa594	590	617	46 ± 2	Life Technologies
CF633	630	650	51 ± 10	Biotium
Atto647N	644	669	55 ± 2	Sigma

Adapted with modifications from ref. [7].

Choice of light microscope

In the Boyden and Zhao labs, we use microscopes such as a Nikon Inverted Eclipse Ti-E/Ti2 epi-fluorescence microscope equipped with a motorized stage, a scientific complementary metal–oxide–semiconductors (sCMOS) camera and a CSU-W1 spinning disk confocal module, for routine imaging of expanded samples. While expansion can improve image resolution for any objective lens, objective lenses with high working distance and high numerical aperture will offer better utility and image quality for expanded samples. We use plate holders to hold 6-well, 12-well or 24-well glass-bottom plates, chosen according to the tissue size after expansion. Comparable resolutions and quality

of images of expanded samples should be obtainable using other conventional diffraction-limited microscopes with analogous setups. Although the protocol described here assumes the usage of a conventional diffraction-limited microscope, the expanded specimen can also in principle be imaged using SRM, such as SIM[33–35], stochastic optical reconstruction microscopy[36], photoactivated localization microscopy[7] and STED microscopy[37,38]. In principle, combining expansion microscopy and SRM techniques can augment the resolution further. However, users need to ensure sufficiently high labeling density to achieve desired resolution. In addition, other factors, such as imaging time, maximum image depth and choice of dye, need to be considered when choosing a super-resolution imaging platform.

Image analysis tools

We use the free software package ImageJ Fiji (http://fiji.sc/Fiji) for image visualization and for simple imaging processing and analysis, such as image registration and estimation of expansion factor. We use customized Matlab code for automated image registration and calculation of expansion factor (Step 15) (https://github.com/zhao-biophotonics/ExPath-reg; examples and libraries are included). Danzl and coworkers also developed similar tools written in Python for expansion and distortion analysis (for code and instructions, please refer to ref. [39]).

Expansion factor calculation

Knowing the expansion factor for a given specimen is necessary to translate the physical scale of a post-expansion image into meaningful biological units. The measurement of expansion factor can easily be done by comparing the size of easily seen features in pre-expansion versus post-expansion images within the same region of interest of the specimen. We recommend using low-magnification images of the specimen before and after expansion, taken on a standard diffraction-limited microscope, for convenient calculation. Although not a requirement, the same objective lens can be used for both pre- and post-expansion images for precise expansion factor calculation. To calculate the expansion factor, simply divide the size of the given feature after expansion by the size of the same feature before expansion.

Materials

Reagents

- Starting material of interest: human tissue sections prepared in a standard pathology or clinical fashion, including FFPE, fresh frozen (and fixed) and chemically stained (e.g., H&E-stained) human tissue slides **! CAUTION** Obtaining ethical approval and conforming to relevant institutional and national regulations is required. Patient consent and IRB review may or may not be required for residual/archived tissue; consult your institution if you have any uncertainties.
- Acetone (used for fixation of fresh frozen tissue) (Fisher Scientific, cat. no. S25904) **! CAUTION** Acetone is highly flammable. Wear gloves and work in a well-ventilated area.
- Formalin, 10% (vol/vol) (Electron Microscopy Sciences, cat. no. 15740) **! CAUTION** Wear gloves and work in a well-ventilated area.
- Xylene (used for coverslip removal from already mounted tissue slides and for deparaffinization) (Thermo Fisher Scientific, cat. no. HC-700-1GAL) **! CAUTION** Xylene is extremely flammable, and proper handling procedures should be employed; work in a well-ventilated area and dispose of the waste as per your institutional guidelines.
- Absolute ethanol (Merck, cat. no. 1009831000) **! CAUTION** Absolute ethanol is flammable. Handle with appropriate safety equipment and measures.
- ddH2O or deionized water (e.g., milli-Q)
- PBS, 1× (Life Technologies, cat. no. 10010023) and 10× (Thermo Fisher Scientific, cat. no. AM9625)
- Sodium citrate tribasic dihydrate (Sigma-Aldrich, cat. no. C8532)
- Blocking buffer, MAXblock Blocking Medium (Active Motif, cat. no. 15252) **▲ CRITICAL** You can use whatever standard blocking buffer works for your specimen and antibodies; since the staining is done before expansion, the staining is not affected by the expansion process and can be done in the way most familiar to you. Some standard immunofluorescence protocols are based on product datasheets for specific antibodies. For example, 1× PBS/5% (vol/vol) normal serum/0.3% (vol/vol) Triton X-100, freshly prepared, is recommended as a blocking buffer for some of the Cell Signaling antibodies (normal serum from the same species as the secondary antibody), while 1% (wt/vol) BSA and 22.52 mg/ml glycine in PBST (1× PBS + 0.1% (vol/vol) Tween 20), freshly prepared, is used in the case of some Abcam antibodies.
- Staining buffer, MAXbind Staining Medium (Active Motif, cat. no. 15253) **▲ CRITICAL** As above, you can use the staining buffer most familiar to you that works for the specimen and antibody of interest.

Table 2 | Examples of primary antibodies validated in ExPath and rExPath

Target	Host	Clonality	Manufacturer	Catalog no.	RRID*
TOM20	Rabbit	Poly	Santa Cruz Biotech	sc-11415	AB_2207533
Collagen IV	Mouse	Mono	Santa Cruz Biotech	sc-59814	AB_1121796
Vimentin	Chicken	Poly	Abcam	ab24525	AB_778824
a-Tubulin	Rabbit	Poly	Abcam	ab15246	AB_301787
VDAC/porin	Mouse	Mono	Abcam	ab14734	AB_443084
KRT19	Rabbit	Poly	Sigma-Aldrich	HPA002465	AB_1079179
ACTN4	Rabbit	Poly	Sigma-Aldrich	HPA001873	AB_1078096
Synaptopodin	Guinea pig	Poly	PROGEN Biotechnik	GP94-IN	AB_2811107
Actin (smooth muscle)/ACTA2	Mouse	Mono	Agilent Technologies	M085129-2	AB_2811108
IgD	Goat	Poly	SouthernBiotech	2030-01	AB_2795623
S100A8	Mouse	Mono	Abcam	ab22506	AB_447111
MCT1	Chicken	Poly	Sigma-Aldrich	AB1286-I	AB_11212410
Tom20	Rabbit	Poly	Sigma-Aldrich	HPA011562	AB_1080326
Nephrin	Rabbit	Poly	Santa Cruz Biotech	sc-376522	AB_11151390
CASP3	Rabbit	Poly	Sigma-Aldrich	HPA002643	AB_1846048
hFOXP3	Goat	Poly	R & D Systems	AF3240	AB_2262812
Granzyme B	Rabbit	Poly	Sigma-Aldrich	HPA003418	AB_1079020
Cytokeratin pan type I/II	Mouse	Mono	Invitrogen	MA5-13156	AB_10983023
GFAP	Chicken	Poly	Abcam	ab4674	AB_304558
INA	Rabbit	Poly	Sigma-Aldrich	HPA008057	AB_1851833
Homer1	Rabbit	Poly	Sigma-Aldrich	HPA036522	AB_2675171
PSD95	Goat	Poly	Abcam	ab12093	AB_298846
Synaptophysin	Rabbit	Poly	Proteintech	60191-1-Ig	AB_10915965
Tyrosine hydroxalase	Chicken	Poly	Abcam	ab76442	AB_1524535
ATPIF1	Rabbit	Poly	MilliporeSigma	ABC137	AB_2811109
CD8	Rabbit	Poly	Abcam	ab85792	AB_10674324
P24	Mouse	Mono	Dako	M0857	AB_2335686
PD-1	Goat	Poly	R & D Systems	AF1086	AB_354588
Hsp 60	Mouse	Mono	Abcam	Ab59457	AB_2121285

*From http://scicrunch.org/resources.

You can also consult the antibody product datasheet for the appropriate antibody diluent. For example, 1× PBS/1% (wt/vol) BSA/0.3% (vol/vol) Triton X-100, freshly prepared, is recommended for some of the Cell Signaling antibodies, while 1% (wt/vol) BSA in PBST, freshly prepared, is used in the case of some Abcam antibodies.

- Washing buffer, MAXwash Washing Medium (Active Motif, cat. no. 15254) ▲ **CRITICAL** As above, you can use the washing buffer most familiar to you that works for the specimen and antibody of interest. 1× TBS/0.1% (vol/vol) Tween-20, kept for ≤1 month at 4 °C, can be used as an alternative.
- Primary antibodies (concentration variable with the antibody used; Table 2 lists examples of primary antibodies validated for use in both ExPath and rExPath methods)
- Secondary antibodies conjugated with fluorescent dyes (concentration variable with the antibody used; see Table 1 for recommended fluorescent dyes)
- DAPI, 1 M (Thermo Fisher Scientific, cat. no. 62248) **! CAUTION** Since it binds to DNA, DAPI is a potential mutagen. Thus, it should be handled with care and properly disposed of.
- Acryloyl-X, SE (AcX); prepare 10-mg/ml stock solution in dimethyl sulfoxide (DMSO) (20-µl aliquots stored in a desiccated environment at −20 °C; Life Technologies, cat. no. A20770)
- Sodium acrylate (Sigma-Aldrich, cat. no. 408220) **! CAUTION** Do not use if the color of acrylate solution appears to be yellow. Get a new batch of sodium acrylate.
- Acrylamide (Sigma-Aldrich, cat. no. A8887) **! CAUTION** Acrylamide is a toxic substance and an irritant.
- *N*,*N*′-Methylenebisacrylamide (Sigma-Aldrich, cat. no. M7279)
- Sodium chloride (Sigma-Aldrich, cat. no. S6191)
- 4-hydroxy-TEMPO (4-HT; Sigma-Aldrich, cat. no. 176141)

- *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED; Sigma-Aldrich, cat. no. T9281)
- Ammonium persulfate (APS; Sigma Aldrich, cat. no. 248614)
- Proteinase K, 800 U/ml (Molecular Biology Grade; New England Biolabs, cat. no. P8107S)
- Trizma base (for preparing the Tris buffer; Sigma Aldrich, cat. no. T1503)
- EDTA disodium salt dihydrate (Sigma-Aldrich, cat. no. E4884)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- Low-melting-point agarose (Sigma-Aldrich, cat. no. 121852)
- Optimal cutting temperature (OCT) solution (Tissue-Tek, cat. no. 4583)
- SlowFade Gold antifade reagent (Invitrogen, cat. no. S36936)

Equipment

- 4 °C, −20 °C and −80 °C storage units
- Falcon conical tubes (15 and 50 ml; Thermo Fisher Scientific, cat. nos. 14-959-49B and 14-432-22)
- Heat-resistant plastic container (for antigen retrieval step; Coplin Staining Jar; Thermo Fisher Scientific, cat. no. 194)
- Microwave (for heating the antigen retrieval solution and low-melting-point agarose)
- Incubators at 37 °C and 60 °C
- Balance
- Laboratory timer
- Eppendorf tubes (Eppendorf, cat. no. 022363204)
- Liquid Blocker PAP Pen (Sigma-Aldrich, cat. no. Z377821)
- Glass slides (VWR, cat. no. 48312-401)
- Cover glass (e.g., VWR micro cover glass, 24 × 60 mm, No. 1 or 1.5 (VWR, cat. no. 16002-252))
- Razor single-edge blades (Fisher Scientific, cat. no. 12-640)
- Parafilm M (Fisher Scientific Parafilm M Wrapping Film, Fisher Scientific, cat. no. S37440)
- pH meter
- (Optional) Green light-emitting diode (LED) light source for side illumination
- (Optional) Bench-top mini-shaker
- Four-well cell culture plate (Thermo Fisher Scientific, cat. no. 167063)
- Six-well black-walled plate with clear glass bottom, glass #1.5H (Cellvis, cat. no. P06-1.5H-N)
- Paintbrushes for handling gels
- Inverted fluorescence or confocal microscope, a long-working-distance 40× objective (optional), computer and image acquisition software (see Equipment setup)

PC and software requirements

- Windows PC or Mac with 8 GB random access memory (RAM) minimum and a video graphic card with a minimum of 1 GB memory
- Fiji/ImageJ (http://fiji.sc/Fiji)
- Optional: MATLAB (http://www.mathworks.com) or Python (https://www.python.org/) (if custom programming is desired)

Reagent setup

AcX stock solution

Prepare 10-mg/ml stock solution in dimethyl sulfoxide (20-µl aliquots can be stored in a desiccated environment at –20 °C for ≥6 months). Use AcX at a final concentration in PBS of 0.03–0.1 mg/ml, depending on the fixative used. In our experience, 0.03 mg/ml is suitable for specimens fixed with non-aldehyde fixatives like acetone or methanol; 0.1 mg/ml is suitable for specimens fixed with aldehyde fixatives. As a rule of thumb, 100 µl of AcX solution is required for a 5-µm-thick tissue specimen of dimensions 5 mm × 5 mm.

Sodium citrate solution

Prepare 20 mM sodium citrate solution at pH 8.0. The solution can be kept for ≤6 months at room temperature (RT, ~22–23 °C).

Monomer stock solutions

Make individual stock solutions of concentrations 38 g/100 ml sodium acrylate, 50 g/100 ml acrylamide, 2 g/100 ml *N*,*N*′-methylenebisacrylamide and 29.2 g/100 ml sodium chloride in water. We recommend using freshly prepared stock solutions for monomer solution preparation (see next step).

Table 3 | Monomer solution preparation from the stock solutions

Chemical	Stock concentration[a]	Amount (ml)	Final concentration[a]
Sodium acrylate	38	2.25	8.6
Acrylamide	50	0.5	2.5
N,*N*′-Methylenebisacrylamide	2	0.50	0.10
Sodium chloride	29.2	4	11.7
PBS	10×	1	1×
Water	-	1.15	-
Total	-	9.4[b]	-

[a]All concentrations are in g/100 ml, except PBS. Optionally, the mechanical strength of gel can be improved by increasing the final concentration of sodium acrylate to 12 g/100 ml and the final concentration of acrylamide to 3.5 g/100 ml while maintaining the rest of the ingredients at the same concentrations. [b]9.4/10 ml (1.06×), with the remaining 6% volume brought up by initiator, accelerator and inhibitor.

Monomer solution

Using the four above monomer stock solutions, prepare a monomer solution with the following final concentrations: 8.6 g/100 ml sodium acrylate, 2.5 g/100 ml acrylamide, 0.1 g/100 ml *N*,*N*′-methylenebisacrylamide and 11.7 g/100 ml sodium chloride in PBS (1× final concentration). Store the monomer solution mix at 4 °C for ≤2 months or at −20 °C for long-term storage (see Table 3).

TEMED accelerator stock solution

Prepare the stock solution of 0.1 g/ml TEMED in water at RT. Store the solution at –20 °C for ≤6 months.

4-HT inhibitor stock solution

Prepare the stock solution of 0.005 g/ml 4-HT in water at RT. Store the solution at –20 °C for ≤6 months.

Ammonium persulfate (APS) initiator stock solution

Prepare the stock solution of 0.1 g/ml APS freshly, every time, in water at RT.

Gelling solution

Mix the following four solutions on ice in the following order (prepare fresh solution): monomer solution (see above), TEMED accelerator solution (from the 10% (wt/vol) stock solution above, so that the final concentration is 0.2% (wt/vol), a dilution of 1:50), 4-HT inhibitor solution (from the 0.5% (wt/vol) stock solution, final concentration 0.01% (wt/vol), a dilution of 1:50) and APS initiator solution (from the 10% (wt/vol) stock solution, final concentration 0.2% (wt/vol), a dilution of 1:50). The initiator solution needs to be added last, ideally right before use, to prevent premature gelation. The mixture should be vortexed to ensure full mixing.

Digestion buffer

Prepare a solution containing (final concentrations) 50 mM Tris, 25 mM EDTA, 0.5% (vol/vol)Triton X-100 and 0.8 M NaCl and then adjust pH to 8.0 at RT. Store the buffer in the refrigerator at 4 °C for ≤6 months.

Low-melting-point agarose solution

Prepare 2% low-melting-point agarose (Sigma-Aldrich, cat. no. 121852) in ddH2O. Heat the agarose-water slurry in the microwave until it starts to boil. Let the solution boil for 1 min or until the solution is clear. Remove the flask from the microwave oven, and gently mix the agarose solution by shaking the flask. It can be stored at RT for ≤6 months.

Equipment setup

Microscope setup

Configure a microscope for the fluorophores you desire to image. One possibility is to configure a four-channel microscope with appropriate excitation light sources and emission filters for the

Table 4 | Example combination of filter sets for four-color imaging

Fluorophore	Excitation (nm)	Dichromatic (nm)	Emission (nm)
DAPI	325–375	400	435–485
Alexa Fluor 488	451–490	497	502–542
Alexa Fluor 546	532–588	595	604–679
Atto 647N	590–650	660	663–738

fluorophores DAPI, Alexa Fluor 488, Alexa Fluor 560 and Alexa Fluor 640 (Table 4). An existing objective lens of your choice can be utilized; one recommended possibility is that a long-working-distance 40× water-immersion objective be used for large-volume wide-field and confocal imaging (examples include Nikon's CFI Apo Lambda S LWD 40×WI and Zeiss's C-Apochromat 40×/1.2 W Corr M27 objectives); another possibility is to use a 20× water immersion objective, which provides a larger field of view.

Procedure

Specimen pre-processing ● Timing 30–40 min including the bench time

1 Prepare the specimens for immunostaining; follow option A for FFPE clinical specimens, option B for H&E-stained and mounted permanent slides, option C for fresh frozen tissue slides and option D for fixed frozen tissue slides.

▲ CRITICAL STEP All washes are at RT, unless indicated otherwise.

(A) **FFPE specimens**

(i) Bake the FFPE tissue slide at 60 °C for 10 min (this step is optional and helpful for easier deparaffinization).

(ii) Sequentially place the specimen in a series of solutions, each time for 3 min, at RT:

Solution	Number of times
Xylene	2
100% ethanol	2
95% ethanol	1
70% ethanol	1
50% ethanol	1
ddH_2O	1

(B) **Stained and mounted permanent slides**

▲ CRITICAL This step works for slides mounted with any organic mounting medium.

(i) Briefly place the specimen in xylene. Then, carefully remove the coverslip with appropriate tools, such as a razor blade.

▲ CRITICAL STEP If the coverslip is difficult to remove, further incubate the slides in xylene at RT until the coverslip is loosened.

? TROUBLESHOOTING

(ii) After coverslip removal, sequentially incubate the slides in a series of solutions, each time for 3 min, at RT:

Solution	Number of times
Xylene	2
100% ethanol	2
95% ethanol	1
70% ethanol	1
50% ethanol	1
ddH_2O	1

(C) **Fresh frozen tissue sections**

(i) If the tissue was frozen but not fixed, fix the tissue on a slide with a desired fixative (for example, fix the tissue section with cold acetone or methanol for 10 min at –20 °C).

(ii) Leave slides at RT exposed to air for 2 min, to let the fixative evaporate.

(iii) Wash the specimen with PBS solution three times at RT, 5 min each wash.

(D) **Fixed frozen tissue slides**

(i) Leave slides at RT for 2 min to let the OCT embedding compound melt (this step may have to be optimized if a non-standard embedding compound is used).

(ii) Wash with PBS solution three times at RT, 5 min each wash.

Heat treatment of the specimen ● Timing 30 min

2 Preheat 20 mM sodium citrate solution (pH 8) by microwaving until it reaches the point of boiling; this usually takes 20–60 s. Place specimens in pre-heated 20 mM sodium citrate solution (pH 8), in a heat-resistant container (e.g., plastic or glass staining Coplin jars), and then transfer the closed container to a 60 °C incubation chamber for 30 min (so that the temperature will slowly decrease from 100 °C to 60 °C).

▲ **CRITICAL STEP** We recommend using the appropriate antigen retrieval solution and heat treatment method for the specimen and antibodies of choice for optimal immunostaining (this information is often provided by the antibody manufacturer, or may be informed by your past experience with the specimen and antibody of interest). Thus, an alternative solution and treatment may be preferable to that described here.

? TROUBLESHOOTING

Immunostaining ● Timing ≥2 h 50 min (9 h to 1 d for conventional staining; 2 h 50 min for rapid staining)

3 Follow option A for conventional immunostaining and option B for rapid immunostaining (≤5-μm-thick tissue sections).

▲ **CRITICAL STEP** Make sure that the whole tissue section is covered by solution during all the steps. To minimize volumes of solution, use a hydrophobic ('PAP') pen to draw a hydrophobic boundary around the tissue section, to confine the solution. The hydrophobic pen inscription does not have to be removed later, before constructing the gelling chamber, since it does not interfere with the specimen or the gel.

▲ **CRITICAL STEP** Note that all the listed blocking, staining and washing media can be replaced with the user's media of choice, based on their past experience or the antibody manufacturer's recommendations, if desired.

(A) **Conventional immunostaining approach**

(i) Block the tissue with MAXblock Blocking Medium for 1 h at 37 °C; we recommend this commercial formulation for sensitive applications and when a mixture of several different antibodies is used for staining of multiple targets (alternative formulations are available; see Reagents).

(ii) Incubate with primary antibodies in MAXbind Staining Medium (alternative formulations are available; see Reagents) at a concentration of 10 μg/ml (or according to the manufacturer's instructions) overnight at 4 °C or for 6 h at RT, depending on the tissue thickness and the antibody.

(iii) Wash three times with MAXwash Washing Medium (alternative formulations are available; see Reagents), each for ≥5 min at RT.

(iv) Incubate with fluorescently labelled secondary antibodies at a concentration of 10 μg/ml in MAXbind Staining Medium (alternative formulations are available; see Reagents) for 2 h at RT (longer incubation times may be needed for thicker (>10-μm) sections and certain antibodies, as per the manufacturer's instructions).

▲ **CRITICAL STEP** For optimal results, we recommend using the concentration of antibody recommended by the manufacturer, or determined empirically.

(v) (Optional) Include 300 nM DAPI with the secondary antibodies, if nuclear visualization in pre-expanded specimens is desired. Nuclear staining with DAPI can also be performed after the incubation with the secondary antibody, for 5 min with 300 nM DAPI at RT.

(vi) Wash in MAXwash Washing Medium (alternative formulations are available; see Reagents) three times, each for 5 min at RT.
? TROUBLESHOOTING

(B) **Rapid immunostaining approach (only for thinner tissue sections, ≤5 μm)**

(i) (Optional, depending on the performance of antibodies) Block the tissue with MAXblock Blocking Medium for 20 min at 37 °C (alternative formulations are available; see Reagents).

(ii) Incubate with primary antibodies in MAXbind Staining Medium (alternative formulations are available; see Reagents) at a concentration of 10 μg/ml (or according to the manufacturer's instructions) for 1 h at 37 °C.

(iii) Wash three times with MAXwash Washing Medium (alternative formulations are available; see Reagents) for 5 min each at RT.

(iv) Incubate with fluorescently coupled secondary antibodies at a concentration of 10 μg/ml in MAXbind Staining Medium (alternative formulations are available; see Reagents) for 1 h at 37 °C.

(v) (Optional) Include 300 nM DAPI with the secondary antibodies, if nuclei visualization in pre-expanded specimens is desired. Nuclear staining with DAPI can also be performed after the incubation with the secondary antibody, for 5 min with 300 nM DAPI at RT.

(vi) Wash in MAXwash Washing Medium (alternative formulations are available; see Reagents) three times, each for 5 min at RT.
? TROUBLESHOOTING

Pre-expansion imaging ● Timing 1–2 h, depending on the tissues and the number of needed images

4 Image sample.
▲ CRITICAL STEP Pre-expansion imaging is recommended when the user aims to measure the expansion factor, or to obtain pre-expansion imaging data.
? TROUBLESHOOTING

Protein anchoring ● Timing 1.5–24 h

5 Incubate each slide with ~250 μl of AcX solution prepared as above in PBS, at a concentration of 0.03 or 0.1 mg/ml AcX (0.03 mg/ml for specimens fixed with non-aldehyde fixatives, 0.1 mg/ml for specimens fixed with aldehyde fixatives).

6 For conventional ExPath, incubate the sample with AcX for ≥6 h at RT (this reaction can be run overnight). For rExPath (≤5-μm-thick tissue sections), incubate the sample for 1.5 h at 37 °C.

In situ polymer synthesis ● Timing 45 min to 3 h

7 Prepare the gelling solution as described in Reagent setup. Follow option A for conventional in situ polymer synthesis; follow option B for rExPath.

(A) **Conventional in situ polymer synthesis**

(i) Add ~250 μl of gelling solution for each slide on top of the tissue section, making sure the whole tissue section is immersed in the solution (the volume of gelling solution will vary according to the tissue area; as a rule of thumb, 250 μl of gelling solution can cover up to a 2 cm × 2 cm tissue area).

(ii) Incubate the specimen for 60 min at 4 °C.

(iii) Aspirate the liquid and replace with fresh gelling solution on each tissue slide.

(iv) Assemble the tissue slide into a gel chamber (Fig. 8a). Construct the gel chamber by placing a coverslip on top of the tissue section, with spacers on either side of the tissue section to prevent compression of the tissue slice. Make spacers from cut coverslips using a diamond knife. For most human tissue sections in clinical settings (4–10 μm thick), pieces of cover glass (VWR micro cover glass, 24 × 60 mm, No.1 or 1.5) can be used as spacers. No. 1.5 coverslips have a height of ~0.16–0.19 mm; by changing the number or types of the coverslips, the height of the spacers can be accordingly adjusted.
▲ CRITICAL STEP Avoid air bubbles trapped inside the chamber. Embedding the sample in an even manner within the gelling solution is important. Try to not disturb the tissue section on the slide during the process since even minute waves in the specimen are amplified during expansion and can become problematic.

(v) Incubate at 37 °C in a humidified environment for 2 h.

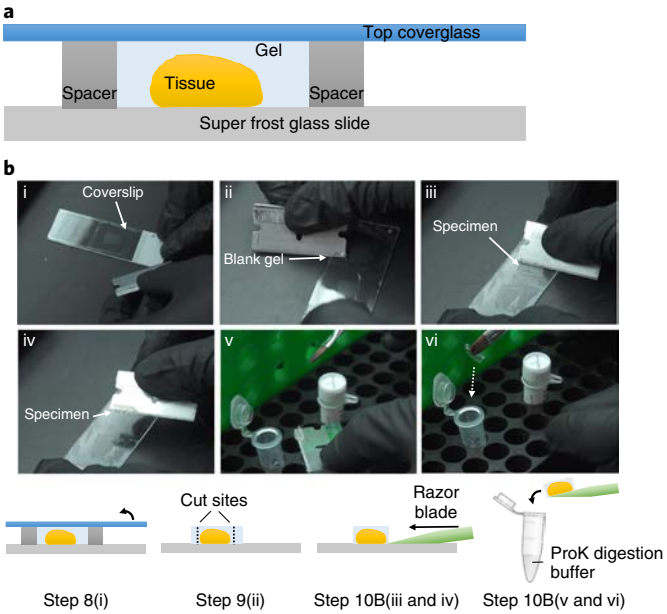

Fig. 8 | Gelling station setup and specimen pre-treatment before proteinase K digestion. a, Schematic of the gelling station setup. **b**, Specimen pre-treatment and handling before proteinase K digestion: i. remove the coverslip; ii. trim the blank gel region surrounding the tissue; iii. and iv. remove the tissue using a razor blade; v. and vi. carefully transfer the gel into the proteinase K–containing digestion buffer.

(B) **Rapid in situ polymer synthesis**

(i) Add ~250 µl of gelling solution for each slide on top of the tissue section and make sure the whole tissue section is immersed in the solution (the volume of gelling solution can vary according to the tissue area; as a rule of thumb, 250 µl of gelling solution can cover up to a 2 cm × 2 cm tissue area).

(ii) Incubate the specimen for 20 min at 4 °C.

(iii) Aspire the liquid and replace with fresh gelling solution on each tissue slide.

(iv) Assemble the tissue slide into a gel chamber (Fig. 8a), as described in option A, Step iv.

(v) Incubate at 60 °C in a humidified environment for 45 min.

■ PAUSE POINT Specimens in the gel chamber, with the coverslip intact, can be stored at 4 °C for ≤4 weeks.

? TROUBLESHOOTING

Proteinase K–based homogenization ● Timing 45 min to 3 h

8 Take off the top cover of the gel chamber using a razor blade placed at the edge of the coverslip (Fig. 8b,i), sliding the blade along the coverslip side touching the gel surface and then gently using the blade to lift the coverslip off the gel surface.

! CAUTION Handle all the glass slides and coverslips with care, to avoid broken glass debris.

9 Trim the tissue-containing gel to minimize volume, using a sharp razor blade, and cut a corner in an off-angle fashion for tracking of orientation throughout later steps (Fig. 8b,ii). If the tissue is too big in size (e.g., >0.6 cm), divide it into multiple pieces using a razor blade, if appropriate.

▲ CRITICAL STEP In the case of tissue microarrays (TMAs), it is important to become familiar with the layout of the TMA, potentially dividing the gelled sample into multiple sections with each section potentially containing multiple cores (e.g., one could divide a gelled TMA into smaller sections, where each section contains four cores in a 2×2 grid). When dealing with TMAs, we recommend following option B of Step 10 to transfer individual sections into properly labeled Eppendorf tubes or small Petri dishes, to keep track of each section in later steps. Cores do not merge together during or after the expansion process, since the entire gel-embedded specimen expands in three dimensions (i.e., the gel between the cores expands just as the gel-embedded cores themselves).

10 Mix proteinase K with the digestion buffer, to give a final concentration of 4 U/ml (1:200 from the 800 U/ml proteinase K stock) just before use. Then, perform the mechanical homogenization step on-slide (option A; Fig. 8b,i and ii) or off-slide (option B; Fig. 8b,i–vi).
▲ CRITICAL STEP Using 4 U/ml of proteinase K generally results in a mechanically homogenized sample with sufficient fluorescent signal for post-expansion imaging. A lower proteinase K concentration may be considered, if significant fluorescent signal loss is observed.

(A) **On-slide digestion (conventional ExPath)**

(i) Transfer the gelled specimen, still on the slide, to a container, such as a four-well cell culture plate.

(ii) Submerge the gelled specimen, still on the slide, in 3 ml of freshly made digestion solution (including proteinase K) and incubate for 3 h at 60 °C or until completion of digestion (e.g., the gel is flat and transparent). Gentle mixing on a shaker may be used at this step (optional) to ensure that the sample is covered with solution (so it does not dry out) and to increase the efficiency of the digestion.
▲ CRITICAL STEP Ensure that the slice is completely submerged in the digestion buffer and ensure that it does not dry out, for example, by sealing the container with parafilm to prevent evaporation and/or by using a shaker. Any container with a cover, such as a small slide box or a plastic well, could be used to incubate the gel in the digestion buffer.

(iii) Normally, the specimen will detach from the glass slide by itself after digestion. If the gel is already floating in the digestion buffer, use a fine, soft paintbrush to pick it up and transfer it into a well of a six-well plate (for inverted microscopes, use black-walled plates with clear glass bottoms) containing PBS. If the gelled tissue is not fully detached, use a razor blade to gently scoop the specimen off the slide into a well of a six-well plate filled with 1× PBS.
▲ CRITICAL STEP It is critical to check for signs that the digestion is complete (i.e., the gelled tissue is detached or mostly detached from the glass slide and becomes transparent, and the gelled tissue remains flat without bending or twisting in the PBS solution). If the digestion does not appear to be complete after 3 h, incubate the gel-tissue hybrid in fresh digestion solution at 60 °C for additional time.

(B) **Off-slide digestion (rExPath)**

(i) Use the razor blade to gently scoop the gelled tissue away from the slide (Fig. 8b, iii and iv).
▲ CRITICAL STEP This step should be performed as soon as the coverslip is removed because partially dried gelled tissue can be difficult to scoop. If the gelled tissue is partially dried, a few drops of digestion buffer can be applied to wet the specimen before proceeding with scooping. Push the razor blade against the glass slide while moving slowly to pick up the gel-tissue hybrid, from its bottom, without damaging it. Tissue damage might occur if the razor blade does not push against the glass slide throughout the whole process.

(ii) Use a paintbrush to carefully transfer the detached gelled tissue into the digestion buffer in a six-well plate (for inverted microscopes, use black-walled plates with clear glass bottoms) or an Eppendorf tube, and incubate for 45 min at 60 °C (Fig. 8b,v and vi). In most cases, a multi-well plate is preferable as it maintains the orientation of the specimen throughout the process. Alternatively, Eppendorf tubes are recommended if you wish to save digestion buffer, and to have better control of evaporation at 60 °C. Gentle mixing on a shaker is recommended but optional, to ensure that the sample is covered with solution (so it does not dry out) and to increase the efficiency of the digestion.
▲ CRITICAL STEP We recommend cutting the gel so it has an asymmetric shape. This enables correct orientation of the gel later, which is with the side of the gel containing the tissue oriented toward the objective lens. Alternatively, the side of the gel containing the tissue can be identified by quickly scanning the focus in z in a fluorescence microscope, to find the specimen within the gel.
▲ CRITICAL STEP Gelled specimens processed in this step may fold or twist or change orientation regardless of whether an Eppendorf tube or a culture plate is used, especially when a shaker is used. Folded/twisted gelled specimens can be unfolded by gently shaking in a saline buffer in a Petri dish or glass bottom multi-well plate. So far, we have

processed hundreds of specimens using Eppendorf tubes without any unsolvable folding/twisting issues.

? TROUBLESHOOTING

Expansion ● Timing 0.5 h

11 Stain the specimen with 300 nM DAPI in PBS buffer for 5 min at RT, and then wash with PBS three times for 5 min each time at RT.

▲ CRITICAL STEP The side of the tissue that was previously in contact with the slide needs to be on the side facing the objective lens to maximize accessibility. If the digestion was performed in an Eppendorf tube, keeping track of the side of the tissue can be challenging at times and verifying it by using a fluorescence microscope is required before expansion. If the gel is cut in an asymmetric shape, the orientation of the tissue can be tracked without using a fluorescence microscope. If you need to transfer or place the expanded gel into a new container, we recommend handling it when the gel is in 1× PBS buffer as the gel is fragile in the fully expanded state in pure water, but much steadier when not fully expanded in 1× PBS buffer. We also recommend using a paintbrush to gently transfer, or flip, the gel, as needed.

■ PAUSE POINT Specimens can be preserved ≤2–3 weeks in PBS with 0.002–0.01% NaN_3 to prevent bacterial growth at 4 °C at this point.

? TROUBLESHOOTING

12 For expansion, remove the PBS and wash the specimens with an excess volume of ddH_2O (we usually use ≥10× the final gel volume; repeat three to five times for 5 min each time at RT). Tissue expansion should reach a plateau after about the third or fourth wash.

▲ CRITICAL STEP The expansion chamber needs to be of adequate size for the specimen. The specimen might need to be trimmed into smaller pieces if no chamber of proper size can be obtained. In general, an expanded gel containing a tissue with a starting diameter <0.6 cm before expansion fits nicely in a glass-bottom six-well plate.

? TROUBLESHOOTING

■ PAUSE POINT Specimens can be preserved ≤2–3 weeks at 4 °C in water with 0.002–0.01% NaN_3 to prevent bacterial growth; the final expansion factor is reversibly reduced by 10% by the addition of NaN_3.

13 (Optional) To prevent drift of the sample during imaging, remove the ddH2O and carefully add 2% low-melting-point agarose solution in ddH2O around and on top of the sample, and then wait until the agarose solution solidifies. Alternatively, the gelled sample can be immobilized on a poly-L-lysine–treated glass slide. To produce a poly-L-lysine–treated glass surface, soak the glass surface with 0.1% (wt/vol) poly-L-lysine in water for 20 min, rinse the coated glass surface with water three times and finally air-dry the glass surface for 1 h in a clean environment before transferring the gelled sample onto the treated glass surface[15].

■ PAUSE POINT After specimen mounting, the sample is stable for ≤2 weeks at 4 °C without the need for parafilm sealing of the receptacle in which the sample is located, and for ≤2 months at 4 °C with parafilm sealing to prevent water evaporation.

Imaging ● Timing variable

14 Image the sample as desired. The user can choose practically any optical microscope for imaging. 40× long-working-distance water-immersion objectives on an inverted microscope are recommended due to the refractive index of expanded specimens being similar to that of water; long-working-distance objectives can help with large-volume imaging. Although the true signal gets diluted by about two orders of magnitude by the expansion process, users can obtain high-quality images by increasing laser/LED power and/or increasing the exposure time of the photosensor/camera.

? TROUBLESHOOTING

Expansion factor measurements ● Timing <30 min

15 Use low-magnification images of the specimen before and after expansion. Calculate the ratio of pre- and post-expansion sizes. One can normalize the quantitative values of measured features by the expansion factor of each specimen, since there is a small degree of sample-to-sample variability in the expansion factor. Alternatively, users can use our customized Matlab code for automated image registration and expansion factor calculation (the code and instruction are available at https://github.com/zhao-biophotonics/ExPath-reg).

Troubleshooting

Troubleshooting advice can be found in Table 5.

Table 5 | Troubleshooting table

Step	Problem	Possible reason	Solution
1	Coverslip cannot be removed from slide (for mounted tissue slides)	Insufficient xylene incubation time when removing the mounting medium	Longer incubation time with xylene
2	Tissue detaches from the slide	(1) The tissue slide is not baked (2) Super-frost glass slides were not used (3) Poor quality of specimen	Use super-frost glass slides for holding tissue sections Bake the tissue slide at 60 °C for 10 min to enhance adhesion
3,4	Dim, uneven or absent fluorescence signal (pre-expanded specimens)	Sub-optimal immunostaining conditions	Optimize immunostaining parameters: temperature, buffer composition and duration for antigen retrieval, the concentration of antibodies, incubation times for staining and the antibodies used
7	No gel is formed	Low concentration of APS and/or TEMED	Add the correct amount of APS and TEMED
		The gelling solution was not replaced with a freshly prepared one, at the indicated time	Ensure the gelling solution is replaced with fresh solution after the initial incubation in the gelling solution at 4 °C. We observed that the gelling reaction becomes inconsistent if the incubation at 4 °C lasts >2 h without replacement of the gelling solution
		The crosslinker was not added, or its concentration is too low (i.e., much less than 0.1%)	Make sure the final concentration of crosslinker is at least 0.1%
	The gel appears to be highly fragile and viscous	Insufficient time for polymerization	Ensure that the incubation time is the correct one. If necessary, try a longer incubation time (e.g., 3 h) to complete the gelling reaction
		The 4-HT concentration is too high	Add the correct amount of 4-HT. If the issue persists, try to reduce the amount of 4-HT by half and perform the gelling process. Note that reducing 4-HT concentration might result in premature gelling during the pre-gelation incubation in the refrigerator
		The quality of sodium acrylate is low	Get a new batch of sodium acrylate
10	The gel is still adhered to the slide	Insufficient digestion	Incubate longer with proteinase K solution or increase the proteinase K concentration; try manually removing the gel from the slide (Step 10B)
	The gel appears to be warped or curved in solution after digestion	Insufficient digestion of the gel due to too high a concentration of AcX	Lower the concentration of AcX by half
		Insufficient digestion of the gel due to sub-optimal digestion conditions	First, make sure the incubation temperature is 60 °C; second, try doubling the proteinase K concentration to see if the result is improved; finally, try to increase the incubation time. We recommend examination of the physical appearance of the gel every 30 min during digestion; as a rule of thumb, if the gel appears to be flat and transparent, that suggests a successful digestion and further incubation is not necessary
11	The gel gets sucked into the pipette tip and damaged	The specimen is transparent and hard to track in the aqueous solutions	Gently shake the specimen chamber and look for the water-gel boundary. Alternatively, illuminate the chamber with a white light LED from various angles to help locate the specimen
12	The expansion factor is less than fourfold	Insufficient dialysis with pure water; using salt-containing buffer instead of water for dialysis; incorrect composition of gelling solution.	Make sure that sufficient buffer exchange with pure water has occurred; make sure that the gelling solution is prepared correctly based on Table 3
	The gel is bigger than the expansion chamber	The gel piece is too big to fit in the chamber	Trim the gel and carefully move a part of it into another expansion chamber

Table continued

Table 5 (continued)

Step	Problem	Possible reason	Solution
14	Dim or absent fluorescence signal after expansion	Less suitable fluorophores were used, such as cyanine dyes Cy5, Cy3 and Alexa Fluor 647	Use the recommended fluorophores (such as Alexa Fluor 488, Alexa Fluor 546 and Atto 647N or CF633)
		The gel may be in the wrong orientation and most high-magnification objectives do not have long enough working distance to image through an entire gel	Use a paintbrush to flip the gel so that the tissue side faces the objective
		Overdigestion by proteinase K	Shorten the digestion time by half or reduce the proteinase K concentration by half and try again until the optimal digestion condition is found
	Focus is drifting	The specimen is moving	Mount the specimen with agarose or treat the glass surface with poly-L-lysine solution before placing the specimen
		The temperature of specimens and optical components of the microscopes are different	Wait until the temperature of the whole system is the same
	Unable to locate the same field of view taken before expansion	Expansion brings in more detailed tissue images, but sometimes this additional information can be confusing. In addition, the expanded specimen may be in a different orientation than that of the pre-expansion image	First, use low-magnification objectives to image the whole tissue section or at least a large portion of the entire specimen. Then, locate the matched field of view of the expanded specimen by comparing the two low-magnification tissue images taken before and after expansion. Finally, center the stage to the matched field of view and acquire images with high-magnification objectives
	The tissue slice is not completely flat in the gel; even minute waves in the tissue slice are amplified with expansion and can become problematic by reducing imaging quality and throughput	The tissue slice partially detached during the process. The tissue slice is not uniformly adhered to the slide	Use a super-frost glass slide to deposit tissue slices Bake the tissue slide at 60 °C for 10 min before the specimen-formatting process Handle gently to minimize disturbance to the tissue section

Timing

Pre-expansion processing:
Step 1, specimen pre-processing: 30–40 min
Step 2, heat treatment of the specimen: 30 min
Step 3, immunostaining: 9 h to 1 d (conventional); 2 h 50 min (rapid)
Step 4 (optional), imaging prior to expansion: 1–2 h
Expansion processing:
Steps 5 and 6, protein anchoring: >6 h, overnight for ExPath; 1.5 h for rExPath
Step 7, in situ polymer synthesis: 3 h for ExPath; 1 h for rExPath
Steps 8–10, proteinase K–based homogenization: 3 h for ExPath; 45 min for rExPath
Steps 11–13, expansion: 30 min
Step 14, imaging: variable depending on the image size
Step 15, estimate expansion factor: <30 min

Anticipated results

It is recommended that the tissue be imaged with a low-magnification objective lens before the in situ polymer synthesis step. First, pre-expansion imaging allows evaluation of the quality of antibody staining. Second, a quick comparison between low-magnification images of pre- and post-expansion samples enables the user to check for completion of the expansion process as well as to calculate the expansion factor. If the ExPath or rExPath process is successful, the pre-expansion image should look highly similar to the post-expansion one in the same field of view (but with lesser resolution), and the expansion factor should be between 4 and 5, if our protocol is followed. Finally, this practice also helps users to locate regions of interest for imaging with high-magnification objectives.

The number of specimens that can be simultaneously processed depends on the user's skills. In general, one can process up to 12 slides at one sitting from the start to the point where the slides are ready for imaging within a few hours using rExPath. As for ExPath, the throughput is about one third of that of rExPath, owing to longer processing times. The number of expanded sections that can be imaged within a single day is dictated by the time spent on the microscope. Based on our experience, each expanded section requires ~20 min for imaging of up to four fields of view on a spinning disk confocal microscope, such as Nikon's Ti2 Eclipse fluorescence microscope, equipped with the CSU-W1 spinning disk confocal imaging module.

High labeling density is critical for the quality of ExPath images. Poor labeling density results in punctate staining patterns and misrepresentations of ultrafine structures within the target. Thus, the user should screen labeling reagents and select the best staining conditions for optimal results. For example, in the case of kidney podocyte foot processes, we found that two specific antibodies against actinin-4 and synaptopodin were both capable of generating sufficient labeling density for resolving tertiary foot processes with feature sizes of ~200 nm, while other similar products available on the market yielded only punctate staining patterns. In addition, even with these two antibodies, heat treatment (Step 2 of the Procedure) was necessary to obtain good results. Another important factor to consider is the digestion condition. Over-digestion may result in reduced labeling density.

We demonstrate here a new variant of ExPath, rExPath, for the fast processing of human specimens. We have tested this new technique in lymph node samples from patients with and without HIV virus (Fig. 6) using both a confocal microscope (Fig. 6a–d) and a wide-field epifluorescence microscope (Fig. 6e–h). rExPath yields results that are highly similar to the original version on 5-μm-thick tissue sections (Fig. 2e–h and Fig. 3), but with significantly reduced processing time. However, users should note that rExPath has not been validated for tissue sections with thickness >5 μm. The performance of rExPath may not be optimal in those circumstances, due to the longer times required for sufficient diffusion of chemicals and antibodies. Therefore, we recommend that users use rExPath on tissue specimens with thickness ≤5 μm, while using ExPath for thicker tissues.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Part of the primary data underlying the figures presented in this article can be found as examples in https://github.com/zhao-biophotonics/ExPath-reg; the rest of the primary data can be provided upon reasonable request from the corresponding authors.

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Author contributions

Y.Z., O.B. and E.S.B. wrote the manuscript. Y.Z., O.B. and F.F. conducted the experiments. Y.Z., O.B., F.F. and E.S.B. analyzed the data. M.C. and S.W. conducted STED imaging for validation of rExPath. J.D. filmed and photographed the ExPath process. G.H.M., N.L.L. and B.D.W. provided de-identified human clinical specimens and helped with the experiments. Y.Z. and E.S.B. oversaw the research.

Competing interests

The authors have filed and obtained patent protection on a subset of the technologies here described (US provisional application no. 62/299,754, 62/463,265 and 62/463,251). E.S.B. helped cofound a company to help disseminate ExM to the community. O.B. is the Co-Founder and CEO of QPathology LLC, Boston, MA.

Additional information

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Key references using this protocol

Zhao, Y. et al. *Nat. Biotechnol.* **35**, 757–764 (2017): https://doi.org/10.1038/nbt.3892
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Data collection	For imaging data collection, we used the commercial Nikon NIS-Elements AR 5.11.00 64-bit
Data analysis	FIJI ImageJ AND https://github.com/zhao-biophotonics/ExPath-reg

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☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

Target Host Clonality Manufacturer Catalog No. RRID*
TOM20 Rabbit poly Santa Cruz Biotech sc-11415 AB_2207533

Collagen IV Mouse mono Santa Cruz Biotech sc-59814 AB_1121796

Vimentin Chicken poly Abcam ab24525 AB_778824

a-Tubulin Rabbit poly Abcam ab15246 AB_301787

VDAC/Porin Mouse mono Abcam ab14734 AB_443084

KRT19 Rabbit poly Sigma Aldrich HPA002465 AB_1079179

ACTN4 Rabbit poly Sigma Aldrich HPA001873 AB_1078096

Synaptopodin Guinea Pig poly PROGEN Biotechnik GP94-IN AB_2811107

Actin (Smooth Muscle) /ACTA2 Mouse mono Agilent Technologies M085129-2 AB_2811108

IgD Goat poly SouthernBiotech 2030-01 AB_2795623

S100A8 Mouse mono Abcam ab22506 AB_447111

MCT1 Chicken poly Sigma Aldrich AB1286-I AB_11212410

Tom20 Rabbit poly Sigma Aldrich HPA011562 AB_1080326

Nephrin Rabbit poly Santa Cruz Biotech sc-376522 AB_11151390

CASP3 Rabbit poly Sigma Aldrich HPA002643 AB_1846048

hFOXP3 Goat poly R & D systems AF3240 AB_2262812

Granzyme B Rabbit poly Sigma Aldrich HPA003418 AB_1079020

Cytokeratin Pan Type I/II Mouse mono Invitrogen MA5-13156 AB_10983023

GFAP Chicken poly Abcam ab4674 AB_304558

INA Rabbit poly Sigma Aldrich HPA008057 AB_1851833

Homer1 Rabbit poly Sigma Aldrich HPA036522 AB_2675171

PSD95 Goat poly Abcam ab12093 AB_298846

Synaptophysin Rabbit poly Proteintech 60191-1-Ig AB_10915965

Tyrosine Hydroxalase Chicken poly Abcam ab76442 AB_1524535

ATPIF1 Rabbit poly Millipore-Sigma Aldrich ABC137 AB_2811109

CD8 Rabbit poly Abcam ab85792 AB_10674324

P24 Mouse Mono Dako M0857 AB_2335686

PD-1 Goat Poly R & D systems AF1086 AB_354588

Hsp 60 Mouse Mono Abcam ab59457 AB_2121285

Validation

All the validation data is available on the manufacturers' website. No further validation was performed by us.