Expansion Microscopy: Improving Imaging Through Uniform Tissue Expansion

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Abstract

Until the past decade, optical microscopy of biological specimens was strongly limited by diffraction and scattering, affecting imaging resolution and depth, respectively. Now, numerous methods are available to overcome each of these limitations, but sub-diffraction limited resolution imaging over large volumes of scattering tissue is still a challenge. This work concerns the development of a new method, Expansion Microscopy (ExM) for achieving effect sub-diffraction-limited optical images in biological specimens. In ExM, the specimen is embedded in a swellable gel material to which fluorescent probes are chemically anchored. The embedded tissue is strongly digested so that it will not hinder uniform expansion driven by the gel. The gel with embedded, fragmented tissue is washed in water, triggering expansion of around 4-fold in each dimension. A variant of the method, ExM with Protein Retention (proExM) is presented that allows proteins themselves, rather than fluorescent probes, to be anchored by a small molecule cross-linker to the gel, so that the method may be carried out entirely with commercial components and standard antibodies.

Thesis Supervisor: Edward S. Boyden
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Table of Contents

Abstract .............................................................................................................. 1

Acknowledgements ............................................................................................ 2

List of Figures and Tables ............................................................................ 4

1. Introduction: Optical and Electron Microscopy in Neuroscience .......... 5
   1.1. Optical Microscopy ...................................................................................... 5
   1.2. Electron Microscopy .................................................................................... 7
   1.3. Overcoming Fundamental Limits in Optical Microscopy ......................... 9
       1.3.1. Optical Clearing to Reduce Scattering ..................................................... 9
       1.3.2. Super-resolution to Circumvent Diffraction ............................................. 9
   1.4. Multi-scale Imaging ................................................................................... 11
   1.5. Expansion Microscopy .............................................................................. 12

2. Expansion Microscopy Development and Method .................................. 13
   2.1. Superabsorbing Polyelectrolyte Gels ........................................................ 14
   2.2. Expansion Microscopy Method ................................................................ 19
   2.3. Characterization and Demonstration ......................................................... 23

3. Expansion Microscopy with Protein Retention ....................................... 33
   3.1. Limitations to In Situ Characterization ..................................................... 34
   3.2. ExM with Protein Anchoring and Reduced Digestion .......................... 35
   3.3. ProExM with Fluorescent Probes .............................................................. 40

4. Conclusion and Future Directions ......................................................... 48
   4.1. Expansion Microscopy Performance ......................................................... 49
   4.2. Protein Retention ...................................................................................... 50
   4.3. Potential Applications ............................................................................. 51

5. Appendix: ................................................................................................. 53
   5.1. Materials and Methods for ExM ............................................................... 53
   5.2. Materials and Methods for proExM ......................................................... 62

6. References .................................................................................................. 70
List of Figures and Tables

Figure 1. Swellable gel material. ................................................................. 16
Figure 2. Expansion factor vs. cross-linking.............................................. 17
Figure 3. Physically expanded brain tissue. .............................................. 20
Figure 4. Optical clearing associated with ExM........................................ 20
Figure 5. Uniformity of proteolysis versus depth..................................... 21
Figure 6. Schematic of tri-functional linker.............................................. 22
Figure 7. Quantification of Expansion Error Using Non-Rigid Registration 25
Figure 8. Nanoscale isotropy of ExM..................................................... 26
Figure 9. ExM in brain tissue................................................................. 28
Figure 10. ExM imaging of antibodies of interest in neuroscience and biology 30
Figure 11. Scalable 3D super-resolution microscopy of mouse brain tissue 32
Figure 12. Post-expansion antibody delivery, after epitope-preserving homogenization 37
Figure 13. Comparison of tissue disruption methods............................. 39
Figure 14. Incomplete homogenization with autoclave and LysC methods 40
Figure 15. Retention of fluorescent signals post-ExM............................... 42
Figure 16. Validation of proExM in different mammalian tissue types........ 44
Figure 17. Optimizing AcX penetration depth in fixed brain tissue........... 46
Figure 18. YFP retention with optimized AcX delivery............................. 47
Figure 19. Workflows for expansion microscopy with protein retention.... 48

Table S1. Fluorescence retention during ExM chemical steps................. 61
Table S2. Chemicals list and suppliers..................................................... 61
Table S3. DNA sequences and modifications used for tri-functional labels 62
Table S4. Monomer solution recipe.......................................................... 62
Table S5. Performance of selected FPs in proExM................................. 69
Table S6. Performance of selected secondary antibody dyes in proExM.... 70
Table S7. Primary antibodies used in Chapter 3.................................... 70
1. Introduction: Optical and Electron Microscopy in Neuroscience

1.1. Optical Microscopy

In the early twentieth century, Santiago Ramon y Cajal used optical microscopy to produce stunning drawings of complete neurons stained with the Golgi silver impregnation method, which produces a dense cell-filling stain that reveals anatomical details down to the elaborate micron-scale dendritic protrusions known as dendritic spines. From these anatomical studies, Cajal deduced the correct direction of the majority of information flow in neural circuits, with each neuron receiving signals in its dendritic branches, summing up those signals in the cell body, and relaying the resulting processed signals to downstream neurons via a single axonal branch.

Since the days of Cajal, the convenience and range of spatial scales accessible by optical microscopy have been supplemented with technological improvements both continuous and disruptive. Electronic photodetectors have replaced the by-hand drawings that Cajal used to document his findings. Materials and manufacturing technologies have gradually made high performance optics commercially available at modest prices, making optical microscopy a workhorse technology used routinely in labs throughout the biological sciences.

An optical microscope, like a camera, focuses rays of light emerging from a focal plane. As microscopes have much higher numerical aperture objective lenses than those used in cameras the depth of field is much narrower and out-of-focus light tends to produce a non-uniform background intensity rather than blurred images of objects throughout the specimen. In thick specimens this out-of-focus light can drastically decrease the signal-to-noise ratio of focused objects. This out-of-focus light can be physically rejected using confocal microscopy, allowing much better discrimination of objects in the axial dimension. Selective plane illumination microscopy (SPIM) achieves a similar result by illuminating the specimen with a plane of light delivered from the side, allowing higher imaging rates and reducing the amount of photodamage.

Fluorescent dyes with high brightness and low bleaching rates have emerged from the field of organic synthesis to enable high (for biology) signal-to-noise ratio imaging of
multiple targets labeled with different colors simultaneously. These dyes have proven especially useful when delivered by probes that bind to specific molecular targets within biological specimens. In particular, the immune system produces antibodies to flexibly recognize a wide variety of potentially novel invading foreign particles, a property that can be harnessed to develop probes that will bind to almost any molecular target of interest. What began as a customized procedure in which researchers would inject an animal with the molecular target of interest, tagged with an immune-stimulating compound, is now practically a commodity—antibodies are commercially available that bind to any of hundreds of thousands of targets. This allows researchers to find a suitable antibody label for essentially any native protein and many other biomolecules at a reasonable cost, with the caveat that antibodies come with varying levels of target affinity and non-specific binding, and can require significant optimization and experimental controls.

An additional class of fluorescent probes has become available with the development of molecular biology tools for manipulating DNA. The genetic code for naturally occurring fluorescent proteins can be introduced to live biological specimens. These proteins can then be used to trace genetically selected cells, fused to particular species of protein, and even anchored directly to the membrane of living cells.

Against this backdrop of new capabilities, two major, fundamental problems in optical microscopy remained until the past decade—diffraction and scattering, limiting the resolution and depth of imaging possible, respectively. For any optical system, which collects a fraction of the light emitted by a specimen, manipulates it, and relays it to a detector, every point emitting light in the specimen produces at the detector a pattern, termed the point spread function (PSF) for that system. Thus, the image produced at the detector can be expressed mathematically as an ideal point-by-point image of the specimen convolved with this spatially extended PSF, resulting in a blurry image of the specimen. The amount of blur depends on the size of the PSF. Well-engineered optical components can reduce the size of the PSF in the image plane but only up to a point—according to the wave theory of light, an optical system capturing only a fraction of the light emitted by a point source will produce diffraction at the edges of the collection optics, which places a lower limit on the size of the PSF. Thus two points sufficiently close to each other in the specimen will produce overlapping PSFs in the image, frustrating efforts to resolve them as distinct points. This
diffraction limit in the plane of the image is approximately half the wavelength of the collected light, divided by the numeric aperture (NA), which is defined by the index of refraction of the imaging media times the sine of the half angle of light collection at the objective lens. For visible light, and a well-designed objective lens collecting close to half the light emitted by the specimen, this results in a resolution limit around several hundred nanometers, or the size of a single synapse in the brain.

In the axial dimension (along the direction of the light path) the PSF is more spatially extended, leading to worse resolution in this dimension and the introduction of out-of-focus light from the entire thickness of the specimen. In confocal microscopy, this out-of-focus light is physically rejected, but the axial resolution is unaffected. The question of which dimension of blurring is the limiting factor in an imaging application depends on the question being addressed. This blur can in principle be computationally removed by deconvolution, but performance of these methods depends on signal-to-noise ratio, which is often relatively low in biological imaging.

Imaging with shorter wavelengths of light, such as ultraviolet or X-ray, would automatically improve diffraction-limited resolution but is not practical for many applications. In particular, X-rays cannot be controlled with nearly the precision and power of glass optics operating in the visible range, resulting in much lower resolution images in practice, in the range of tens of microns, and do not have the full spectrum of distinguishable colors available with visible fluorescence dyes. Closer to the visible spectrum, UV dyes typically have worse brightness and photostability performance than those in the visible range, though UV microscopy can be useful in some applications where the intrinsic UV absorption of various biomolecules is of interest.

1.2. Electron Microscopy

Electrons have much shorter wavelengths than visible light or X-rays, being essentially particle-like for imaging purposes, and though they can be very damaging to biological specimens they are easier to focus than X-rays by using electrostatic fields in free space. Thus, in the twentieth century, the optical diffraction limit was circumvented through the development of electron microscopy, especially in the transmission mode (TEM). In hard materials, electron microscopes can produce images with atomic-level resolution. Cryo-electron tomography achieves high resolution images of proteins and protein
complexes by observing many copies of the same macromolecule, from different angles\textsuperscript{14}. Due to lower signal-to-noise ratio and susceptibility to beam damage, TEM of intact biological specimens is typically limited to several nanometer resolution\textsuperscript{15}, two orders of magnitude better than the optical diffraction limit. Unfortunately, this resolution boost relative to optical microscopy comes with more elaborate and specialized specimen preparation procedures, more limitations on ease of antibody staining—especially multi-color staining, and requires embedding specimens in a polymeric resin and slicing them to less than 100 nm thick using a specialized diamond knife apparatus\textsuperscript{16}.

With the resolution of TEM, and chemical methods to stain for lipid and protein density\textsuperscript{17}, it is possible to clearly visualize many features of the machinery responsible for the transmission of signals across a synapse, including the vesicles that store and release small molecule neurotransmitters from the pre-synaptic side, the tightly controlled gap or synaptic cleft between pre- and post-synaptic neurons, a dense array of proteins responsible for transducing the chemical signal into both electrical and chemical signals on the post-synaptic side, and a complex set of proteins that scaffold all these components\textsuperscript{18,21}. Many synapses are formed not directly on a dendritic branch of the post-synaptic neuron, but on a micron-scale bulb known as a dendritic spine, attached to the branch by a thin fiber, termed the dendritic spine neck. These structures affect electrical signaling by adding a high electrical impedance along the path to the dendritic branch and partially isolating the dendritic spine. Multiple synapses can be housed on the same spine head, sometimes carrying signals from distant parts of the brain and applying different signal transformations, suggesting a complex signal processing function confined to micron-sized compartment\textsuperscript{21}. A single neuron may have easily thousands of such compartments. In addition to these details of neuronal structure, increasing evidence points to the importance of non-neuronal glial cells in neural circuit development, maintenance and function, again with structural features beyond the resolving power of diffraction-limited optical microscopes. TEM has played a large role in the initial exploration of these structures.
1.3. Overcoming Fundamental Limits in Optical Microscopy

1.3.1. Optical Clearing to Reduce Scattering

Until the past decade, optical microscopy for biological specimens has seen little technical innovation on two limitations: the diffraction limit on resolution (as discussed above), and scattering. Anyone who has eaten French fries out of a thin paper bag can appreciate that the paper is opaque not because of light absorption but because of light scattering—when oil from the fries soaks into it, the paper becomes transparent by preventing scattering. The organic fibers of the paper normally form a complex network permeated by air, producing a strong, random spatial variation in the index of refraction, on size scales similar to the wavelength of visible light. This results in many refractions in random directions, or scattering. This random scattering of light rays prevents light from penetrating far through the material. By introducing oil to fill the space between fibers, the index of refraction is homogenized. Similarly, biological tissue consists of high-index lipid and protein structures immersed in a relatively low-index aqueous environment, introducing scattering and impeding clear resolution of structures more than a few tens of microns from the surface. Much as cooking oil clears the paper bag, more specialized high index fluids have long been used to partially match the complex variation of index in biological tissues.

The past decade has seen a renewed effort to optimize this technique for better clearing and simultaneous preservation of fluorescent signals. In parallel, a novel technique has been developed to achieve optical clearing in entire intact organs, and even entire intact animals, by embedding them in a material familiar to biologists—a polyacrylamide gel material similar to that used to analyze biological homogenates by electrophoresis. Like the polymeric resins used for TEM, this material locks native biological molecules in place, but also has large enough pores for lipids to be strongly extracted, resulting in homogenization of the tissue refractive index to that due to proteins and carbohydrates alone, which can be effectively matched with a single index matching fluid.

1.3.2. Super-resolution to Circumvent Diffraction

In the past decade, sub-100nm resolution has been extended to optical imaging through the emergence of several “super-resolution” microscopy methods. Preceding the work reported here, there were three broad approaches to far field super-resolution
microscopy—structured illumination microscopy (SIM), stochastic point source localization (e.g. STORM) and point scanning sub-diffraction limited excitation (e.g. STED). Each has its own benefits and drawbacks. Near-field microscopy is not subject to diffraction, but is only sensitive to surface features, a major limitation on utility in biological studies.

**Structured Illumination Microscopy (SIM).**

In SIM, a wide-field illumination beam is modulated with a sine wave in one dimension at a high spatial frequency. The phase and direction of this sine wave are varied over several values, with the final super-resolved image computed from the resulting frames. The resulting resolution is about twice as good as the diffraction limit. Intuitively, as a sinusoidal pattern of illumination intensity is passed over a point source, the brightness of the imaged point spread function also varies sinusoidally. The variation of this brightness as a function of the known spatial phase of the illumination sinusoid is strongest at the point that the sinusoid varies about its average value, providing a sensitive measure of the true location of the point source. This method requires no special fluorophores or specimen preparation but does require algorithmic reconstruction. Like deconvolution, it suffers in low signal-to-noise ratio or thick scattering specimens.

**Stochastic point localization.**

These methods (e.g. PALM, STORM) take advantage of the fact that many fluorescent dyes switch stochastically during illumination (or under control of another wavelength of light, as with fluorescent proteins used in PALM) between a light and non-fluorescent dark state. By tuning the fraction of molecules in the dark state during any given exposure, it is possible to ensure that most molecules in the fluorescent state are separated from each other by at least the diffraction limit. The point spread function for each molecule can thus be imaged separately and its center identified to much better than the diffraction limit. Many such exposures can be acquired, with different random subsets of dye molecules in the fluorescent state in each frame, and eventually a full image can be assembled with resolution far exceeding the diffraction limit. The need to collect many exposures for each frame in the final image limits the acquisition speed, depending on how sparse the fluorophores are to begin with, and the need to image single fluorophores means that high performance optical systems are required. The need for single molecule imaging, and the bleaching of fluorophores outside the imaged focal plane limit the application of this
technique to imaging longer than the scattering depth away from the specimen surface. Effective fluorophore blinking can also be achieved by localizing fluorophores to antibodies not by a permanent chemical bond, but by a short DNA oligonucleotide with a tunable bound-unbound fraction, similarly resulting in a sparse subset of probes bearing a fluorophore in any given exposure\textsuperscript{33}.

\textbf{Sub-diffraction limited excitation beam.}

These methods (e.g. STED, RESOLFT) produce an effectively sub-diffraction limited excitation beam by superimposing a diffraction limited excitation point with a donut-shaped de-excitation pattern, where the dark spot in the middle of the donut is the only part of the pattern that is not de-excited prior to fluorescence collection. While most of the donut is beyond the point of saturating the de-excitation effect, the donut hole can be made effectively much narrower than the diffraction limit. This effectively sub-diffraction limited excitation beam is then rastered over the specimen as in confocal microscopy. In STED, de-excitation is achieved by stimulated emission, requiring specialized equipment to produce high laser powers, and also limiting the application of this technique to thick tissues\textsuperscript{34}. In RESOLFT, the fluorophores used are engineered fluorescent proteins that can be switched to a dark state by illumination at modest powers, but requires expression of these fluorophores separately for each target of interest.

1.4. Multi-scale Imaging

In order to affect the neuron’s firing rate, electrical signals generated by the synaptic machinery must propagate to the cell body through the dendritic arbor, possibly with many branch points with varying levels of electrical impedance matching. This process depends strongly on the detailed geometry of the dendritic arbor\textsuperscript{35}, which extends over much larger volumes than those typically imaged in the ultrathin (sub-100nm) slices of TEM or existing super-resolution methods. These existing super-resolution methods are most often demonstrated in cultured cells and thin (~10 micron) tissue slices, due to the higher stringency on optical system quality (e.g. greater required signal-to-noise ratio) they require compared to routine fluorescence imaging and, in the case of RESOLFT, the difficulty of expressing fluorescent proteins in live animals compared with cultured cells.

Due to the interest in reconstructing large volume images of neural tissue with high resolution, several methods have been developed to circumvent the volume limitations of
electron and super-resolution optical microscopy. Large volumes of tissue can be automatically cut into an array of ultra-thin slices, which can be imaged by TEM. 3D images can be reconstructed from these slices and neuronal axons and dendrites traced using semi-automated algorithms. Sub-diffraction limited optical microscopy can, itself, be performed on serially arrayed ultrathin tissue slices. If scanning electron microscopy (SEM) is used rather than TEM, slicing and imaging can be integrated into one device that alternates scanning the specimen surface with slicing the imaged material off, eventually scanning through the entire volume. Staining can be optimized to cover the whole brain uniformly prior to slicing. SEM can also be integrated with focused ion beam milling (FIB-SEM) for the automated removal of as thin as 15nm layers of material after imaging each block face.

Correlated light and EM.

It is possible to obtain both fine ultrastructure and molecular annotation by carrying out diffraction-limited optical microscopy followed by electron microscopy of the same specimen, with the fluorescent signal converted into an electron-dense signal, known as correlative light and electron microscopy. This approach can be extended to large volumes using Array Tomography, in which ultrathin tissue slices are arrayed out, as in serial electron microscopy, but before electron microscopy the slices are stained with fluorescent antibodies and imaged by optical microscopy, potentially with multiple rounds of antibody staining and imaging. This allows ultrastructure to be annotated with multi-color molecular information. Although lateral resolution is in this case subject to the diffraction limit, the axial resolution is much better than the diffraction limit because the slices themselves are a fraction of an optical wavelength thick. Electron microscopic reconstructions can also be paired with in vivo characterization of neural function using, for example, an optical reporter of neural activity, yielding a powerful tool for studying neural circuits.

1.5. Expansion Microscopy

In this work, I present a method developed to image sub-diffraction-limited structures in biological tissue in the context of thick tissue slices, in which the specimen is physically expanded prior to imaging on a diffraction-limited microscope. This technique requires no special equipment, imaging conditions, or staining methods, other than a need for longer exposure times to compensate for lower signals after expansion. Unlike other
super-resolution methods, however, physical expansion is not compatible with in vivo imaging. Expansion is achieved by embedding the antibody-stained specimen in a swellable material, chemically anchoring molecules of interest to the material, digesting the specimen to remove mechanical impediments to uniform expansion, and washing the gel-embedded specimen in water to drive expansion. The specimen then swells by a factor of approximately 4-fold in each dimension, absorbing >50 times its weight in water. Once expanded, the specimen is more than 99% water, with highly uniform refractive index, rendering it transparent and index-matched to water. This enables imaging through a thick tissue volume using standard water-immersion optics. A small molecule cross-linker bearing a protein-anchoring group and a gel-polymerizable group enables native proteins, antibodies and fluorescent proteins to be retained in the gel. Fluorescently labeled antibodies and fluorescent proteins retain about half their fluorescence, enabling super-resolution imaging using only commercially available chemicals and diffraction-limited confocal microscopes.

2. Expansion Microscopy Development and Method

*This section reflects work conducted with Fei Chen and published previously*.

In optical microscopy, fine structural details are resolved by using refraction to magnify images of a specimen. In Expansion Microscopy (ExM), the entire specimen is physically expanded, bringing structures smaller than the diffraction limit into the size range resolvable with a diffraction-limited microscope. Biological specimen expansion is driven by a swellable material that is synthesized in situ, throughout the approximately 60% water volume of the tissue. Fluorescent dye molecules are delivered by antibodies to label molecules of interest, and chemically anchored to the swellable material. The mechanical structure of the specimen is then disrupted by a strong proteolytic treatment, allowing uniform expansion driven by the swellable material. The uniformity of expansion is expected to degrade at some length scale—molecules are not expanding on the atomic scale, and any spatial information at a scale finer than the distance between gel cross-links will be lost due to random fluctuations of the polymer chains. The present method uses four-fold expansion to achieve an effective resolution of about 70nm using diffraction-limited water immersion optics, a distance over which numerous crosslink-to-crosslink polymer chain lengths will display statistically averaged chain fluctuations. The uniformity of expansion at length scales
from macroscopic down to the finest resolvable features is demonstrated by imaging and registering the same fields of view before with an existing super-resolution method and after expansion, and by measuring the size of well-defined sub-cellular structures with expected features in the sub-diffraction-limited range. ExM is demonstrated in both cultured cells and intact brain tissue, performing three-color super-resolution imaging of \( \sim 10^7 \mu \text{m}^3 \) of the mouse hippocampus with a conventional confocal microscope.

2.1. Superabsorbing Polyelectrolyte Gels

At the heart of expansion microscopy is the material driving uniform expansion of the specimen, a crosslinked polymer network consisting primarily of sodium polyacrylate, which can hold many times its solid weight in water to produce a gel. This material was the first polyelectrolyte gel to be thoroughly characterized, by Tanaka and coworkers starting in the 1970’s\(^{47,48} \), and is now widely used in commercial applications requiring strong absorption of water, such as diapers and for water retention in soil\(^{49} \). Sodium polyacrylate was selected for use in expansion microscopy based on the thorough characterization available in the scientific literature, the ready commercial availability of its monomeric precursors, and the simple chemical procedures involved in its synthesis—factors also likely influencing its widespread adoption for commercial uses.

**Polyelectrolyte gels**

The main precursor to a polyacrylate gel is the monomer sodium acrylate, an electrolyte containing a carboxylate group attached to a double-bonded carbon-carbon pair. The carbon-carbon double bond is susceptible to attack by a free radical (unpaired electron), whereupon the double-bonded electron pair supplies one electron to form a bond with the attacking radical, and one to generate a new radical at the end of the growing chain. The new radical can then attack another monomer, growing the polymer chain one monomer at a time. This is the radical polymerization chain reaction used to create common plastic materials including polystyrene and polyethylene, although the radical reactivity of acryloyl monomers (including acrylate, acrylamide and other modifications of the carboxy moiety) can be quite different from these examples due to the proximity of the carbonyl (double bonded carbon-oxygen) group.

Much as rubber must be cross-linked with sulfur-sulfur bonds through vulcanization to form the tough material used to make tires, in order to form a polymeric material that can
remain solid while retaining many times its solid weight in water, the polymer chains must be
crosslinked to each other to form a continuous network. In the case of a polyacrylate gel,
this is achieved by including a cross-linker containing two acryloyl groups, which can add to
two polymer chains independently, forming a cross-link. It is also possible for a single
growing polymer chain to react first to one acryloyl of the cross-linker, and then the other,
forming a loop rather than a cross-link—a network defect.

Once the polymer network is formed, the material contains a high density of
negative charges from the carboxylate groups chemically attached to the polymer backbone,
and an equal density of positive counterions free to diffuse within the macroscopic volume
defined by the polymer network. The polymerization reaction can be carried out in the
presence of excess free ions from sodium chloride, which provide additional shielding of the
mutual repulsion of the fixed negative charges for each other, allowing the polymer chains to
adopt a random walk-like collapsed conformation (Figure 1i). When this material is washed
with salt-free water, the excess ions are washed away, reducing charge shielding and
increasing the mutual repulsion of the polymer chain for itself. This forces the chains into
more extended conformations, driving macroscopic expansion of the material (Figure 1ii).

The free counterions to the fixed negative carboxylates also exert a swelling effect
related to osmosis\textsuperscript{50,51}. In the textbook example of osmosis, solutes are confined to a region
of space defined by a membrane that is permeable to water but not the solute species. If the
membrane is elastic, water will enter the enclosed volume, causing it to swell while diluting
the impermeant solute. Likewise in the polyelectrolyte gel, the counterions must remain
confined to the volume of the polymer network to ensure overall charge neutrality, but that
volume can increase by absorbing water and forcing the polymer chains to adopt more
extended conformations. The overall degree of swelling is then determined by a balance of
the increase in entropy of the counterions accessing a greater volume, the decrease in
entropy of the polymer chains adopting more extended conformations, and the change in
enthalpy as extended polymer chains interact less with each other and more with the solvent.
Figure 1. Swellable gel material.
Schematic representation of a collapsed polyelectrolyte polymer network (i) in high salt, showing crosslinker (dot) and polymer chain (line), and expanded polymer network (ii) after dialysis with H2O.

Gel network uniformity

Conveniently, polyacrylate gels tend to have better uniformity than their nearest non-electrolyte analog, polyacrylamide. As the polymer forms, the osmotic effect that drives macroscopic expansion also influences the growing chains to adopt more extended conformations on the micro-scale than they otherwise would. This in turn reduces the chance of a growing chain to react twice to the same cross-linker, reducing the network defect rate.

Because polyacrylate and related copolymers are thoroughly studied in the context of polymer physics, there is a wealth of information available to guide the design of a suitable material for tissue expansion. Perhaps the most important consideration is the uniformity of the polymer network and the distribution of its cross-links—the swelling extent is strongly dependent on the cross-link concentration (Figure 2), with more cross-linking resulting in a tougher material with a smaller degree of expansion when equilibrated with salt-free water. Variations of the cross-linker concentration on the micron scale could potentially result in non-uniformities in expansion that would not be apparent at the macroscale.
Figure 2. Expansion factor vs. cross-linking.
Linear expansion factor for gels cast without specimens, as a function of the concentration of cross-linker used. Error bars represent standard deviation (n = 4 samples).

Another contributor to cross-linker inhomogeneity is the differing radical reactivities of the monomer acrylate and the uncharged cross-linker. These differences result in complex, temporally non-uniform reaction kinetics. In particular, the reaction rate for adding a monomer to the end of a growing polymer chain depends on the identities of both the monomer and the radical at the growing end of the chain. The effect on polymer composition as a function of time throughout synthesis, due to the non-unity ratios between these four distinct rates, can be predicted using the Mayo-Lewis model\textsuperscript{54}. For a polymer synthesized from two different monomers, this model describes the instantaneous change in free monomer concentrations by considering the reaction rates for adding a monomer of each type to a growing chain capped with an activated radical of each monomer type. The reactivity ratio for each monomer is defined by considering a growing chain terminated with that monomer in its radical form, and finding the ratio of reaction rates for that chain to add another monomer of the same vs the other type.
In the case of acrylic acid and acrylamide, a close chemical analog of the cross-linker used, the reactivity ratio of acrylate is less than 0.5 and that of acrylamide is greater than 1 for the reaction conditions relevant here\textsuperscript{55}, which means that growing chains terminated with either an acrylate or and acrylamide both have a greater propensity to add acrylamide than acrylate. The result is that acrylamide will be rapidly incorporated at the beginning of the reaction and the ratio of free acrylamide to acrylate monomer will decrease during the reaction, altering the composition of the resulting polymer as a function of time. Considering the results of calculating the polymer composition as a function of the fraction of the more reactive acrylamide monomer in the feed stock (Fig. 4-2 in \textsuperscript{56}) the relative enhancement of acrylamide in the polymer versus the monomer stock is greater for low concentrations of acrylamide compared to stocks with a more equal amount of the two monomers. This will result in greater heterogeneity during the course of polymerization. Indeed, the acrylamide-based crosslinker used in polyacrylate gels is present at roughly 1 mol\% of total monomer, resulting in non-uniform incorporation of cross-linker as polymer chains are synthesized. We thus reasoned that including a significant mole fraction of acrylamide itself in the monomer precursor would result in faster and more homogenous reaction kinetics.

**Controlling reaction with polymerization inhibitors**

To achieve uniform expansion it is critical that the precursor solution have sufficient time to permeate the specimen completely. For cultured cells one cell monolayer thick this is not an issue, as the small molecule precursor diffuses uniformly over distances of microns in seconds. The precursor solution contains dissolved oxygen from the atmosphere, a potent inhibitor of polymerization\textsuperscript{57}, resulting in a lead time of several minutes (at room temperature) before the precursor begins to gel. This leaves plenty of time to apply the solution to cells and move them to the recommended temperature for gelation.

In a slice of tissue hundreds of microns in thickness, the diffusion time can no longer be ignored. It is possible to get a rough estimate of the time required for diffusion to approach completion in a thick tissue slice, using the rule of thumb that the characteristic diffusion time is approximately the diffusion distance squared, divided by the diffusivity (Section 3.2.5 in \textsuperscript{58}). Using a rough value of $10^{-5}$ cm$^2$/s for small molecules in water (Fig. 3.11 in \textsuperscript{58}) and a tissue thickness of 250 microns as an example, the characteristic time would be about 1 minute. Assuming that the actual diffusive path length in modestly permeabilized
tissue would be several times greater than that in vitro, and a requirement of several characteristic time constants to approach equilibrium, delaying polymerization by 30 minutes should allow adequate penetration of the precursor solution.

The ideal compound to solve this problem would be one that would react and permanently terminate free radical initiators and short polymers, and once consumed not further affect the rate of polymerization (i.e. an ideal inhibitor\textsuperscript{59}), and must be water soluble. In living radical polymerization, radical polymerization kinetics are controlled by the addition of a stable radical compound that reversibly caps growing polymer chains, significantly slowing their growth. One family of compounds commonly used for this purpose is the cyclic nitroxides, encompassing 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) with various substitutions. These are included in excess to the radical initiator in order to achieve slower, controlled polymerization of all growing chains simultaneously, with reduced termination between chains.\textsuperscript{60} We reasoned that a low concentration of such a stable radical would react to short growing oligomers in the early stages of polymerization, strongly slowing their growth and essentially preventing them from participating in the gel network. Once the stable radical was consumed, subsequently initiated chains would then grow at the normal, much faster rate, resulting in a similar polymer network but with an induction time controlled by the amount of inhibitor to allow the precursor solution to diffuse throughout thick tissue specimens. While these radical syntheses are commonly carried out in organic solvents, the 4-hydroxylated version, 4-hydroxy-TEMPO is water soluble and thus suitable for use in Expansion Microscopy.

### 2.2. Expansion Microscopy Method

The ExM process starts with brain tissue that is chemically fixed and permeabilized according to standard immunofluorescence protocols (Figure 3, left), into which the gel precursor solution is infused: the electrolytic monomer sodium acrylate\textsuperscript{48,49}, the co-monomer acrylamide and the crosslinker N-N'-methylenebisacrylamide. After triggering \textit{in situ} free radical polymerization with ammonium persulfate (APS) initiator and tetramethylethylenediamine (TEMED) accelerator in the presence of salt, the embedded brain tissue is treated extensively with protease to homogenize the mechanical characteristics of the tissue-polymer composite. Following proteolysis, dialysis of the processed specimen in deionized water resulted in a 4-fold linear expansion, without distortion at the level of gross
anatomy (Figure 3, right). The brain tissue thus expanded through this process is transparent (Figure 4), since it consists almost entirely of water. We found the digestion to be uniform throughout the slice (Figure 5).

**Figure 3. Physically expanded brain tissue.**
Photographs of fixed mouse brain tissue section before (left) and after (right) ExM processing. After-expansion image is taken under intense side illumination. Scale bars: 5mm

**Figure 4. Optical clearing associated with ExM.**
Expansion significantly reduces scattering of the sample, since the sample is mostly water. A 200 μm fixed brain slice is opaque primarily due to scattering (A). However, the post-ExM sample is transparent (B). We quantified the transmittance of light through the tissue pre- (blue) vs. post- (orange) ExM processing (C).
Figure 5. Uniformity of proteolysis versus depth.
Residual YFP fluorescence as a function of depth in the slice, after digestion and expansion. Proteolysis during the ExM process significantly reduces endogenous YFP fluorescence in expanded samples of Thy1-YFP mouse brain. The residual YFP fluorescence can thus be imaged as a proxy for proteolytic homogeneity. (A) Maximum intensity projection in the X dimension of 25 Z stacks of pyramidal cells in the cortex of Thy1-YFP-H brain slices after overnight digestion and subsequent expansion, and processed to exclude high-brightness nuclei and background as described in the Methods. The length in the Z dimension is 400 μm in post-expanded units (almost all of the thickness of the 100 μm slice after ~4.5x expansion). (B) Mean fluorescence intensity across the stacks of (A), normalized by overall mean fluorescence, plotted as a function of Z. Error bars, mean-normalized stack-to-stack standard deviation (n = 25 Z stacks, 2 brain slices). Dotted line, visual reference for fluorescence equal to the mean. The lack of systematic variation of YFP fluorescence vs. depth demonstrates the uniformity of proteolytic digestion throughout the slice.

In order to use the swellable polymer discussed above to drive tissue expansion, it is necessary to chemically anchor dye molecules to the material, so that the tissue can be enzymatically dissolved while the staining pattern is retained. The conceptually simplest way to achieve this is to use a modified version of the fluorescently labeled secondary antibodies that are normally used in immunofluorescence microscopy, such that the dye molecules are themselves also decorated with a monomer analog that will be incorporated into the swellable material. Small molecule fluorophores are commonly available with one site that can be readily reacted to form a conjugate with another item, such as an antibody. This attachment chemistry could be, for example, an NHS-ester, which reacts readily with the
primary amine side chain of the amino acid lysine. Thus, dye anchoring could be achieved by using a tri-functional crosslinker that would attach to the dye, and a secondary antibody, and carry a radical reactive monomer.

The commercial availability of chemically modified DNA oligonucleotides offers a route to avoid custom chemical synthesis. Conveniently, methacrylamide modifications are used to anchor DNA oligonucleotides into polyacrylamide gels\(^{61}\), and thus are perfectly suited to anchor to the acrylate-acrylamide copolymer used in this work. As DNA oligonucleotide synthesis is a highly modular technology, it is straightforward to order a single strand labeled with both the methacrylamide monomer and a fluorescent dye. A complementary strand can be modified to attach to the secondary antibody, providing the third chemical function. Thus, the fluorescent tag is targeted to a biomolecule of interest, yet remains anchored covalently to the polymer network throughout the ExM process (Figure 6).

![Figure 6. Schematic of tri-functional linker.](image)

Schematic of fluorescent label that can be targeted to a biomolecule, anchored to the gel, and retained after protein digestion. (right) Schematic of antibody stained microtubules (green) embedded within the polymer network (orange). Oligonucleotide labels (upper left) are hybridized to complementary oligonucleotides covalently attached to secondary antibodies and incorporated during gel synthesis. (lower left) Fluorophores (green) introduced via the DNA label, and hybridized (blue) to the oligo-bearing secondary antibody bound via the primary antibody (gray) to the microtubules (purple), are incorporated into the gel (orange lines) via the methacryloyl group (orange dot) and remain after digestion of all proteins (dotted lines).
2.3. Characterization and Demonstration

Expansion is bound to be non-uniform below some length scale, not smaller than the distance between polymer crosslinks but potentially on longer, observable scales as well. To test for this we image the same specimens before and after expansion, using either laser scanning confocal microscopy (LSM) or super-resolution SIM (SR-SIM). Structures with known shape and size can also be imaged and measured to confirm uniformity on observable length scales.

**Characterization in cells**

We performed fluorescence imaging using ExM, examining microtubules in fixed HEK293 cells labeled with the DNA-based tri-functional label and imaged with confocal laser scanning microscopy pre- vs. post-ExM processing. The post-ExM image (Figure 8b) was registered to the pre-ExM image (Figure 8a) via a similarity transformation, resulting in visually indistinguishable images. To quantify the isotropy of ExM, we calculated the deformation vector field between the images via a non-rigid registration process (Figure 7).

From this vector field, we quantified the root-mean-square error of feature measurements post-ExM, and found that the errors in length were small (<1% of distance, for errors larger than the imaging system point spread function size) (Figure 8c, n = 5 samples). Unless otherwise noted, all distances measured in the post-expansion specimen are reported divided by the expansion factor.

We next compared pre-ExM images taken on a conventional super-resolution microscope to post-ExM images taken on a confocal microscope. Specifically, we labeled features traditionally used to characterize the performance of super-resolution microscopes, including microtubules and clathrin coated pits, and imaged them with a super-resolution structured illumination microscope (SR-SIM) pre-ExM, and a spinning disk confocal post-ExM. Qualitatively (Figure 8d, e), the images were similar, and quantitatively (Figure 8), measurement errors were again on the order of 1%, and well within the point spread function size of the SR-SIM microscope (n = 3 samples). Microtubule networks appeared more sharply resolved in ExM (Figure 8g) than with SR-SIM (Figure 8f). ExM clearly resolved individual microtubules that could not be distinguished with SR-SIM (Figure 8h). Microtubules imaged with ExM presented a full width at half maximum (FWHM; representative microtubule shown in Figure 8j) of $83.6 \pm 5.3$ nm (mean ± standard
deviation, \( n = 24 \) microtubules from 3 samples). This FWHM reflects the resolution of ExM convolved by the width of the labeled microtubule. To estimate the resolution of ExM, we deconvolved (as in \(^6\)) our observed microtubule FWHM by the known immunostained microtubule width (55 nm\(^3\)), conservatively ignoring the width of the tri-functional label, and obtained a resolution for ExM of \( \sim 60 \) nm. This conservative estimate is comparable to the diffraction limited confocal resolution (\( \sim 250 \) nm lateral resolution \(^6\)) divided by the observed expansion factor (\( \sim 4.5 \)).
Figure 7. Quantification of Expansion Error Using Non-Rigid Registration.

We quantified the error of ExM by deforming the post-ExM image via a non-rigid registration process to attempt an exact match to the pre-ExM image (in contrast to the similarity transform used in Fig. 2A, 2B and elsewhere). We used a B-spline based non-rigid registration algorithm (see Methods) which generates a vector field that maps the post-ExM image (A, magenta) to the pre-ExM image (A, green). In (A) the overlap between the pre- and post-ExM images appears white, and the deformation vector field (white arrows) is plotted with vector magnitudes scaled by factor of 2 for visibility. The post-ExM image after deformation (B, magenta) colocalizes with the pre-ExM image (B, green; overlap appears white). Using this deformation field, we can calculate the error of ExM for various length measurements. This is schematized in (C): the magenta lines, representing structures in the post-ExM image, are mapped to the green lines, representing the pre-ExM image, via the vector field depicted by black arrows. Measurement L' along the line segment A'B' in the post-ExM image is mapped to measurement L along the line segment AB in the pre-ExM image. The ExM error is calculated as |L-L'|, i.e. the difference between the deformation vectors AA' and BB'. Using the deformation field generated we can calculate the ExM error between all extracted features in the pre- vs. post- images. Scale bars: 1 μm.
Figure 8. Nanoscale isotropy of ExM.
Expansion microscopy physically magnifies with nanoscale isotropy, enabling super-resolution imaging on diffraction-limited microscopes. Throughout this figure, we compared images acquired via a conventional microscopy method (blue scale bars), vs. images acquired post-expansion (orange scale bars). (A), (B) LSM image of microtubules (40x, 1.30 NA) (A) and post-expansion LSM image of the same sample (20x, 0.8 NA) (B). (A) and (B) are maximum intensity projections (MIPs) of 1.5 μm thickness (in pre-ExM distance units). (C) Root-mean-square (RMS) length measurement error of pre- vs. post-ExM confocal images of cultured cells (blue line, mean; shaded area, standard deviation; n = 5 samples). (D), (E) SR-SIM image of immunostained microtubules (D) (100x, 1.40 NA used here and throughout the rest of this figure), and post-expansion image of the same sample taken on a spinning disk confocal (E). (D) and (E) are MIPs of 1 μm thickness (in pre-ExM distance units). (F), (G) Magnified views of boxed regions of (D) and (E) respectively. (H) Profiles of microtubule intensity taken along the blue and orange dotted lines in (F) and (G), and plotted using the same colors, demonstrating ExM resolution vs. SR-SIM resolution. (I) RMS length measurement error of ExM vs. SR-SIM images (blue line, mean; shaded area, standard deviation; n = 5 samples). (J) Line profile of the width of a representative microtubule (blue line), with Gaussian fit (black dotted line). (K), (L) SR-SIM image of immunostained clathrin coated pits (CCPs) (K), and post-expansion image of the same sample imaged with spinning disk confocal (L). (K) and (L) are MIPs of 500 nm thickness (in pre-ExM distance units). (M), (N) Magnified views of a single CCP in the boxed regions of (K) and (L) respectively. The SR-SIM image (M) is depicted interpolated such that the pixel size is the same as that of the ExM image (N). (O) Scatterplot of radii of individual CCPs measured via angular averaging of the radial intensity profile. The CCP radius measured from the
ExM image is plotted against the radius measurement in SR-SIM. The green line represents the line \( y = x \), and the shaded green region represents half-pixel width of digitization error in both \( x \) and \( y \). Scale bars for pre- vs. post-ExM images: (A) 20 \( \mu \text{m} \), (B) 20 \( \mu \text{m} \) (physical size post-expansion, 81.6 \( \mu \text{m} \)); (D) 2 \( \mu \text{m} \), (E) 2 \( \mu \text{m} \) (9.1 \( \mu \text{m} \)); (F) 500 nm, (G) 500 nm (2.27 \( \mu \text{m} \)); (K) 2 \( \mu \text{m} \), (L) 2 \( \mu \text{m} \) (8.82 \( \mu \text{m} \)); (M) 100 nm, (N) 100 nm (441 nm).

A comparison of clathrin-coated pits imaged with ExM vs. SR-SIM provides an independent demonstration of the nanoscopic isotropy and high resolution of ExM. Clathrin coated pits were well resolved (Figure 8k, l). ExM resolved the central nulls of the pits better than SR-SIM (Figure 8m, n). Clathrin-coated pit radii measured via ExM and SR-SIM were highly correlated, with a slope of 1.001 (total least squares regression, confidence interval .013 with \( P < 0.05 \), \( n = 50 \) pits from 3 samples) (Figure 8o). 49 of the 50 points lay within a half-pixel distance of the unity slope line, suggesting that variation in the ExM vs. SR-SIM comparison was within the digitization error of the measurement.

**Characterization in tissue**

We next applied ExM to fixed brain tissue. Slices of brain from Thy1-YFP-H mice expressing cytosolic YFP under the Thy1 promoter in a subset of neurons were stained with a tri-functional label bearing Alexa 488, using anti-GFP primary antibodies (which also bind YFP). Slices expanded ~4x, similar to the expansion factor in cultured cells. We compared pre- vs. post-ExM images taken on an epifluorescence microscope. As with cultured cells, the post-ExM image (Figure 9b) was registered to the pre-ExM image (Figure 9a) via a similarity transformation. The registered images closely matched, although some features moved in or out of the depth of field, due to the axial expansion post-ExM. Quantitatively, post-ExM measurement errors (Figure 9c, \( n = 4 \) cortical slices) were 2-4%.
Figure 9. ExM in brain tissue.

Super-resolution imaging of synapses and neurons in intact mammalian brain tissue using ExM. (A), (B) Widefield fluorescence images of Thy1-YFP mouse brain (e.g., cortex and hippocampus), showing anti-GFP staining (white) pre- (A) vs. post- (B) expansion (4x, 0.13 NA used for both of these panels). (C) Root-mean-square (RMS) length measurement error for pre- vs. post-ExM images of brain slices (blue line, mean; shaded area, standard deviation; n = 4 samples). (D), (E) Confocal fluorescence images of boxed regions in (A) and (B) respectively, stained with presynaptic (anti-Bassoon, blue) and postsynaptic (anti-Homer1, red) markers, in addition to anti-GFP (green), pre- (D) vs. post- (E) expansion (40x, water immersion, 1.15NA used for both of these panels, and throughout the rest of the figure). (F) is a maximum intensity projection of 4 z-slices chosen to match the axial extent in (D) as closely as possible. (F), (G) Details of boxed regions in (D) and (E). (G) is a maximum intensity projection of 4 z-slices chosen to match the axial extent in (F) as closely as possible. (H) Detail of single representative synapse highlighted in (G). (H) is a single z-slice. (I) Staining intensity of the synapse of (H) as a function of position along the long axis of the white boxed region in (H) for Bassoon (blue) and Homer1 (red). Gaussian fits to these plots are included as dotted black lines. a.u., arbitrary units. (J) Bassoon-Homer1 separation (n = 277 synapses from 4 cortical slices), calculated from the means of their respective Gaussian fits. Scale bars for pre vs. post-ExM images: (A) 500 μm, (B) 500 μm (physical size post-expansion 2.01 mm); (D) 5 μm, (E) 5 μm (20.1 μm); (F) 2.5 μm, (G) 2.5 μm (10.0 μm) and (H) 250 nm (1.00 μm).
We synthesized tri-functional labels with different colors and oligonucleotides, to enable multicolor ExM. We obtained pre- (Figure 9d) vs. post-ExM (Figure 9e) images of Thy1-YFP-H mouse cortex with ExM labels directed against YFP (green) and the pre- and post-synaptic scaffolding proteins Bassoon (blue) and Homer1 (red). In the pre-ExM image, Bassoon and Homer1 staining form overlapping spots at each synapse (Figure 9f), while the post-ExM image (Figure 9g) shows clearly distinguishable pre- and post-synaptic labeling. We quantified the distance between the Bassoon and Homer1 scaffolds, as measured by ExM. We fit the distributions of Bassoon and Homer1 staining intensity, taken along the line perpendicular to the synaptic cleft (Figure 9h, boxed region), to Gaussians (Figure 9i). The Bassoon-Homer1 separation, calculated as the difference between the means for the Gaussian distributions of each color, was $169 \pm 32.6$ nm (Figure 9j), $n = 277$ synapses from 4 cortical slices), similar to a previous study using STORM in the ventral cortex and olfactory bulb, which obtained $\sim 150$ nm separation$^{66}$.

ExM uses chemically modified secondary antibodies, but the target-specific primary antibodies are unmodified. The method is thus expected to be compatible with any choice of primary antibody. A selection of antibody targets are shown in Figure 10.
Figure 10. ExM imaging of antibodies of interest in neuroscience and biology.
Confocal images of expanded Thy1-YFP mouse brain cerebral cortex sections stained with anti-GFP (green) and antibodies against other proteins (red) as follows: (A) GAD65/67, (B) ChAT, (C) CaMKII, (D) GABA, (E) Lamin A/C, (F) NMDAR2a/b. Scale bars: (A) 10 µm in pre-expansion units (physical size post-expansion, 45 µm); (B) 10 µm (47 µm); (C) 10 µm (40 µm); (D) 10 µm (44 µm); (E) 10 µm (43 µm); (F) 10 µm (43 µm).
Demonstration of large volume super-resolution imaging

To explore whether expanded samples, scanned on fast diffraction-limited microscopes, could support scalable super-resolution imaging, we imaged a volume of the adult Thy1-YFP-H mouse brain spanning 500 µm x 180 µm x 100 µm (a typical slice thickness for immunohistochemistry, with three labels (Figure 11a; anti-GFP, green; anti-Homer1, red; anti-Bassoon, blue). The diffraction limit of our confocal spinning disk microscope (with 40x, 1.15 NA, water immersion objective), divided by the expansion factor, yields an estimated effective resolution of ~70 nm laterally and ~200 nm axially. Shown in Figure 11a is a 3D rendered image of the dataset (see Movie S1 in 46 for animated rendering). Zooming into the raw dataset, nanoscale features emerge (Figure 11b-d). We performed a volume rendering of the YFP-expressing neurons in a subset of CA1 stratum lacunosum moleculare (slm), revealing spine morphology (Figure 11b and Movie S2 in 46). Focusing on a dendrite in CA1 slm, we observed the post-synaptic protein Homer1 to be well localized to dendritic spine heads, with the presynaptic molecule Bassoon in apposition (Figure 11c and Movie S3 in 46). Examination of a mossy fiber bouton in the hilus of the dentate gyrus reveals invaginations into the bouton by spiny excrescences of the opposing dendrite, as observed previously via electron microscopy73 (Figure 11d and Movie S4 in 46). Thus, ExM enables multiscale imaging and visualization of nanoscale features, but across length scales relevant to understanding large-scale neural circuits.
Figure 11. Scalable 3D super-resolution microscopy of mouse brain tissue.

(A) Volume rendering of a portion of hippocampus showing neurons (expressing Thy1-YFP, shown in green) and synapses (marked with antibodies against Bassoon (blue) and Homer1 (red)), with regions named at top. The 3D rendering is downsampled 8-fold from the acquired resolution. (B) Volume rendering of neurites in CA1 stratum lacunosum moleculare (slm) showing dendrites, and spines. (C) Volume rendering of representative dendritic branch in the slm of hippocampal area CA1. (D) Close-up of a mossy fiber bouton in the hilus of the dentate gyrus. Panels (i-iii) represent selected z-slices of the bouton showing its morphology and associated synapses. Scale bars: (A) 100 μm in each dimension; (B) 52.7 μm (x), 42.5 μm (y), and 35.2 μm (z); (C) 13.5 μm (x), 7.3 μm (y), and 2.8 μm (z); (D) (i-iii) 1 μm.
3. Expansion Microscopy with Protein Retention

This section reflects work conducted with Fei Chen, Kiryl Piatkevich, Yongxin Zhao and Jay Yu, and currently accepted for publication at Nature Biotechnology.

In Expansion Microscopy, fluorescent dyes are chemically anchored to a polymer network while the underlying network of biological macromolecules is disrupted with strong digestion. This process homogenizes the mechanical properties of a specimen-gel hybrid, allowing uniform expansion and effective sub-diffraction limited imaging, and homogenizes the optical properties of the expanded specimen, allowing aberration-free imaging throughout thick specimens. The expanded specimen is also homogenized in terms of the chemical environment, substituting the >99% water gel environment for the complex, crowded environment of biological specimens, which feature numerous types of biomolecule in close proximity. These molecules appear at various levels of clustering, from the soluble enzymes that participate in the Kreb cycle, to the highly organized complex of proteins positioned in the post-synaptic density and the long branched carbohydrates that make up the glycocalyx. If biomolecules themselves, rather than just dyes, are chemically anchored to the polymer network, this homogenization of the chemical environment may enable improvements to in situ assays that currently suffer from false positive and negative results, and low quantitation compared with in vitro methods, because of the chemical complexity of the in situ environment.

Anchoring proteins into the swellable material is simple to do with commercially available crosslinkers, but due to the tradeoffs between digestion required for uniform expansion and the retention of biomolecule functional integrity, characterization of biomolecules in the expanded state is not currently a user-ready, optimization-free technology. In the specific case of fluorescent probes (fluorescent probes and fluorescently labeled secondary antibodies), these can be functionally anchored to the material and uniformly expanded, avoiding the need for the custom tri-functional linker implemented using DNA oligonucleotides in the approach presented in Chapter 2, above.
3.1. Limitations to In Situ Characterization

Antibody staining has become a powerful technology for characterizing biological specimens at the protein level, both in situ (in intact biological specimens, including tissue slices) and in vitro (including purified proteins and tissue homogenates). The ease with which these antibodies can now be acquired commercially, for virtually any target researchers wish to study, has greatly expanded their range of application since their introduction as a research tool, but also masks important issues associated with using them and interpreting the results. While the study of DNA and RNA allows the relatively straightforward design of probes by simple base-pair complementation with predictable binding affinities, affinity probes for proteins must be developed in an evolutionary manner with diversification and selection. This can be done in live animals, harnessing their natural immune response, or in engineered enzymatic or microbial systems\textsuperscript{74,75}, but in any case results in a complex mix of binders with unpredictable on-target and non-specific or off-target binding affinities.

In vitro assays benefit from reduced complexity compared with the in situ environment of fixed biological specimens, and also use methods to identify or further reduce the incidence of false positive binding results. In Western blotting, proteins are separated according to basic physical properties (usually size) using gel electrophoresis, transferred to a film, and then antibody stained, so that only results that match the physical characteristic of the target as determined by electrophoresis are counted as a hit\textsuperscript{76}. In ELISA, sequential binding of the target by two distinct antibodies prevents hits due to non-target molecules containing a motif that binds to only one of the antibodies used\textsuperscript{77}. The proximity-dependent ligation assay (PLA), like ELISA, depends on the target binding two separate antibodies but in this case the antibodies bear short oligonucleotides, whose colocalization is detected by oligonucleotide ligation and subsequent binding of an amplifiable fluorescent oligonucleotide probe whose affinity is selective for the ligated pairs\textsuperscript{78}. PLA can also be used to detect instances of nanoscale proximity of two different targets\textsuperscript{79}.

In addition to false positives due to non-specific binding of antibodies, correct binding of antibodies can be hindered by the local environment of the antibody-binding epitope. Primary antibodies and structurally closely related nanobodies bind to a segment of their target measuring several nanometers on a side\textsuperscript{80}, but occupy a volume of approximately 2x12x15nm\textsuperscript{81}. Each primary antibody is then bound by several secondary antibodies of the
same size. The accessibility of the target to the primary and of the primary to the secondaries can depend on local steric hinderance, an effect that is likely to be strongest at sites with densely scaffolded proteins like synapses. These scaffolded proteins are directly involved in signal transduction and thus are of particular interest in neuroscience. These issues will be particularly acute if simultaneous labeling of multiple targets is desired.

The optimization and validation required for high-quality routine antibody staining\textsuperscript{3,82}, and the difficulty of staining low density targets in crowded environments, such as neurotransmitter receptors, can introduce experimental delays. The optimization steps required for multiple targets may not be compatible with each other\textsuperscript{83}. In the context of super-resolution imaging, it is particularly important to consider the low/variable antibody labeling density to avoid poor or spurious results\textsuperscript{84–86}. Large-scale studies seeking to map the distribution of molecular targets in the brain will require reliable optimization-free antibody staining to get an unbiased view without requiring specific controls for each individual stain.

3.2. ExM with Protein Anchoring and Reduced Digestion

Acryloyl-X is a chemical crosslinker with one moiety (N-hydroxy succinimidyl ester) that reacts robustly to proteins via primary amines and another (acryloyl) that can be incorporated into a growing polymer chain in a radical chain reaction synthesis. This crosslinker is used to anchor proteins in vitro into a gel\textsuperscript{87}, but can also be used to anchor the proteins of intact tissue into the swellable material used in Expansion Microscopy.

Although a nonspecific protease was used in the original protocol to break native biological protein structures down to the smallest constituents possible, this is not strictly necessary. Conceptually, biomolecules and complexes of any size can be anchored to the gel and separated from each other. If the size of separated pieces is less than the effective resolution of imaging post-expansion, this will not affect the uniformity of expansion on an observable scale. On the other hand, with direct anchoring of proteins to the gel, if a single such separated complex, protein, or protein fragment bears two or more anchor points, it will contribute additional cross-linking beyond that due to the material itself, reducing the local expansion extent. Thus, an ideal technology would have sufficient disruption to cleave proteins on average at least once for every anchor point, rather than the stronger goal of total dissolution from the first iteration of the Expansion Microscopy method.
Borrowing from denaturing SDS-PAGE\textsuperscript{88} and antigen retrieval protocols\textsuperscript{89}, we treated gel-embedded tissues in an alkaline detergent-rich buffer for one hour in an autoclave, and found \(~4\times\) expansion of Thy1-YFP mouse brain samples (Figure 12a, showing endogenous YFP pre-treatment; Figure 12b, showing post-expansion labeling with anti-GFP). We found that antibodies could indeed be delivered successfully post-expansion (Figure 12c-e). As a second treatment strategy, we exposed gel-embedded tissues to LysC, which cuts proteins at Lys residues (in contrast to nonspecific proteinase K)\textsuperscript{90-91}.

Post-expansion staining in both cases was highly variable depending upon antibody identity (e.g., compare lamin A/C examined with three different protocols (Figure 12f(i-iii), to images obtained in the original ExM protocol, Figure 10). For some antibodies, post-expansion staining appeared to result in brighter signal compared to pre-gelation staining (Tom20, Figure 12g(i) vs h(i) (autoclaved); GFP, Figure 12g(ii) vs. h(ii) (autoclaved); PSD-95, Figure 12g(iii) vs. Figure 12h(iii) (LysC)). However, the variability (Figure 13) and incomplete homogenization (Figure 14) suggested that the strong proteolysis of the original ExM protocol was necessary for reliable expansion.

We explored less disruptive methods than nonspecific proteolysis, such as antigen retrieval and the usage of targeted proteases, and found that these strategies enabled many retained native proteins to be antibody labeled after expansion. We directly compared the staining brightness for antibody targets using these methods, and found stronger signal with post-disruption than pre-gelation staining, for several targets. However, these methods require optimization on a per-target basis and in some cases result in defects in uniform tissue expansion.
Figure 12. Post-expansion antibody delivery, after epitope-preserving homogenization.
Wide-field fluorescence images of Thy1-YFP-expressing mouse brain hemisphere slice before expansion (a), and after autoclave treatment and antibody staining (b). (c-h) Confocal micrographs of cortex from Thy1-YFP-expressing mouse brain treated with different disruption methods and antibodies, with anti-GFP (green, staining YFP) as a reference. (c) Autoclave method followed by staining against bassoon (blue) and homer (red). (d) Autoclaving followed by myelin basic protein staining. (e) Autoclaving followed by vimentin (red) and glial fibrillar acidic protein (blue) staining. (f) Staining for Lamin A/C after autoclave (i) or LysC (ii) treatment, or with secondary antibodies applied after LysC homogenization (with primaries previously anchored to the gel using AcX) (g-h) Comparison of staining before gelation (g) versus after disruption (h) using the autoclave method for Tom20 (i) and YFP (ii, shown in the red channel in the bottom panel because the endogenous YFP is green), and after disruption using LysC for homer (red) and PSD-95 (blue) (iii). Scale bars: (a) 1mm, (b) 1mm (3.96mm), (c-h) 5µm (~21µm).
Figure 13. Comparison of tissue disruption methods.
(Figure 13, Previous page) Comparison of immunostaining methods with autoclave, LysC, and pre-gelation antibody treatment. Confocal images of Thy1-YFP expressing mouse cerebral cortex, immunostained pre-gelation followed by AcX treatment, gelation, and proteinase K digestion (proExM), column (i). Thy1-YFP brain samples immunostained after AcX treatment and gelation followed by autoclave treatment, column (ii), or by LysC digestion column (iii). Autoclave and LysC specimens all have YFP stained with anti-GFP (green) in addition to TOM20 (row (a)), homer (red) and bassoon (blue) (row (b)), homer (red) and post-synaptic density 95 (PSD95, blue) (row (c)), glutamic acid decarboxylase (GAD) 65/67 (row (d)), myelin basic protein (MBP, row (e)), and vimentin (red) and glial fibrillary acidic protein (GFAP, blue) (row (f)). Scale bars; 5μm (~20μm).

![Image](image_url)

**Figure 14. Incomplete homogenization with autoclave and LysC methods.**
Fluorescence images of Thy1-YFP expressing mouse cerebral cortex, with YFP stained with anti-GFP using confocal imaging after autoclave treatment and antibody staining, showing a discontinuous neurite not residing at the surface of the imaged volume (a), and using widefield imaging after LysC treatment and antibody staining, showing defects in the expansion regions containing white matter tracts (b). Scale bars; (a) 5μm (~20μm), (b) 0.5mm (~2mm).

3.3. ProExM with Fluorescent Probes

To develop an optimization-free method that is compatible with existing labeling methods, we demonstrate that genetically encoded fluorescent proteins and conventional fluorescently labeled secondary antibodies that are directly anchored to the gel exhibit preserved fluorescent signals even when subjected to the nonspecific proteolytic digestion from the original ExM protocol. Furthermore, an increase in digestion temperature allowed this protocol to generalize to a diversity of mammalian tissue types with varying mechanical properties. proExM is thus a simple extension of standard histological methods used to prepare samples for imaging. We demonstrate multi-color super-resolution (~70 nm)
imaging of cells and mammalian tissues with proExM on conventional confocal microscopes.

**Fluorescent protein retention**

We sought to devise a strategy that would combine the convenience of direct protein anchoring with strong proteinase K treatment. It is known that green fluorescent protein (GFP) exhibits extraordinary stability to proteases\(^2\),\(^3\). We hypothesized that GFP and GFP-like fluorescent proteins (FPs) might retain their fluorescence after the proteolytic digestion of the original ExM method, if they were retained in the polymer-specimen composite using AcX. We discovered that treatment with AcX followed by the standard ExM workflow, including proteinase K digestion, can preserve GFP fluorescence in the expanded gel with high efficiency (approx. 50%). Because of the utility of this protocol, we termed the process of AcX treatment of a fixed specimen, followed by gelation, strong digestion, expansion, and imaging as protein retention expansion microscopy (proExM).

We systematically examined persistence of fluorescence for various FPs in the proExM workflow. We selected 20 widely used FPs with spectra ranging from the blue to the near-infrared. Selected FPs were fused to histone proteins and expressed in human embryonic kidney (HEK293FT) cells. To assess FP performance in proExM, we compared images of live cultures vs. after-proExM images of the same cells. Most FPs retained more than 50% of their live fluorescence intensity after proExM (n = 4 samples each; Figure 15b), comparable to the persistence of small-molecule fluorophores in the original ExM protocol\(^4\).

**Antibody signal retention**

Having seen that FPs could persist sufficiently to report signals even after a strong digestion process, we next sought to determine if other signals might persist. We discovered that proExM anchors, and preserves the fluorescence of, commercial fluorescently conjugated secondary antibodies. Following gelation and digestion, specimens labeled with secondary antibodies bearing a variety of small-molecule fluorophores retained ~50% of their initial brightness (n = 3 samples each; Figure 15c). In the original ExM protocol, custom conjugation of secondary antibodies to enable labeling with a gel-anchorable fluorophore was required\(^1\). proExM, in contrast, allows commercial secondary antibodies to be used in place of these custom formulations.
Figure 15. Retention of fluorescent signals post-ExM.
Retention of fluorescent protein (FP) and antibody fluorescence signals in proExM and proExM of FP fusions. (a) Representative images of selected FP-histone fusion proteins in live HEK293FT cells (upper row) and in the same cells after proExM treatment (lower row); iRFP was expressed as N-terminal fusion with nuclear localization sequence (NLS). (b) Quantified fluorescence of experiments as in panel a, after proExM treatment (crosshatched bars; mean ± standard deviation; n = 4 transfection replicates each). Open bars, literature values of the brightnesses of these fluorophores, normalized to the brightness of EGFP. (c) Retention of fluorescence for selected dyes conjugated with antibodies, after proExM treatment (mean ± standard deviation, n = 3 samples each), in mouse brain slice. (d) Super-resolution structured illumination microscopy (SR-SIM) image of immunostained microtubules after the anchoring step vs. (e) post-expansion image of the same sample acquired with a spinning disk confocal microscope. (f) Root mean square (RMS) length measurement error as a function of measurement length for proExM vs SIM images (blue line, mean shaded area, standard deviation; n = 4 samples). Scale bars: (a) 5 μm, (d) 5 μm, (e) 5 μm (physical size post-expansion, 20.5 μm).

Characterization of isotropy

To assess the performance of proExM in three-dimensional tissues, we performed proExM on four different mouse tissue types (brain, pancreas, lung and spleen, (Figure 16a-d). Mouse brain expressing YFP under the Thy1 promoter (Thy1-YFP) in a sparse subset of neurons expands without distortion at the millimeter scale following treatment with proteinase K (Figure 16a, top vs. bottom). Pancreas, spleen and lung have different mechanical properties than brain (e.g., more connective tissue), which hinders expansion following room temperature proteinase K digestion. We antibody stained the intermediate filament vimentin as a marker of connective tissue to examine the isotropy of expansion in these diverse tissue types. We observed that, with a slight modification in the digestion temperature to the optimum of the proteinase K enzyme (60°C for 4 hours), proExM
allows for expansion of pancreas, lung, and spleen tissue, with excellent preservation of tissue morphology at the millimeter length scale (Figure 16b-d, top vs. bottom). High-resolution diffraction-limited microscopy of the tissue before (Figure 16e, f) vs after proExM (Figure 16e, g) shows the resolution improvement of proExM. We quantified the isotropy of expansion by measuring the root-mean-square (RMS) error of feature measurements after proExM in the microscale (<100 μm) for pancreas, lung and spleen tissue. The RMS errors were small (1-3% of the measurement distance) and similar among all three of the tissue types (Figure 16h) at this length scale.

To examine the isotropy of expansion at the nanoscale, we performed SR-SIM (Figure 16i, j) and proExM confocal imaging (Figure 16i, k) on vimentin staining in the pancreas. Again, we observed small RMS errors on the order of 1-5% of the measurement length for measurements between 0 and 25 microns (Figure 16l, n = 4 fields of view from 2 samples). We performed a similar analysis on mouse brain cortical tissue stained with antibodies against Tom20, a mitochondrial marker, and imaged with SR-SIM before (Figure 16m, n) and confocal after (Figure 16o) proExM processing using proteinase K digestion at room temperature. RMS errors for this tissue type were between 1-3% of the measurement length, between 0 and 40 microns (Figure 16p, n = 3 specimens).
Figure 16. Validation of proExM in different mammalian tissue types.
(a-d) Low magnification, wide-field images of pre-expansion (top) and post-expansion (bottom) samples of Thy1-YFP mouse brain (a) and vimentin-immunostained mouse pancreas (b), spleen (c), and lung (d). (e) Composite fluorescence image of Tom20 in Thy1-YFP mouse brain imaged with super-resolution structured illumination microscopy (SR-SIM) (green) and proExM (purple) with conventional confocal microscopy with distortion vector field overlaid (white arrows). (f) Pre-expansion SR-SIM image showing boxed region in (a). (g) Post-expansion confocal image of (f). (h) RMS length measurement error as a function of measurement length for proExM vs SR-SIM pre-expansion for Tom20 staining in Thy1-YFP mouse brain (blue line, mean; shaded area, standard deviation; n = 3 mouse brain cortex samples). (i) High magnification, wide-field fluorescence composite image of vimentin in mouse pancreas before (green) and after (purple) expansion with distortion vector field overlaid (white arrows, see methods). (j) Pre-expansion wide-field image showing boxed region in (i). (k) Post-expansion image of (j). (l) Root mean square (RMS) length measurement error as a function of measurement length for proExM vs
widefield pre-expansion images for the different tissue types in (b-d) (blue line, mean; shaded area, standard deviation; n = 3 samples from pancreas, spleen, and lung). (m) Composite fluorescence image of vimentin in mouse pancreas imaged with super-resolution structured illumination microscopy (SR-SIM) (green) and proExM (purple) with conventional confocal microscopy with distortion vector field overlaid (white arrows). (n) Pre-expansion SR-SIM image showing boxed region in (m). (o) Post-expansion confocal image of (n). (p) RMS length measurement error as a function of measurement length for proExM vs SR-SIM pre-expansion for vimentin staining in pancreas (blue line, mean; shaded area, standard deviation; n = 4 fields of view from 2 samples). Scale bars: (a) top 200 μm, bottom 200 μm (physical size post-expansion, 800 μm), (b-d) top 500 μm, bottom 500 μm (2.21 mm, 2.06 mm, 2.04 mm, respectively), (e, f) 10 μm, (g) 10 μm (40 μm), (h) 10 μm, (i) 5 μm, (k) 5 μm (20.4 μm), (m) 5 μm, (n) 5 μm, (o) 5 μm (20.65 μm).

The concentration of acryloyl-X (AcX) used to treat tissue for ProExM is much lower than the final concentration that reacts to primary amines in the tissue. As a result, AcX must partition strongly into the tissue, a process that takes multiple diffusion time constants. Over these longer times the inactivating hydrolysis side reaction of AcX becomes a serious problem, limiting the uniformity of AcX reacted throughout the tissue. For tissue slices thicker than 100 μm, this problem can be mediated by delivering AcX at lower pH (reducing the hydrolysis rate) and for a longer time (Figure 17). With these optimizations, proExM can be applied to 500 μm thick (Figure 18), making it compatible with live brain slice preparations, without further sectioning. The monotonic fall-off in retained YFP intensity in Figure 18i versus depth suggests that this is due to optical aberations, such as index mismatch between the aqueous specimen and the non-immersion objective lens. Incomplete retention of YFP would result in reduced signal only around the middle of the specimen. A schematic of the proExM and post-expansion staining methods is presented in Figure 19.
Figure 17. Optimizing AcX penetration depth in fixed brain tissue.
(Figure 17, Previous page) (a) Chamber assay for measuring penetration depth of a NHS-ester mixture (99% AcX + 1% NHS-biotin, which has similar molecular weight and charge as AcX) from the side of a tissue slice. After overnight treatment with the NHS-ester mixture, slices were retrieved, washed and treated with fluorophore-conjugated streptavidin to visualize penetration of NHS-ester mixture. (b) Representative image of a 100-μm-thick mouse brain slice stained under the chamber assay conditions. Scale bar 1mm. (c) Fluorescent intensity along the line-cut represented as the white dashed line in b. The distance over which the intensity drops from maximum to half of its value (D_{1/2}) is a characteristic length for the depth of NHS-ester penetration. (d, e) Staining with MES-based saline (NIBS; 100 mM MES + 150 mM NaCl) yields significantly improved depth of NHS-ester penetration than phosphate-based saline (PBS) over all pH levels tested. Scale bar 1 mm. (f, g) Staining at 4°C yields moderately greater depth of penetration than at RT. Scale bar 1 mm.

Figure 18. YFP retention with optimized AcX delivery.

(h) Representative images of native YFP fluorescence in a 500-μm-thick Thy1-YFP mouse brain slice, before (left) and after (right) proExM. Scale bar 1 mm (pre-expansion units). (i) Confocal imaging demonstrates YFP fluorescence retention at the center of the 500-μm-thick slice after an overnight AcX treatment with MBS, pH 6.0. Scale bar 100 μm (post-expansion units).
Figure 19. Workflows for expansion microscopy with protein retention.  
Three basic sample processing workflows were explored in this paper.  
**Top**, samples are chemically fixed and stained with antibodies, using conventional immunostaining protocols,  
before AcX treatment at room temperature and subsequent ExM processing (gelation, proteinase K treatment, and expansion in water).  
**Middle**, samples expressing fluorescent proteins (FPs) are chemically fixed (and optionally permeabilized) before AcX treatment, and subsequent ExM processing.  
**Bottom**, samples treated with AcX, followed by gelation, are then processed with a gentle homogenization procedure (e.g., alkaline hydrolysis and denaturation, or digestion with LysC), and finally antibody staining in the expanded state.

4. Conclusion and Future Directions

Optical microscopy has developed into a major technology for carrying out investigations in all branches of biology. Numerous technological advances have made it a powerful and flexible tool for assessing specimens at many length scales, readily spanning from the macroscopic down to the diffraction limit. In the past decade, even the diffraction limit has been circumvented by several super-resolution optical microscopy methods, giving visual access to sub-diffraction-limited structures in cultured cells and thin tissue slices. In the same time frame, the problem of scattering has received substantial attention, giving ready access to larger volumes of tissue. The gap in length scales—sub-diffraction-limited imaging in thick tissue slices—has remained a particularly challenging area despite these advances. Expansion Microscopy is a new method for effective super resolution imaging where the specimen itself is physically expanded prior to imaging on a diffraction-limited
microscope, resulting in a >99% aqueous tissue-gel hybrid allowing imaging of sub
diffraction-limited structures in thick tissue slices without customized equipment or
demanding imaging conditions, with the caveat that it is not compatible with in vivo
imaging.

4.1. Expansion Microscopy Performance

Using Expansion Microscopy, fixed cells and tissues can be physically magnified,
with isotropic nanoscale resolution (~70 nm lateral resolution), through in situ polymer
synthesis and chemically driven polymer swelling. We achieved roughly two orders of
magnitude of volumetric expansion, facilitated by anchoring fluorescent tags to the polymer
network, followed by disruption of endogenous biomolecular structures to homogenize the
mechanical properties of the sample. We discovered that ExM preserves nanoscale features
through multiple independent comparisons, including before-and-after comparisons of
stereotyped features, as well as comparisons to SR-SIM microscopy.

Super-resolution imaging methods are slower than their diffraction limited
counterparts because they must resolve more voxels per unit volume. ExM achieves this
effect by expanding the voxels physically. The speed of voxel acquisition compares favorably
to other high-speed super-resolution methods such as parallel RESOLFT. The physical
magnification of ExM, however, enables super-resolution imaging with several fundamental
new properties. The dimming effect on signals is partially offset by the concomitant dilution
of autofluorescence and reduced loss due to scattering, though longer exposure times are
typically useful, depending on the brightness of the unexpanded specimen. The expansion is
isotropic, meaning that axial resolution is improved by the same factor as lateral resolution.
Because the magnification is not dependent on the fluorophores involved, ExM can achieve
super-resolution with multiple standard fluorophores, on a diffraction-limited microscope.
Super-resolution imaging is often performed within ~10 μm of the sample surface due to
low signal-to-noise, scattering and refractive index mismatch. Here, we demonstrated 3-color
super-resolution imaging of a large volume of brain tissue over an axial extent of 100 μm
with a standard spinning disk confocal microscope. Because the ExM-processed sample is
almost entirely made of water, eliminating scattering, ExM may empower very fast imaging
methods like light sheet fluorescence microscopy to become super-resolution methods.
Since the sample is physically larger, any mechanical errors in post-expansion sectioning,
microscope stage movement, thermal drift, and other sources of measurement error, are effectively divided by the expansion factor, making the microscopy more robust.

The fundamental resolution of ExM is likely a complex function of physical parameters such as the spatial density of cross-links of the polymer. The high performance of ExM suggests that despite statistical fluctuations in polymer chain length at the molecular scale, at the nanoscale distances here examined, these fluctuations average out, yielding the observed isotropy. By tuning the materials used for ExM, yet higher resolutions may be possible, as well as further degrees of expansion.

4.2. Protein Retention

In the original ExM protocol, we conjugate DNA oligonucleotides to secondary antibodies in order to enable attaching fluorophores to the polymer at sites of antibody binding. proExM eliminates the need for such custom-made antibody labels for ExM; instead, FPs and fluorescent secondary antibodies treated with the cross-linker can be directly visualized after expansion. Thus, proExM does not require end-users to carry out conjugation reactions, resulting in a faster protocol. Like any protocol involving antibody staining, the diffusion of unmodified antibodies will need to be considered. A key feature of ExM is that it requires neither custom hardware nor fluorophores for super-resolution imaging; here we continue this line of technology development by additionally enabling the secondary antibodies to be conventional as well. Preservation of endogenous fluorescence allows for the use of transgenic animals, viral expression vectors, and transfection of FPs, all without immunostaining. Distortion was low, perhaps because both the polymer and digestion methods were the same as our original expansion protocol; only the anchoring method was different.

As with the original ExM protocol, samples processed with proExM are optically clear and index matched to water. This allows for super-resolution imaging deep into samples, on conventional fluorescence microscopes, limited only by working distance of the objective lens. Like ExM, proExM does not require specialized hardware, extensive post-processing, or other enabling aspects of conventional super-resolution methods.
4.3. Potential Applications

Electron microscopy has revealed many structures on sub-diffraction-limited size scales that may play important roles in information processing in neural circuits. These structures can be studied in cell culture and thin tissue slice contexts using existing sub-diffraction-limited optical microscopy techniques but their arrangement in the full context of a dendritic branch, full dendritic arbor, or multi-neuron circuit is a greater challenge. Expansion Microscopy is especially well-suited to access the resolution and volume scales needed to address questions regarding such structures in their native circuit context. Because ExM, and particularly proExM requires no specialized equipment, stringent imaging conditions, or image post-processing, this approach may prove useful not only for large scale hypothesis-free mapping studies, but also for independent use by researchers dedicated to understanding particular structures and circuits. What follows is an overview of some of these microcircuit features and how they might be involved in neural computations.

Individual synapses are not the smallest unit of neural processing. The position of neurotransmitter receptors within a synapse and even at extra-synaptic locations on the same dendritic spine can influence how signals are transmitted to the post-synaptic neuron\textsuperscript{96,100}. Compartmentalization of signaling within a single dendritic spine can be tightly controlled. Two pathways can converge on the same second messenger system, but have distinct signaling properties due to spatial segregation within a dendritic spine coupled with an inhibitory mechanism to keep the spatial extent of second messenger diffusion confined to individual sub-compartments, all on the micron scale\textsuperscript{101,102}. These sub-spine components are held in place by scaffolding systems\textsuperscript{103}, where single mutations of scaffolding proteins can result in complex disease-like phenotypes\textsuperscript{104}.

Glia, non-neuronal brain cells, are increasingly known to participate not only in passive roles relating to cellular homeostasis but also in signal processing functions\textsuperscript{105} including direct signaling with neurons\textsuperscript{106} and regulating synaptic connectivity\textsuperscript{107}. For example, in tripartite synapses, pre- and post-synaptic neural processes are partially encased in a sheath formed by an astroglial cell. The extent of ensheathment may affect the amount of neurotransmitter that spills out of the synapse into the surrounding extra-cellular space\textsuperscript{108}, which can affect signaling away from the synapse of origin\textsuperscript{109}. Activated microglia can modify neural connectivity by displacing individual pre-synaptic contacts\textsuperscript{110}. 

51
Hippocampal, cerebellar, thalamic and olfactory bulb glomeruli feature complex arrangements of synapses encased in a glial ensheathment. In the case of the olfactory bulb, each glomerulus is a compact structure that collects the inputs from a single class of olfactory receptor, along with numerous lateral inhibitory connections from neighboring glomeruli. These structures are known to play an important role in olfactory signal processing but they are too small for their internal synaptic structure to be resolved on diffraction-limited scopes. Thalamic glomeruli feature a primary post-synaptic dendritic spine with numerous inputs from several distant parts of the brain, all converging on a single glomerular structure. Thus, thalamic glomeruli are positioned to carry out a multi-modal signal integration function at the micron scale, before signals are reaching the dendritic shaft, let alone a neuronal cell body and the action potential-generating machinery of the neuron. A specific example with a putative logical function is the spine-triad hypothesized to perform an AND-NOT function. Glial cells are also known to be involved in synaptic pruning during development, an important process that is disrupted in autism.

The fibers carrying signals to and from the neuronal cell body begin several microns in diameter, but tend to narrow towards their distal ends. Fine fibers can be under 1 micron, making them impossible to trace using diffraction-limited optical microscopy in all but very sparsely stained specimens. In this case, it is the axial resolution in particular that prevents adequate imaging. Expansion Microscopy can play a role in tracing the circuitry of more densely labeled specimens. In particular, there are bundles of distal dendrites and axons that converge from distant brain regions to form organized structures that are too fine to resolve by diffraction limited microscopy. The function of these structures is unknown, and require in vivo activity mapping to fully understand, but ExM can play an important role in determining the fine anatomy of such structures in the context of the circuits they are embedded in. In short, there are numerous promising applications for the resolution and volume imaging capabilities of Expansion Microscopy. The ease and economy of use of the technique will lower the barrier for researchers pursuing these questions to independently adopt the method and apply it to their own hypothesis-driven studies.
5. Appendix:

5.1. Materials and Methods for ExM

A complete list of chemicals and supplier catalog numbers used can be found in Table S2.

**Labels for ExM:** DNA sequences on secondary antibodies were synthesized with 5’ amine modification (Integrated DNA Technologies) and conjugated to the antibodies (Jackson ImmunoResearch, Affinipure donkey antibodies) using a commercial kit (Solulink, Antibody-Oligonucleotide All-in-One Conjugation Kit). For the tri-functional label, the oligonucleotides were synthesized with a 3’ amine modification and a 5’ Acrydite modification (Integrated DNA Technologies), then conjugated to dyes (Alexa 488, Atto 565 and Atto 647N) modified with NHS-ester chemistry per the dye manufacturer’s directions (see Table S3 for sequences). We found that Cy5 undergoes strong bleaching during polymerization, most likely due to the radical reactivity of its simple linear conjugated backbone, while other fluorophores tested retained at least 50% of their brightness (Table S1). Tri-functional labels were purified via reverse-phase HPLC, lyophilized, and re-suspended in ddH₂O.

**Cultured cell preparation and staining:** HEK293-FT cells (Invitrogen) were cultured in Culturewell Chambered Coverglasses (Invitrogen) per manufacturer’s instructions. All solutions below were made up in 1x phosphate buffered saline (PBS), and incubations carried out at room temperature. To preserve microtubule ultrastructure, cells were fixed, as in 115, in 3% formaldehyde/0.1% glutaraldehyde for 10 minutes, followed by reduction with 0.1% NaBH₄ for 7 minutes, and quenching in 100 mM glycine for 10 minutes. For clathrin, cells were fixed in 4% formaldehyde for 10 minutes followed by quenching in 100 mM glycine for 10 minutes. Cells were permeabilized with 0.2% Triton X-100 for 15 minutes at room temperature and blocked with 5% normal donkey serum for one hour. Specimens were incubated with primary antibodies (Sheep anti-Tubulin, Cytoskeleton ATN02; Rabbit anti-Clathrin, Abcam AB21679) in blocking buffer at a concentration of 10 μg/mL for 1-4 hours, and then washed in PBS three times for 5 minutes each. Specimens were incubated with DNA-labeled secondary antibodies in DNA hybridization buffer (2x
saline-sodium citrate (SSC) buffer, 10% dextran sulfate, 1 mg/mL yeast tRNA, 5% normal donkey serum) at a concentration of approximately 10 μg/mL for 1-4 hours, then washed in PBS as for primary. Specimens were incubated with tri-functional labels in hybridization buffer at a concentration of 0.5 ng/μL for each oligonucleotide overnight, then washed three times in 1x PBS.

**Brain tissue preparation and staining:** All procedures involving animals were in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Massachusetts Institute of Technology Committee on Animal Care. All solutions below were made up in 1x phosphate buffered saline (PBS), and incubations carried out at room temperature unless otherwise noted. Mice, wildtype (C57BL/6, obtained from Taconic) and transgenic expressing cytosolic YFP under the Thy1 promoter (Thy1-YFP-H strain on C57BL/6, obtained from Jax), were anesthetized with isoflurane and perfused transcardially with ice cold 4% paraformaldehyde. Brains were dissected out, left in 4% paraformaldehyde at 4°C for one day, and then sunk in 30% sucrose with 100 mM glycine for one day. Slices greater than 30 μm thick were sliced on a vibratome (Leica VT1000S); slices 30 μm thick were frozen in -40°C isopentane cooled with dry ice, embedded in M-1 embedding matrix (Thermo Scientific) and sliced on a cryotome (Leica CM1850UV). Slices were permeabilized and blocked with 0.1% Triton X-100 and 2% normal donkey serum (slice blocking buffer) for at least six hours. Slices were incubated with primary antibodies in slice blocking buffer at a concentration of 10 μg/mL for 6-24 hours depending on slice thickness and antibody, and then washed in slice blocking buffer four times, for thirty minutes each time, changing solutions in between. Slices were incubated with DNA-labeled secondary antibodies in hybridization buffer plus 0.1% Triton X-100 at a concentration of approximately 10 μg/mL for 6-24 hours depending on slice thickness and antibody, then washed in slice blocking buffer as for primary. Specimens were incubated with tri-functional labels in hybridization buffer plus 0.1% Triton X-100 at a concentration of 0.5 ng/μL per oligonucleotide for 6-12 hours, then washed in slice blocking buffer as for primary. Slices used were 30 μm (for the bleaching experiments of Table S1), 200 μm (Figure 3, Figure 4), or 100 μm (all other figures with slices) thick.
For Figure 9 and Figure 11, slices were stained with primary antibodies Chicken anti-GFP, Millipore AB16901; Rabbit anti-Homer1, Synaptic Systems 160003; Mouse anti-Bassoon, Abcam AB82958. For Figure 10, antibodies used were Rabbit anti-GAD65/67, Chemicon AB1511; Rabbit anti-ChAT, Millipore AB143; Rabbit anti-CaMKII, Epitomics 2048-1; Rabbit anti-GABA, Sigma A2052; Mouse anti-Lamin A/C, Cell Signaling Technology #4777; Rabbit anti-NMDAR2a/b, Millipore AB1548.

**In situ polymer synthesis:** Monomer solution (1x PBS, 2 M NaCl, 8.625% (w/w) sodium acrylate, 2.5% (w/w) acrylamide, 0.15% (w/w) N,N'-methylenebisacrylamide) was mixed (see Table S4), frozen in aliquots, and thawed before use. Prior to embedding, monomer solution was cooled to 4°C to prevent premature gelation. Concentrated stocks (10% w/w) of ammonium persulfate (APS) initiator and tetramethylethylenediamine (TEMED) accelerator were added to the monomer solution up to 0.2% (w/w) each. For slices, the inhibitor 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (4-hydroxy-TEMPO) was added up to 0.01% (w/w) from a 0.5% (w/w) stock to inhibit gelation during diffusion of the monomer solution into tissue sections. Stained cells or tissue slices were incubated with the monomer solution plus APS/TEMED (and 4-hydroxy-TEMPO for slices) at 4°C for one minute, 30 minutes or 45 minutes for cultured cells, 30 µm/100 µm slices and 200 µm slices respectively, and then transferred to a humidified 37°C incubator for two hours. Slices were incubated with at least 100-fold excess volume of monomer solution.

Specimen-free gels of Figure 2 were cast with the same recipe as for cultured cells except with a variable amount of cross-linker, in forms constructed from two coverslips separated by a silicone gasket 1 mm thick, with circular holes 5.2 mm in diameter. Gels were expanded as with cultured cells, but without needing digestion. diameters of expanded gels were measured using Vernier calipers to find the linear expansion factor.

**Digestion and expansion:** Proteinase K (New England Biolabs) was diluted 1:100 to 8 units/mL in digestion buffer (50 mM Tris (pH 8), 1 mM EDTA, 0.5% Triton X-100, 0.8 M guanidine HCl) and applied directly to gels in at least ten times volume excess. For cells, gels were formed in a Culturewell Chambered Coverglass (Invitrogen), and the chamber walls removed before adding digestion buffer in order to improve access of enzyme to the embedded cells. For tissue slices, gel chambers were constructed with two pieces of
coverglass separated by spacers on either side of the tissue section (for 30 and 100 μm sections, #1 coverglasses were used for spacers and for 200 μm sections, two #1 coverglasses were used for spacers). The gels were then incubated in digestion buffer for at least 12 hours. Digested gels were next placed in excess volumes of doubly de-ionized water for 0.25-2 hours to expand, with longer times for thicker gels. This step was repeated 3-5 times in fresh water, until the size of the expanding sample plateaued.

**Acrydite covalent anchoring efficiency:** Labeled DNA oligonucleotides (A1 and A2, in Table S3) were ordered from IDT without the 5’ acrydite modification, for the anchoring efficiency experiments of Table S1. Specimen-free gels (n = 4 gels) were cast with the same recipe as for cultured cells with addition of DNA labels with and without acrydite modification (0.25 ng/μL of each strand). Following gelation, gels were dialyzed in 20x volume of 10x PBS for 1 day to allow unincorporated DNA labels to equilibrate in concentration with the dialysate. Samples were taken from the dialysate after equilibration and measured on a fluorescence plate reader (Spectramax M5e). A fluorescence standard curve made from serial dilutions of label oligonucleotides was used to determine fluorescence concentration in the dialysate. The incorporation efficiency of non-acrydite labels was confirmed to be zero by fluorescence microscopy of the gels following dialysis equilibration; the ratio of fluorescence intensity inside the gel to the dialysate was 0.99 ± 0.07 (n = 4 samples). Acrydite incorporation efficiency was calculated as 1 – (dialysate concentration with acrydite/dialysate concentration without acrydite).

**Chemical bleaching during gelation:** Fluorescence intensity in stained brain slices was measured before vs. after expansion for the bleaching experiments of Table S1. Wild type brain slices (30 μm thick) containing sections of mouse hippocampus were stained with anti-GABA primary (Sigma A2052) antibody, DNA secondary antibodies, and tertiary DNA bearing either Alexa 488, Atto 565, or Atto 647N as described above. Epifluorescence images of the brain slice were taken with 4x 0.13 NA objective with tiling to cover the entire slice, pre-gelation. Following in situ polymer synthesis as described above, epifluorescence images of the slice were taken again with identical imaging conditions. A region of interest in the hippocampus was used to determine the loss of fluorescence during gelation.
Imaging:

*Cultured cells.* Super-resolution structured illumination microscope imaging was performed on a Deltavision OMX SIM microscope with 100x 1.40 NA (Olympus) oil objective. Stained cells were imaged with SlowFade Gold (Invitrogen) antifade reagent for suppression of photobleaching and refractive index matching for pre-expansion imaging. Pre-expansion imaging was performed on a Zeiss Laser Scanning Confocal (LSM710) with 40x 1.30 NA oil objective at 1 Airy unit and Nyquist sampling.

Post-expansion imaging was performed on a Perkin Elmer spinning disk (CSU-10 Yokogawa) confocal or a Zeiss LSM 710. Briefly, expanded samples were placed in glass-bottom six-well plates (In Vitro Scientific) and held in place by surrounding with low-melting point agarose. Images were taken at with 1 Airy unit and Nyquist sampling on the LSM 710 on a 20x 0.8 NA (Zeiss) air objective. Images on the Perkin Elmer were taken on a 100x 1.40 NA (Zeiss) oil objective.

Figure 8A, B are maximum intensity projections (MIPs) of 1.5 μm thickness (in pre-ExM distance units). Figure 8D, E are MIPs of 1 μm thickness. Figure 8K, L are MIPs of 500 nm thickness. The SR-SIM image of Figure 8M is depicted interpolated such that the pixel size is the same as that of the corresponding ExM image of Figure 8N.

*Brain slices.* To quantify expansion factor for tissue slices, specimens were imaged pre-ExM on a Nikon Ti-E epifluorescence microscope with a 4x 0.13 NA air objective. Otherwise, tissue slices were imaged using an Andor spinning disk (CSU-X1 Yokogawa) confocal system with a 40x 1.15 NA water immersion objective (Nikon) or, for Figure 5 and Figure 10E, the Zeiss LSM 710 with 40x 1.1 NA water objective. For pre-ExM confocal imaging, stained slices were treated with an anti-fade buffer (0.05% (w/w) p-phenyldiamine, 20 mM Tris (pH 8.5), 100 mM NaCl). Expanded slices were, for Figure 9 and Figure 11, sandwiched between coverslips of appropriate size (e.g., 45 x 60 mm), forming a chamber which was then backfilled with water and sealed with epoxy. Specimens encapsulated in this way were stable for at least a few days.

Figure 9E, G are maximum intensity projections (MIPs) of 500 nm thickness (in pre-ExM distance units), chosen to match the axial extent captured in Figure 9D, F, respectively, as closely as possible. Figure 9H is a single z-slice.
For large volume imaging of the mouse hippocampus (Figure 11), the encapsulated expanded specimen was tiled with an array of 12 by 5 z-stacks with ~20% overlap at the boundaries (Nikon). The tiled stacks were downsampled by a factor of 8 for 3D rendering and reconstructed with the ImageJ stitching plugin\textsuperscript{116}. Imaging of these 60 confocal z-stacks, in three colors, with filter switching between each color (required for the low-crosstalk imaging using CMOS cameras in the spinning disk microscope), took ~27 hours.

**Optical clearing measurements:** Transmission measurements of tissue sections before and after expansion were performed using transmitted white light illumination on a Nikon Ti-E inverted microscope with pass-band filters (480/20, 520/20, 572/20, 610/38) to quantify wavelength dependence, as presented in Figure 4. Briefly, 200 μm thick tissue sections were imaged before expansion and after expansion with a 4x 0.13 NA objective with tiling to cover the entire area of the slice. Light transmission was calculated by measuring the transmission intensity of a circular region of interest centered on the tissue slice normalized by the average light transmission absent of the tissue slice.

**Post-ExM Residual YFP fluorescence measurements:** Unlabeled brain slices (100 μm) from Thy1-YFP-H mice were gelled, digested and expanded as described above, to result in the digestion penetration experiments of Figure 5. Slices were digested with proteinase K for 16 hours. Following digestion, residual YFP fluorescence in cortical pyramidal cells was imaged on a confocal microscope (LSM 710, 40x 1.10 NA water) with pinhole opened to maximum extent (16 Airy units; 16 μm optical sectioning) to collect the dim residual YFP fluorescence. A collection of 25 Z stacks from 2 brain slices were taken across most of the expanded slice thickness (400 μm). To quantify the residual fluorescence at each Z position, the stacks were processed in a custom Matlab script. Briefly, for each image, salt and pepper noise was removed with a [5x5] median filter and the image was segmented into three regions with two thresholds generated via Otsu’s method\textsuperscript{117}. The lowest segment of pixel intensities was the background, the middle segment consisted of most of the processes and cell bodies, and the highest segment consisted of a subset of nuclei in which much higher YFP concentrations were present (e.g., see Figure 9D for an example of such a hyper-bright nucleus). The middle segment of pixel intensities was chosen for the analysis. For each stack, the processed images were averaged for each Z position. The average intensity for each Z position was normalized by the overall average intensity.
across all Z positions and all stacks for a given brain slice. The normalized average intensity as a function of Z is plotted in Figure 5 along with the mean-normalized stack-to-stack standard deviation for each Z position.

**Microscopy analysis.**

*Spinning disk confocal image processing.* Standard flatfield correction was performed as needed due to uneven illumination background. Specifically, background was subtracted with a 200 pixel wide ‘rolling ball’ algorithm as implemented in ImageJ (i.e., Figure 8E, L, Figure 11).

*Expansion degree calculation.* The expansion degree was determined by choosing two landmarks that could be clearly identified in both pre- and post-expansion images, measuring the distance between these landmarks, and calculating the ratio of this measurement pre- vs. post-expansion. These manually chosen points were used to register the images to each other using a similarity transform (i.e., translation, rotation, and scaling) and the resulting registered images were inspected visually to confirm reasonable registration over the entire specimen.

*Non-rigid registration for analysis of measurement errors.* Pre- and post-ExM images were first histogram equalized (i.e., for the entire histogram of pixel intensities across each image; Matlab) to each other. Masks were generated to exclude regions with no features by applying a Gaussian blur with a standard deviation of 8 pixels and manually choosing an intensity threshold below which to exclude pixels that were part of the background. (Gaussian blur was used only to generate masks, not for subsequent image processing.) Non-rigid registration between the images was performed using a B-spline-based registration package in Matlab\textsuperscript{118} using manually selected control points carrying a penalty weight of 1. Registration was performed in four stages with B-spline grids increasing in density from 64 pixels per grid point to 8 pixels per grid point. Analysis of measurement error was performed as schematized in Figure 7.

*Microtubule full width at half maximum (FWHM):* Intensity profiles perpendicular to microtubule orientation was taken averaging over a line profile width of 10 pixels (~150 nm). Intensity profiles were fit to a Gaussian using the Matlab ‘fit’ function and the FWHM calculated from the Gaussian fit.
Quantification of clathrin coated pit (CCP) radii: As performed for Figure 80. Super-resolution structured illumination microscope (SR-SIM) and ExM images of CCPs were first aligned via similarity transform using 2 control points across the field of view. CPPs were identified by visual inspection of ExM z-stacks, and were selected with a rectangular region of interest (ROI, ~2x diameter of pit) so that there were no neighboring pits or background punctate staining within an ROI. For each pit selected in the analysis, its ROI was used to crop the ExM image and corresponding SR-SIM image. A maximum intensity projection of the ExM image corresponding to the same depth of field as the SR-SIM image was chosen to adjust for different optical sectioning thickness, corresponding to ~2 ExM planes (~60 nm/plane in pre-expansion units) for each SR-SIM z-plane (125 nm/plane) chosen. CCP radii were calculated with angular averaging of a radial line profile originating from the centroid of each CCP, with the centroid calculated from the Otsu-binarized image. The angular radial line profile was fit using the Matlab 'fit' function to the sum of 2 Gaussians. The CCP radius was determined to be the half maximum of the fitted angular line profile.

Synapse quantification: Synapses of Figure 9 were identified by visual inspection of ExM z-stacks. Candidate instances of closely apposed Bassoon and Homer1 antibody-stained spots were selected from a maximum intensity projection of each stack. Each candidate was then inspected in the original z-stack and selected for inclusion in the analysis if it did not meet any of the following rejection criteria: synapses that were not oriented perpendicular to the imaging plane were recognized when the stained spots shifted continuously between consecutive z-slices, and were rejected; synapses with coincident punctate background staining were rejected; complex assemblies of synapses (e.g., with multiple pre- or post-synaptic terminals) were rejected; synapses that were excessively curved (e.g., relative to the 10-pixel line width, see below) were rejected. For each synapse selected for inclusion in the analysis, a line profile perpendicular to the synaptic cleft was chosen. The staining intensity for Bassoon and Homer1 was analyzed along each line profile, averaging over a width of 10 pixels (~300 nm). The resulting intensity distributions were fit to Gaussian distributions with a DC offset using the Matlab ‘fit’ function. Any synapses with a resulting goodness of fit, for either Homer1 or Bassoon, of less than 0.9 were rejected. The Bassoon-Homer1 separation was calculated as the separation between the means of the two distributions for each synapse.
### Table S1. Fluorescence retention during ExM chemical steps.

<table>
<thead>
<tr>
<th>Fluorescence Retention After Gelation</th>
<th>Percent Retention</th>
<th>Standard Deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa 488</td>
<td>57.2</td>
<td>2.9 (n = 2 slices)</td>
</tr>
<tr>
<td>Atto 565</td>
<td>76.2</td>
<td>0.5 (n = 2 slices)</td>
</tr>
<tr>
<td>Atto 647N</td>
<td>58.5</td>
<td>2.8 (n = 2 slices)</td>
</tr>
</tbody>
</table>

### Covalent Anchoring Efficiency During Gelation

<table>
<thead>
<tr>
<th>Percentage Anchored</th>
<th>Standard Deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrydite DNA</td>
<td>87.2</td>
</tr>
</tbody>
</table>

### Table S2. Chemicals list and suppliers.

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Supplier</th>
<th>Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ExM Gel or Preparation</td>
<td>Sodium Acrylate</td>
<td>Sigma 408220</td>
</tr>
<tr>
<td></td>
<td>Acrylamide</td>
<td>Sigma A9099</td>
</tr>
<tr>
<td></td>
<td>N,N'-Methylenebisacrylamide</td>
<td>Sigma M7279</td>
</tr>
<tr>
<td></td>
<td>Ammonium Persulfate</td>
<td>Sigma A3678</td>
</tr>
<tr>
<td></td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
<td>Sigma T7024</td>
</tr>
<tr>
<td></td>
<td>4-Hydroxy-TEMPO</td>
<td>Sigma 176141</td>
</tr>
<tr>
<td>Fluorescent Dyes</td>
<td>Alexa 488 NHS ester</td>
<td>Life Technologies A-20000</td>
</tr>
<tr>
<td></td>
<td>Atto 565 NHS Ester</td>
<td>Sigma 72464</td>
</tr>
<tr>
<td></td>
<td>Atto 647N NHS Ester</td>
<td>Sigma 18373</td>
</tr>
<tr>
<td>Hybridization Buffer</td>
<td>Dextran Sulfate</td>
<td>Millipore S4030</td>
</tr>
<tr>
<td></td>
<td>SSC</td>
<td>Life Tech. 15557</td>
</tr>
<tr>
<td></td>
<td>Yeast tRNA</td>
<td>Roche 10109495001</td>
</tr>
<tr>
<td></td>
<td>Normal Donkey Serum</td>
<td>Jackson Immunoresearch 017-000-001</td>
</tr>
<tr>
<td>Fixation and Permeabilization</td>
<td>Paraformaldehyde</td>
<td>Electron Microscopy Sciences 15710</td>
</tr>
<tr>
<td></td>
<td>Glutaraldehyde</td>
<td>Electron Microscopy Sciences 16020</td>
</tr>
<tr>
<td></td>
<td>Triton X-100</td>
<td>Sigma 93426</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>Sigma 50046</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>Life Technologies 70011-044</td>
</tr>
<tr>
<td>Protein Digestion</td>
<td>Proteinase K</td>
<td>New England Biolabs P8107S</td>
</tr>
<tr>
<td></td>
<td>Ethylenediaminetetraacetic acid</td>
<td>Sigma EDS</td>
</tr>
<tr>
<td></td>
<td>Guanidine HCl</td>
<td>Sigma G3272</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl</td>
<td>Life Technologies AM9855</td>
</tr>
</tbody>
</table>
Table S3. DNA sequences and modifications used for tri-functional labels.

All DNA sequences were ordered from Integrated DNA Technologies, and adapted from \cite{119}. Each strand for antibody conjugation consists of two 20bp domains separated by two bases of A/T. Two complementary tri-functional label domains hybridize to each strand conjugated to an antibody. Each antibody strand corresponds to one color of dye.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody A</td>
<td>CCGAATACAAAGCATCAACG GGTGACAGGGGATCAAAATCT</td>
<td>AA 5' Amine</td>
</tr>
<tr>
<td>Antibody B</td>
<td>TACGCCCTAAGAAATCCGAAC GCATTACGTCCTCATAAGT</td>
<td>TT 5' Amine</td>
</tr>
<tr>
<td>Antibody C</td>
<td>GACCCTAAGCATACATCGTC GACTAGTATAACTGGATTT</td>
<td>TT 5' Amine</td>
</tr>
<tr>
<td>A1</td>
<td>CGTTGATGGCTTGTTATTCCG</td>
<td>5' Acrydite 3' A488</td>
</tr>
<tr>
<td>A2</td>
<td>AGATTGTGATCCCTGTACACC</td>
<td>5' Acrydite 3' A488</td>
</tr>
<tr>
<td>B1</td>
<td>GTTCCGGAATGTTATGAGGCGTATG</td>
<td>5' Acrydite 3' Atto 565</td>
</tr>
<tr>
<td>B2</td>
<td>ACTTATGAGGCAGTGAATGCT</td>
<td>5' Acrydite 3' Atto 565</td>
</tr>
<tr>
<td>C1</td>
<td>GACGATGTATGCTTATGAGGGTCA</td>
<td>5' Acrydite 3' Atto 647N</td>
</tr>
<tr>
<td>C2</td>
<td>CAATCCAGTTATCAGTAGTCT</td>
<td>5' Acrydite 3' Atto 647N</td>
</tr>
</tbody>
</table>

Table S4. Monomer solution recipe.

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock concentration*</th>
<th>Amount (mL)</th>
<th>Final concentration*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium acrylate</td>
<td>38 (33% w/w)</td>
<td>2.25</td>
<td>8.6</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>50</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>N,N'-Methylenebisacrylamide</td>
<td>2</td>
<td>0.75</td>
<td>0.15</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>29.2</td>
<td>4</td>
<td>11.7</td>
</tr>
<tr>
<td>PBS</td>
<td>10x</td>
<td>1</td>
<td>1x</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>9.4**</td>
<td></td>
</tr>
</tbody>
</table>

*All concentrations in g/100 mL except PBS

**This solution is stored as 0.94 mL aliquots, with initiator, accelerator and inhibitor added to bring the final volume up to 1 mL per aliquot immediately before each experiment.

5.2. Materials and Methods for proExM

**Fluorescent Protein Screening** (Figure 15a, b). Most of the mammalian plasmids were obtained from Addgene (Table S5). To construct the remaining ones, pmKate2-H2B-N1 and pPATagRFP-H2B-N1 plasmids the respective genes were PCR amplified as \textit{AgeI/NotI} fragments and swapped with the LSSmOrange gene in pH2B-LSSmOrange-N1 (Addgene). To generate NLS-iRFP fusion protein, a PCR-amplified \textit{AgeI/NotI} fragment
encoding gene of iRFP was swapped with LSSmKate2 gene in pNLS-LSSmKate2-N1 (Addgene plasmid #31871). HEK293FT (Invitrogen) and HeLa (ATCC CCL-2) cells were cultured in DMEM medium (Cellgro) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin (Cellgro), and 1% sodium pyruvate (BioWhittaker). Cells were transfected using TransIT-X2 transfection reagent (Mirus Bio) according to the manufacturer’s protocol. Wide-field imaging of live HEK293FT cells was performed 24 h after transfection using a Nikon Eclipse Ti inverted microscope equipped with 10x NA 0.3 objective lens, a SPECTRA X light engine (Lumencor) with 390/22 nm, 438/24 nm, 475/28 nm, 510/25 nm, 585/29 nm, and 631/28 nm exciters (Semrock), and a 5.5 Zyla camera (Andor), controlled by NIS-Elements AR software. Immediately after live cell imaging cell cultures were fixed with 4% paraformaldehyde for 10 min, and permeabilized with 0.1% Triton-X for 15 min, washed 3 times for 5 minutes with PBS (Cellgro) and treated with 0.1 mg/ml AcX (LifeTechnologies) for at least 6 h, gelled and digested with proteinase K overnight as described below (see “AcX treatment” and “Gelation, digestion and expansion” sections).

Following digestion, the samples were processed by extensively washing with PBS, and then shrunk in 1 M NaCl and 60 mm MgCl₂ (except for YFP, which is chloride sensitive²⁰, and thus was measured in the expanded state). Registration of pre- and post-sample processing images was carried out with an implementation of the SIFT/RANSAC algorithm, in MATLAB. Automatic Otsu thresholding via CellProfiler²¹ of fluorescent nuclei allowed for automated measurement of fluorescent intensity in the same set of cells before and after sample processing. Intensity measurements for each nucleus before and after sample processing were normalized by segmented area to account for fluorophore dilution (area was used since epifluorescent optical sectioning mitigates the axial expansion effect on brightness).

Quantification of fluorescent dye retention during ProExM. Fluorescent secondary antibodies (goat anti-rabbit, 10 µg/mL) were purchased from commercial vendors (see Table S6 for list of fluorescent secondary antibodies). Retention (Figure 15c) was quantified via before-after proExM imaging mouse cortex as described below. Cortical sections of wild type (only used for Alexa 488 due to Thy1-YFP crosstalk) and
Thyl-YFP brain slices (50 μm thick) were stained with anti-Homer primary antibody (Synaptic Systems; see Table S7), and different secondary antibodies described in Table S6. Epifluorescence images of brain slices were taken with 4x 0.13 NA objective pre-gelation. Following proExM gelation and digestion, the brain slices were washed extensively with PBS (3 x 30 min), and epifluorescence images of the slice were taken again with identical imaging conditions. A region of interest in the cortex was used to determine the loss of fluorescence during proExM processing. Intensity measurements before and after sample processing were normalized by segmented area to account for fluorophore dilution.

Structured illumination microscopy pre-expansion imaging. HeLa cells were fixed with 4% paraformaldehyde for 10 min, washed 3 times for 5 minutes with PBS, and permeabilized with 0.1% Triton-X for 15 min. Microtubules in fixed HeLa were stained with primary antibodies (Sheep Anti-Tubulin, Cytoskeleton ATNO2) in blocking buffer 1x PBS with 0.1% Triton X-100 and 2% normal donkey serum (PBT) at a concentration of 10 μg/mL for 1-4 hours and then washed in PBS three times for 5 minutes each. Specimens were then incubated with secondary antibodies (Donkey Anti-Sheep Alexa 488, Life Technologies, 10 μg/mL) in PBT for 1-4 hours and then washed in PBS three times for 5 minutes. 50 μm brain tissue slices were prepared and stained with primary and secondary antibodies (Rabbit Anti-Tom20, Santa Cruz Biotech sc-11415 and Goat Anti-Rabbit Alexa 568 (Life Technologies)) as described below. Super-resolution structured illumination microscope imaging was performed on a Deltavision OMX Blaze (GE healthcare) SIM microscope with 100x 1.40 NA (Olympus) oil objective. Stained cells were imaged with SlowFade Gold (Invitrogen) antifade reagent for suppression of photobleaching and refractive index matching for pre-expansion imaging.

Measurement Error Quantification. The same fields of view were imaged pre- and post-expansion. Post-expansion images were first registered to the corresponding pre-expansion images by rotation, translation and uniform scaling. In case the specimen tilt changed between pre- and post-expansion imaging, this was corrected using a 3D rotation without scaling using the Fiji 3D Viewer package. These scaled images were
then registered again to the pre-expansion images, but this time with a B-spline-based non-rigid registration package in Matlab\textsuperscript{22} to capture any non-uniformities in the expansion process. Control points for registration were automatically generated using scale-invariant feature transform (SIFT) keypoints\textsuperscript{23}. SIFT keypoints were generated using the VLFeat open source library\textsuperscript{24}, and random sample consensus (RANSAC) was used to estimate a geometric transformation limited to rotation, translation, and scaling. The vector deformation field mapping the scaled post-expansion image to the pre-expansion image expresses the shift of each point in the post-expansion image relative to an ideal uniform expansion. By subtracting the resulting vectors at any two points, we find the relative localization error in using the post-expansion image to measure the distance between those two points. We sample the entire population of possible point-to-point measurements and find the root-mean-square error for such measurements as a function of measurement length.

**Mouse perfusion.** All solutions below were made up in 1x phosphate buffered saline (PBS). Mice were anesthetized with isoflurane and perfused transcardially with ice cold 4% paraformaldehyde. Brains were dissected out, left in 4% paraformaldehyde at 4°C for one day, before moving to 100 mM glycine. Slices (50 μm, and 100 μm) were sliced on a vibratome (Leica VT1000S) and stored at 4°C until staining.

**AcX treatment.** Acryloyl-X, SE (6-((acryloyl)amino)hexanoic acid, succinimidyl ester, here abbreviated AcX; Thermo-Fisher) was resuspended in anhydrous DMSO at a concentration of 10 mg/mL, aliquoted and stored frozen in a desiccated environment. AcX prepared this way can be stored for up to 2 months. For anchoring, cells and tissue slices are incubated in AcX diluted in PBS at a concentration of 0.1 mg/mL for > 6 hours, at room temperature. For thick tissue (> 100 microns), AcX penetration depth and labeling uniformity can be improved by incubating the sample at lower pH, at lower temperature, and in a 2-(N-morpholino)ethanesulfonic acid (MES)-based saline (100 mM MES, 150 mM NaCl; Figure 17). Tissue slices can be incubated on a shaker or rocker to ensure mixing during the reaction.
**Gelation, digestion and expansion.** For AcX anchored fluorescent proteins and antibody staining, the following steps - gelation, digestion and expansion - can be performed as described previously. Briefly, monomer solution (1x PBS, 2 M NaCl, 8.625% (w/w) sodium acrylate, 2.5% (w/w) acrylamide, 0.15% (w/w) N,N'-methylenebisacrylamide) was mixed, frozen in aliquots, and thawed before use. Monomer solution was cooled to 4°C before use. Concentrated stocks (10% w/w) of ammonium persulfate (APS) initiator and tetramethylethylenediamine (TEMED) accelerator were added to the monomer solution up to 0.2% (w/w) each. For slices, the inhibitor 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (4-hydroxy-TEMPO) was added up to 0.01% (w/w) from a 0.5% (w/w) stock to inhibit gelation during diffusion of the monomer solution into tissue sections. Cells or tissue slices were incubated with the monomer solution plus APS/TEMED (and 4-hydroxy-TEMPO for slices) at 4°C for one minute, 30 minutes for cultured cells, and brain slices respectively, and then transferred to a humidified 37°C incubator for two hours for gelation.

Proteinase K (New England Biolabs) was diluted 1:100 to 8 units/mL in digestion buffer (50 mM Tris (pH 8), 1 mM EDTA, 0.5% Triton X-100, 1 M NaCl) and incubated with the gels fully immersed in proteinase solution overnight at RT (this step can also be performed at 37°C for 4 hours). Digested gels were next placed in excess volumes of doubly de-ionized water for 0.25-2 hours to expand, with longer times for thicker gels. This step was repeated 3-5 times in fresh water, until the size of the expanding sample plateaued.

**Fluorescence microscopy after expansion.** Post-expansion confocal imaging of cells was performed on an Andor spinning disk (CSU-X1 Yokogawa) confocal system with a 60x 1.40 NA oil objective (Figure 15). To quantify expansion factor for tissue slices and low-magnification before vs. after comparisons, specimens were imaged pre-ExM on a Nikon Ti-E epifluorescence microscope with a 4x 0.13 NA air objective (Figure 16a-d and Figure 14b). For Figure 16a-b, tissue slices were imaged on Nikon Ti-E epifluorescence microscope with a 10x 0.45 NA. Otherwise, all other tissues presented were imaged using an Andor spinning disk (CSU-X1 Yokogawa) confocal system with a 40x 1.15 NA water immersion objective (Nikon) with the exception of Figure 12, Figure
14a and Figure 13, where a Zeiss LSM 710 with 40x 1.1 NA water objective. The Zeiss LSM 710 with 10x 0.3 NA air lens was used for Figure 18i.

To stabilize the gels against drift during imaging following expansion, gels were placed in glass bottom 6 well plates with all excess liquid removed. If needed for immobilization, liquid low melt agarose (2% w/w) was pipetted around the gel and allowed to solidify, to encase the gels before imaging.

ProExM of different tissue types. Standard histology preparations of mouse normal fresh frozen tissue sections, postfixed with cold acetone, of pancreas, spleen and lung (5-10 μm) were obtained from US Biomax (MOFTS036, MOFTS051, and MOFTS031, respectively). Tissues were blocked with 1x PBS with 0.1% Triton X-100 and 2% normal donkey serum (PBT) for 30 minutes before antibody staining. Tissues were stained with primary chicken anti-vimentin (Abcam) for 4 hours at RT and then washed four times 30 minutes with PBT. Slices were incubated with secondary antibodies for 2 hours at RT (Anti-Chicken Alexa 488, Life Technologies). Pre-expansion imaging was performed as described above. Tissues were incubated with 0.05 mg/mL AcX in PBS at RT overnight before gelation, digestion and expansion described above with the exception that digestion was performed at 60°C for 4 hours.

Antibody staining of endogenous proteins. Specimens, either before gelation or after autoclave or LysC treatment, were incubated in 1x PBS with 0.1% Triton X-100 and 2% normal donkey serum (PBT) at room temperature (RT) for 2 hours for blocking, and in the case of pre-gelation specimens, permeabilization. Specimens were incubated with primary antibodies at 3 μg/mL in PBT, for 4 hours (RT), and then washed four times 30 minutes with PBT. Specimens were incubated with secondary antibodies at 20 μg/mL in PBT, for 4 hours (RT), and then washed four times at least 30 minutes with PBT. Secondary antibodies used were: goat Anti-Chicken Alexa 488 (Life Technologies), goat Anti-Rabbit Alexa 546 (Life Technologies) and goat Anti-Mouse CF633 (Biotium), except that goat Anti-Chicken Alexa 546 (Life Technologies) was used for Figure 12e, g(ii), h(ii) and goat Anti-Rabbit Alexa 488 (Life Technologies) was used for Figure 12e.
Specimen disruption using autoclave. After gelation, gels were recovered from gelation chambers and washed in 1M NaCl. Gels were washed for 15 minutes in Disruption Buffer (100mM Tris base, 5% Triton X-100, 1% SDS), then placed in fresh Disruption Buffer and treated by autoclave on liquid sterilization mode with a temperature of 121°C held for one hour. This treatment must be carried out in an autoclave-safe vessel such as polypropylene tubes. Gels were then transferred to well plates for antibody staining and imaging and washed in PBT (1xPBS, 2% normal donkey serum, 0.1% Triton X-100) to remove Disruption Buffer.

Mild digestion with LysC. After gelation, gels were pre-treated in HBSS buffer (with calcium and magnesium, ThermoFisher Scientific) with 600 U/ml collagenase type II (Life Technologies) in 37°C for 2-4 hours. Gels were then washed for 5 minutes in LysC digestion buffer (25 mM Tris-HCl, 1 mM EDTA, pH 8.5) and incubated with 33 μg/ml LysC (Promega) in 37°C for at least 8 hours. Finally, gels were washed in LysC digestion buffer 3x for 30 mins each and were subjected to immunostaining with identical steps that have been described above.

Animal care. All methods for animal care and use were approved by the Massachusetts Institute of Technology Committee on Animal Care and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. One adult male rhesus macaque (Macaca mulatta) weighing 12kg was used for this study, as well as 1 C57BL/6 mouse, 4 Emx1-Cre mice, and 10 Thy1-YFP mice, ages ~1-3 months old. Mice were used without regard for gender.

Macaque procedures. Virus injections were performed with sevoflurane anesthesia using stereotactic coordinates to target 8 injection sites. Viruses (AAV8,) were centrifuged and loaded into 10μL gas-tight syringes (Hamilton) that had been back-filled with silicone oil (Sigma). A total of 3μL of virus was infused into the brain at two locations (deep then 500 μm superficial) at a rate of 100-200 nL/minute using stereotactic micromanipulator arms (David Kopf Instruments) and UMP3 micro-syringe injector pumps (World Precision Instruments). After each injection, the needle and syringe were
left in place for 10 minutes before withdrawal. Blunt 33G needles were used for all injections. 1mg Dexamethasone was also administered to prevent brain swelling. Euthanasia took place 4 weeks after viral injection. An overdose of pentobarbital was administered prior to perfusion with phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA). The brain was then extracted, blocked, and stored in a 20% glycerol with 0.1% sodium azide solution, and finally cut into 40μm microtome sections.

<table>
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<tr>
<th>Protein</th>
<th>Ex max, nm</th>
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<sup>a</sup> mKate2 gene from Addgene plasmid 37132 was swapped with LSSmOrange gene in Addgene plasmid 37133.

<sup>b</sup> cloned as N-terminus fusion with nuclear localization sequence.

<sup>c</sup> since EYFP is particularly sensitive to the high Cl⁻ used to shrink the gel<sup>20</sup>, retention of EYFP fluorescence was measured in fully expanded gel.
Table S6. Performance of selected secondary antibody dyes in proExM.

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Table S7. Primary antibodies used in Chapter 3.

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6. References


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