

Iterative Expansion Microscopy using Lipid and Protein Labels for Nano-scale Imaging of Brain Circuits

by

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B.A. Statistics, University of California, Berkeley (2013)

Submitted to the Program in Media Arts and Sciences, School of Architecture and Planning, in partial fulfillment of the requirements for the degree of

Master of Science

at the

Massachusetts Institute of Technology

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Abstract:

Expansion microscopy(ExM) and iterative expansion microscopy(iExM) enables nanometer resolution by physically expanding fixed specimens using a conventional diffraction limited optical microscope. Here, we introduce a polymer-anchorable lipid intercalating label that binds to membranes of fixed tissue to perform ExM and iExM, which we call membrane expansion microscopy (mExM) and membrane iterative expansion microscopy (imExM), respectively. The technology is also compatible with immunohistochemistry to visualize proteins of interest (i.e. nuclear pore complex, Golgi apparatus, etc.). With the technology, we can reconstruct structural and molecular information at a resolution between approximately 15nm to 20 nm.

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Introduction

Issues in brain connectivity are known to be the source of various critical diseases and mental disorders. For example, scientists have found decreased connectivity and hippocampus changes^{1,2} in brains affected by Alzheimer's disease. Depression can also cause atypical increases and decreases in connectivity³. Given these findings, researchers are heavily interested in mapping the nervous system at the level of synaptic connections, a field known as connectomics. Furthermore, the precise functioning of neurons and neuronal circuits is largely unknown⁴, meaning that a complete map of the synaptic network may not be enough to fully understand how the brain works⁵. Developing a brain map, along with understanding functionality, may lead to a clearer understanding of how neuronal circuits are formed, shape behaviors, or cause neurological diseases⁵. Although the importance of this research is widely recognized, progress has been slow.

Traditionally, electron microscopy (EM), an imaging process, has been used to map nervous systems. However, this tool has two major limitations. First, scalability: it took approximately 7 years to complete neuronal maps of *C. elegans*⁶. Approximately 302 neurons and 3,000 neuron connections were identified in *C. elegans* using EM⁶. Meanwhile, the human brain has approximately 100 billion neurons and therefore 100 trillion connections, with those connections and patterns being highly diverse across individuals^{5,7,8}. For this reason, mapping the human brain with EM is impractical. The second limitation is damage to biomolecular information. Many proteins are destroyed

during the EM preparation procedure, and therefore it is difficult to obtain biomolecular information (i.e. proteins). This limitation makes it even more difficult to determine the function of each neuron and neuronal circuits, even if the nervous system was successfully mapped.

Recent developments in both imaging technology and machine learning have been adopted to address these problems. Super-resolution imaging techniques, such as Stochastic Optical Reconstruction Microscopy (STORM), Photoactivated localization microscopy (PALM), and Structured Illumination Microscopy (SIM), have successfully visualized biomolecules at 10~20 nanometer resolution⁹. However, scalability issues persist, as it requires multiple images to construct one final image, requiring a significant time investment to acquire large fields of view¹⁰. Meanwhile, EM approaches have been scaled up with machine learning. With terabytes of EM imaging datasets and manually-traced neurons, neurons in raw EM images can now be digitally segmented with high accuracy¹¹. Computers have now completely replaced manual neuronal tracing. However, even if machine learning can enable a complete mapping of neural circuits, EM imaging limitations regarding biomolecule identification remain. It is clear that new tools must be developed to achieve both neuronal connection mapping and biomolecule analysis.

One potential solution is expansion microscopy (ExM)¹². The technology physically expands chemically fixed specimens, enabling biomolecule visualization at ~70

nanometer resolution throughout the brain and using a conventional diffraction limited optical microscope¹³. A chemically fixed specimen can be expanded multiple times to achieve ~20 nanometer resolution with an additional technology called iterative expansion microscopy (iExM)¹⁴. The technology is scalable as it successfully validated with thick tissue slices (~200 μm)¹³, while other approaches, such as EM and STORM, require the tissue size to be 30~60 nanometers¹⁵ and ~20 nanometer¹⁶, respectively. However, the technology is not able to visualize the membrane since most of the existing membrane probe contains fluorophores, which will degrade during the expansion microscopy procedure¹³. This limits the technology to identify the boundaries of neurons along with biomolecule of interest. Hence, developing the membrane probe that can be directly applied to the current expansion microscopy scheme will overcome the limitation by producing EM-like images with conventional microscopy to map the brain along with annotating protein of interest¹⁷. With the probe, the technology may enable the identification and functional understanding of each neuron and neuronal circuit in a scalable fashion.

In this thesis, to address this need, a new membrane tag (pGk5b) is introduced, and applied to the current expansion microscopy scheme, namely membrane expansion microscopy (mExM) and iterative membrane expansion microscopy (imExM). Finally, optimized mExM and imExM are introduced.

Overview of current Expansion Microscopy Scheme

Expansion Microscopy

Expansion Microscopy (ExM) is a recently developed nanoscale resolution imaging technology that uses fast, conventional diffraction-limited microscopy by physically expanding biological samples isotropically through a simple chemical process^{12,13}. ExM involves five sequential steps that can be done with standard lab equipment and commercially available reagents. First, we fix the biological specimens with standard chemical fixation methods, such as 4% paraformaldehyde (PFA) or 4% PFA and 0.1% glutaraldehyde (GLUT) solution. We then anchor the biomolecules of interest (ie. proteins and RNAs) to a swellable polymer network, which is constructed by gelation step in the ExM procedure, inside of biological specimens. We treat the sample with a chemical compound (digestion solution) to mechanically homogenize the sample by enzymatic digestion. Finally, we add water to the sample. After the aforementioned steps, the sample typically expands ~4.5x times linearly, resulting that the biomolecules of interest are evenly separated, and the effective resolution under conventional confocal microscope is now 60-70 nanometer (300 nanometer / 4.5)

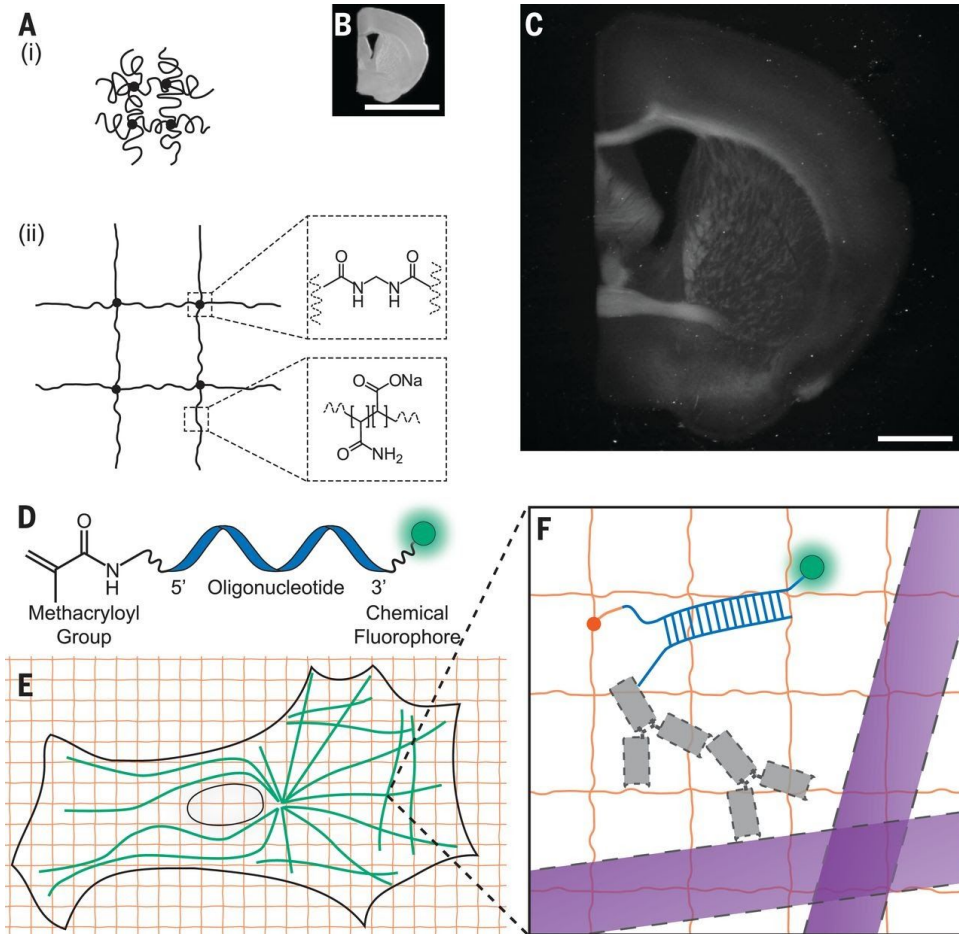


Figure 1. Concepts of expansion microscopy. **(A)** Schematic of (i) collapsed polyelectrolyte network, showing crosslinker (dot) and polymer chain (line), and (ii) expanded network after H₂O dialysis. **(B)** Photograph of fixed mouse brain slice. **(C)** Photograph, post-ExM, of the sample (B) under side illumination. **(D)** Schematic of a label that can be anchored to the gel at the site of a biomolecule. **(E)** Schematic of microtubules (green) and polymer network (orange). **(F)** The label of (D), hybridized to the oligo-bearing secondary antibody top (top gray shape) bound via the primary (bottom gray shape) to microtubules (purple), is incorporated into the gel (orange lines) via the methacryloyl group (orange dot) and remains after proteolysis (dotted lines). Scale bars, (B) and (C) 5 mm. Schematics are not to scale. Modified from F.Chen et al. (2015)¹². Reproduced with permission.

After the very first concept of ExM was introduced, many different versions and improved versions of ExM were reported by many other groups. For example, expansion fluorescence in situ hybridization (ExFISH)¹⁸ enables to visualize both RNA and proteins in nanoscale resolution, expansion sequencing (ExSEQ)¹⁹ allows both

long-read targeted and untargeted in situ RNA sequencing. ExM have also been successfully expanded in various ranges of different samples including E.Coli²⁰, cultured cells^{12,21}, mice cortex¹³, hippocampus and many other brain regions^{13,21}, as well as drosophila, zebrafish and C.elegans. ExM with other super-resolution imaging technology has also been established such as expansion lattice lightsheet microscopy (ExLLSM)²⁰ which enables even higher resolution.

Iterative Expansion Microscopy

Iterative Expansion Microscopy (iExM) is a technology that enables a second round of expansion after the first expansion by ExM, resulting in a ~20x (~4.5 x ~4.5) linear expansion of original state. Unlike ExM, iExM uses a chemoselective cleavable crosslinker to construct a first swellable polymer network inside of biological specimens, such as N,N'-cystaminebisacrylamide (BAC), N,N'-(1,2-Dihydroxyethylene) bisacrylamide (DHEBA) and (+)-N,N'-Diallyltartramide (DATD). After the enzymatic digestion and adding water to expand the sample, the sample is re-embedded with a similar polymer network that was used to formulate the first polymer network in order to maintain expanded state. Once the sample is re-embedded, a second swellable polymer network is constructed inside of the sample by using the crosslinker that has not been used for the first crosslinker. The first and second crosslinker has to be chosen in terms of cleaviability. For example, if one used DATD crosslinker to formulate the first polymer network, the second polymer network has to be made with N,N'-methylenebis

(acrylamide) (BIS). Then, the biomolecules of interest in the first polymer network transfers to the second polymer network by using chemical modifiers and linkers. Once the information is transferred, the first polymer network is cleaved by appropriate chemical solution. Here, since the cleavability of the first and second crosslinker is orthogonal to each other, the structural integrity of the second polymer network cannot be disturbed by a cleavage solution. For example, if one used DATD crosslinker to formulate the first polymer network, it can be cleaved by 0.30mM sodium metaperiodate in 0.1M acetate buffer (ph 5.5). Finally, we add water to expand the sample, resulting the 20x linear expansion. The final effective resolution is ~25 nanometer which is slightly lower than expected resolution of 15 nanometer, due to the size of labels (ie. antibodies)¹⁴ .

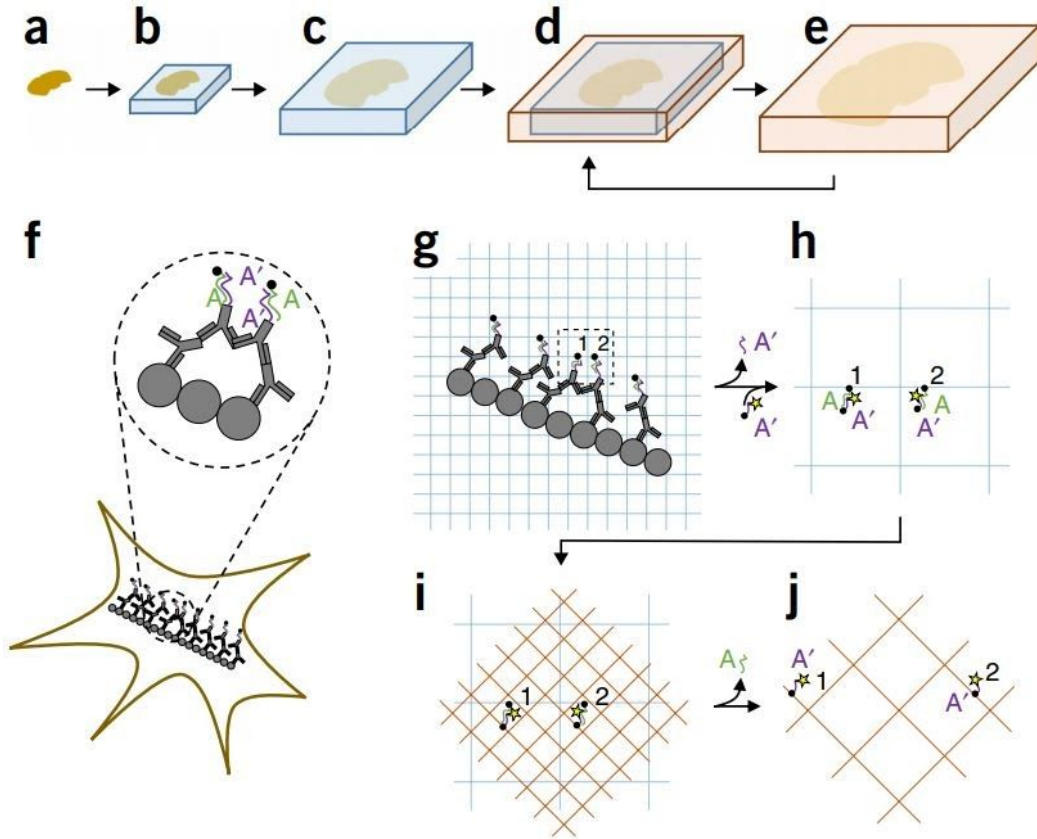


Figure 2. Concept of iterative expansion microscopy. (a–e) Schematic of iterative expansion. (b) First, a swellable polyelectrolyte gel network containing a cleavable crosslinker is formed throughout a specimen, then (c) it is mechanically homogenized and expanded. (d) After expansion, a second swellable polyelectrolyte gel network is formed throughout the first, and then (e) it is expanded after dissolving the first gel. (f–j) Molecular view of the iExM process. (f) Biomolecules of interest (gray circles) are first labeled with a primary antibody (shown also in gray) followed by a secondary antibody conjugated to a DNA (purple, sequence A') molecule, then a complementary DNA (green, sequence A) bearing a gel-anchoring moiety (acrydite, black dot), as in our original ExM procedure¹. (g) The sample (two example biomolecules are labeled "1" and "2", to be followed throughout subsequent diagram panels) is embedded in a cleavable swellable polyelectrolyte gel (blue mesh). This gel incorporates the DNA of sequence A at the gel-anchoring site, and it is expanded. (h) A DNA oligo with the original A' sequence (purple strand) bearing a fluorophore (yellow star) and a new gel-anchoring moiety (acrydite, black dot) is hybridized to the anchored A-sequence DNA (green). (i) A second swellable gel (orange mesh) is formed that incorporates the final fluorophore-bearing DNA oligo (sequence A', purple). (j) The gel expands the labels away from each other after digesting the first and re-embedding gel through crosslinker cleavage. Modified from Chang et al. (2017)¹⁴. Reproduced with permission.

Membrane Expansion Microscopy (mExM)

Traditional membrane labeling probes, such as Dil, DiO and DiD, have been widely adopted to study neuronal pathways in both live and fixed tissues. Such labels contain long hydrophobic chains and fluorophores, which tends to diffuse within membranes, allowing fine labeling of neuronal projections^{22,23}. Recent developments in membrane labeling probes such as mCLING²⁴ contain hydrophilic moieties to allow them to even more freely diffuse throughout the tissues²⁴. Aforementioned probes, however, cannot be directly applied to the current ExM scheme due to following reasons: (1) attached fluorophores degrade when the sample goes through free-radical polymerization during the ExM procedure, (2) those probes does not have enough chemical handles to perform iExM to achieve even higher resolution. To overcome issues with existing membrane probes with the current ExM scheme, we designed a new membrane probe by considering following criterion: (1) optimal amphiphilicity to maximize the lipid membrane intercalation and diffusion throughout the tissue, (2) a chemical handles for fluorophores conjugation after gelation, so that the fluorophores won't be degraded during the free radical polymerization and to minimize the molecular weight to support the diffusion throughout the tissue, (3) a multiple of gel anchorable sites for binding probe to the swellable polymer network inside of the sample to expand multiple times.

Our membrane-intercalating probe consists of a chain of lysines. Primary amines that are contained in the side chains of lysine enable binding to a swellable polymer network by using acryloyl-X (AcX). Positively charged amines also support interactions with

negatively charged membranes²⁵. We placed a lipid tail on the amine terminus of the lysine chain to achieve lipid membrane intercalation. We investigated two different types of lipid tails: (1) palmitoyl, and (2) farnesyl tails, and observed that the palmitoyl lipid tail provides denser labeled results than the ones with farnesyl lipid tails. We placed glycine between the lipid tail and the chains of lysines to provide mechanical flexibility²⁶. To achieve fast diffusion and dense labeling, we limited the molecular weight of the label to be ~1kDa, resulting in the label to have 5 lysines in the backbone. Instead of L-lysines, we decided to use D-lysines as L-lysine could be degraded during the proteinase K digestion steps in the ExM procedure. We added biotin to the carboxy-terminus of the terminal lysine, as the biotin reacts with one of four binding sites of fluorescently labeled streptavidin, and remaining binding sites can be used for binding extra biotinylated fluorophores to boost the brightness. As a result, the final formulation of membrane probe for ExM is palmitoyl-G-KKKKK-biotin (pGk5b).

To establish better lipid retention in the context of ExM, we explored various conditions of chemical fixation, as 4% PFA fixation alone cannot fix most lipids²⁷. We first explored using PFA with glutaraldehyde (GLUT) as it helps to fix lipids²⁷. With a mice brain tissue slice, we observed that 4% PFA and 0.1% GLUT provides the best lipid retention in the context of ExM. High concentration of GLUT can potentially preserve most lipids, but this fixes the sample too strong so that the sample cannot be fully homogenized in the digestion steps in ExM, which leads to a low expansion factor (~2x expansion factor). We also explored a wide range of temperatures and observed significant lipid loss at high temperature. We thus decided to perform all procedures in 4°C for the tissue

processing since the lipids are more likely to be in ordered state in a low temperature, so the loss of lipids are minimized since their diffusivity is limited²⁸. As a result, we perfuse mice with 4% PFA and 0.1% GLUT solution in 4°C, slice the brain where the buffer tray is filled with ice-cold 1xPBS, fix the tissue slices with 4% PFA and 0.1% GLUT solution in 4°C. We then incubate the tissue with AcX solution at 4°C, and do pre-gelation steps with standard ExM monomer solution at 4°C. Next, we incubate the sample in a chamber at 37°C to polymerize the sample. Once the sample is fully polymerized, the samples are homogenized with enzymatic digestion buffer (proteinase-K), and we label the lipids with fluorescent streptavidin.

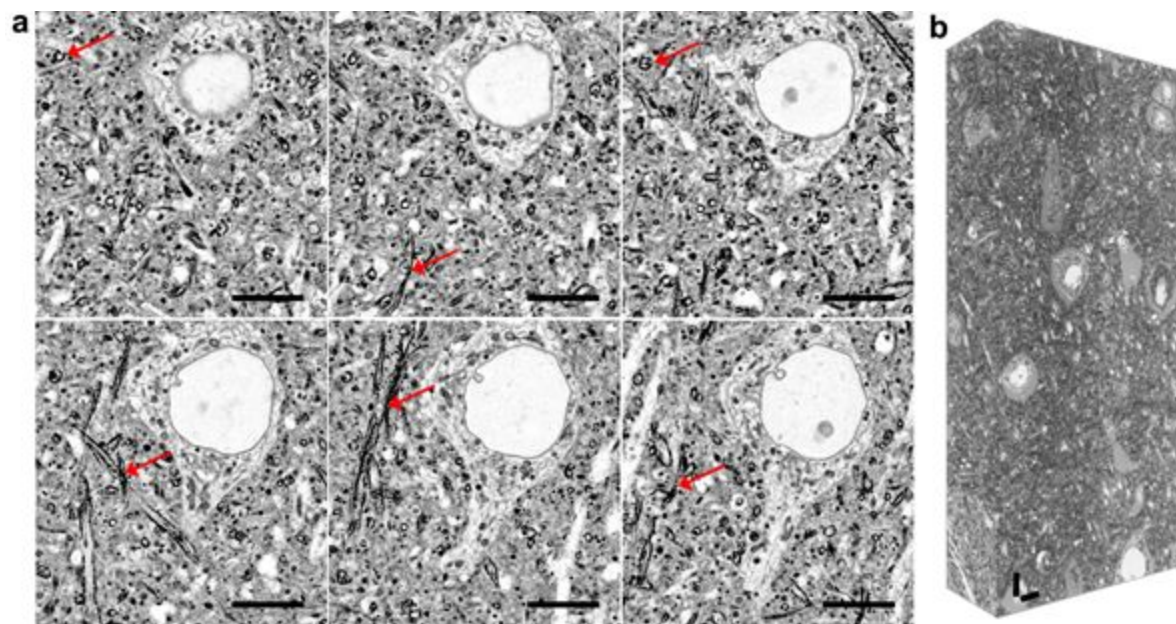


Figure 3. Membrane expansion microscopy (mExM) of fixed mice brain tissue. (a) mExM enables the labeling of membranes in thick pieces of mouse brain tissue. Here shown are six serial sections from a 3D image stack taken with a confocal spinning disk microscope. Axons can be identified by their high contrast due to the increased concentration of lipids in myelin (details shows with red arrows). (b) mExM processed tissue imaged with light-sheet microscopy. Scale bars represented in pre-expansion units: 10µm. Modified from Emmanouil D. Karagiannis et al. (2019)²⁹

mExM with Immunohistochemistry

Standard immunostaining protocols start with permeabilization of the sample with detergents, such as Saponin, Triton-X and Tween20. Those detergents allow the antibodies to penetrate throughout the cells in the tissues. However, detergents will also solubilize lipids, resulting in significant loss of lipids to label. To overcome this limitation, we fix the membrane probe by applying 0.1% PFA, so that the lipid probe remains in the original space during the detergent treatment. This method gave us a low expansion factor (x2.8). Alternatively, inspired by iExM, we constructed a cleavable non-expandable gel (similar to “re-embedding gel” in iExM¹⁴) inside of the tissue, and then anchor the membrane probe and proteins to the polymer network by using AcX. We then treat the sample with antibodies, create expandable gel inside of the sample and transfer the information to the expandable gel. After cleaving the first non-expandable gel and immersing the sample in the water to expand, we observed that the antibody signal was weak although lipid staining was preserved. We hypothesized that this is attributed to diffusion limitation of non-expandable gel.

Inspired by tissue proteomics protocols for formalin-fixed paraffin-embedded (FFPE) tissues^{30,31,32}, we developed a novel post-expansion antibody staining protocol, meaning that we first label the membrane with pGk5b, anchor lipid probes as well as proteins to ExM gel, and mechanically homogenize the sample with high temperature. The difference between previous post-expansion antibody staining protocol for ExM and our

method is that we use a DTT and SDS in a Tris buffer (pH 8.0) instead of the antigen retrieval buffer. With the DTT and SDS buffer, we boil the sample for 30 minutes 100°C and for 2 hours at 80°C. Such protocol is similar to fixation reversal protocols in formalin-fixed paraffin-embedded (FFPE) tissues, we named the buffer as “fixation reversal” (FR) buffer.

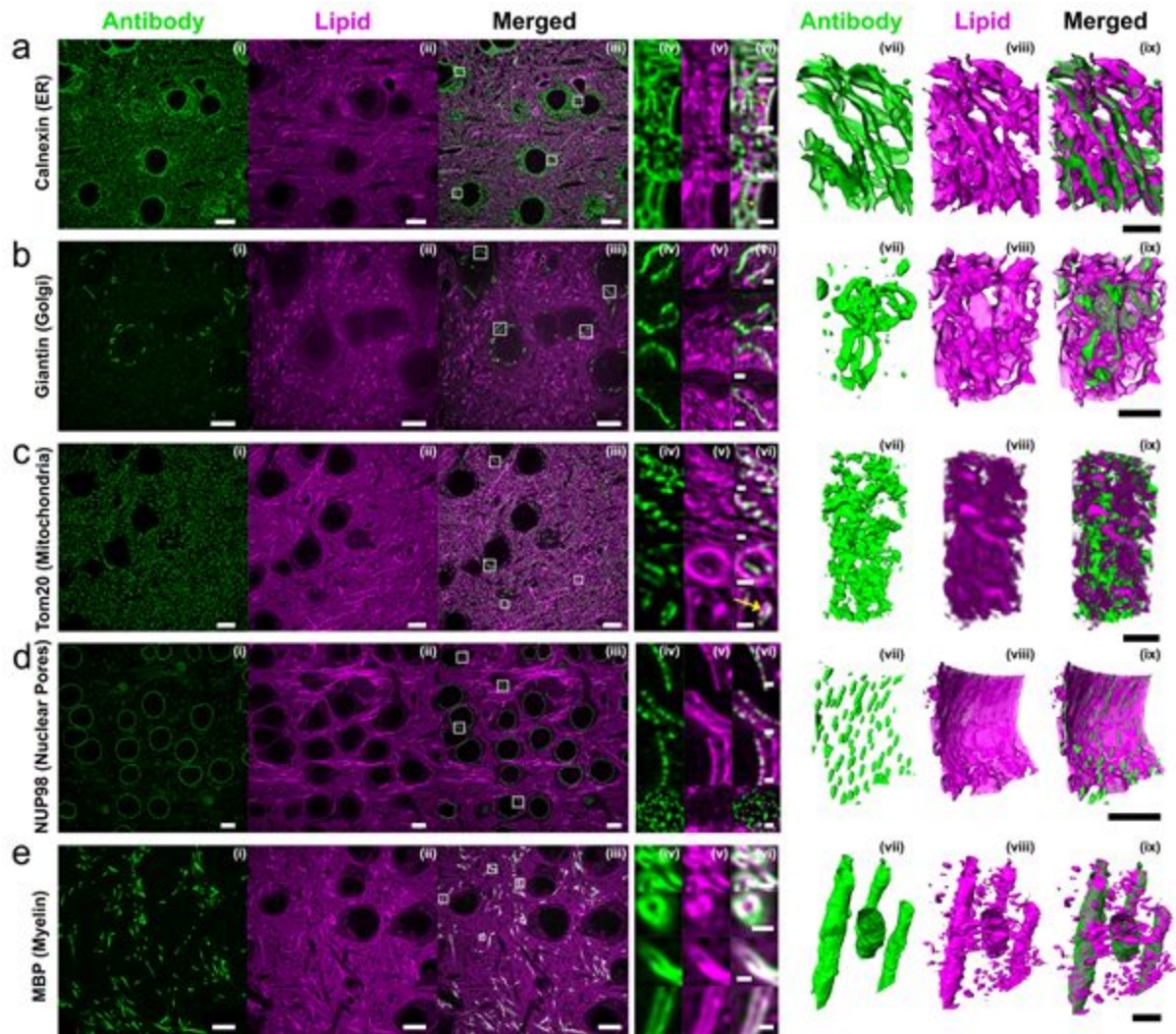


Figure 4. mExM with immunohistochemistry to visualize protein of interest along with lipids. After fixation and labeling with 100µM pGk5b, mouse brain tissue is gelled and processed in the FR buffer (0.5% PEG20000, 100mM DTT, 4% SDS, in 100mM Tris pH8) by heating at 100°C for 30min, then 80°C for 2hr. After washing in PBS the gels are labeled with antibodies for (a) an endoplasmic reticulum surface protein (Calnexin),

(b) a Golgi apparatus marker (Giantin), (c) a mitochondrial membrane protein (Tom20), (d) a nuclear pore complex component (NUP98), and (e) myelin (Myelin Basic Protein). (i-ii) Individual signals for antibodies (green) and membranes (magenta) and (iii) the overlay of (i) and (ii). (iv-vi) Details from the regions of (iii) indicated by squares. (iv) Antibody signal (green), (v) membrane signal (magenta) and (vi) the overlay of (iv) and (v). (vii-ix) Spatial visualization in 3D of lipid and antibody co-labeling with mExM. The membrane label is in magenta and antibodies in green. (a) Calnexin stains for rough endoplasmic reticulum and co-localizes with the membrane signal. Calnexin also co-localizes with the nuclear membrane signal (yellow arrows in vi). (b) Giantin is expressed on the surface of the Golgi apparatus and also co-localizes with membrane signals. (c) Mitochondrial staining is prevalent throughout the tissue and the Tom20 antibody signal overlaps with membrane labels. Tom20 appears to cluster at the mitochondrial membrane (arrow in vi). (d) Nuclear pore complexes span throughout the nuclear membrane. (e) Myelin basic protein co-localizes with the membrane signal and exhibits dense labeling, corresponding to the amount of lipid in highly myelinated regions of axons. Scale bars represented in pre-expansion units: (i-iii) 10 μ m, (iv-vi) 1 μ m. (vii-ix) Scale bars represented in pre-expansion units: 5 μ m. Modified from Emmanouil D. Karagiannis et al. (2019)²⁹

We performed antibody staining in the context of mExM (Fig. 4a, bottom row). We used antibodies against specific organelles to visualize membrane-localized proteins. We used calnexin, giantin, Tom20 and Nup98 for the endoplasmic reticulum (ER) (Fig. 4a), Golgi apparatus (Fig. 4b), mitochondria (Fig. 4c), and nuclear membrane (Fig. 4d), respectively. We also stained myelin with an antibody against myelin basic protein (Fig. 4e). As we can see on Figure 4, we were able to observe clear colocalization of antibody signals and the lipid signals.

To quantify the colocalization of antibodies and the membrane label, we applied our membrane label to cells including HeLa cells and expanded the samples, which we virally expressed membrane intercalating GFP in mitochondria and Endoplasmic Reticulum. In all cases we achieve co-localization of above 90% (n=5 for HeLa cells,

n=5 for HeLa cells) between the membrane signal and the organelle surface expressed GFP.

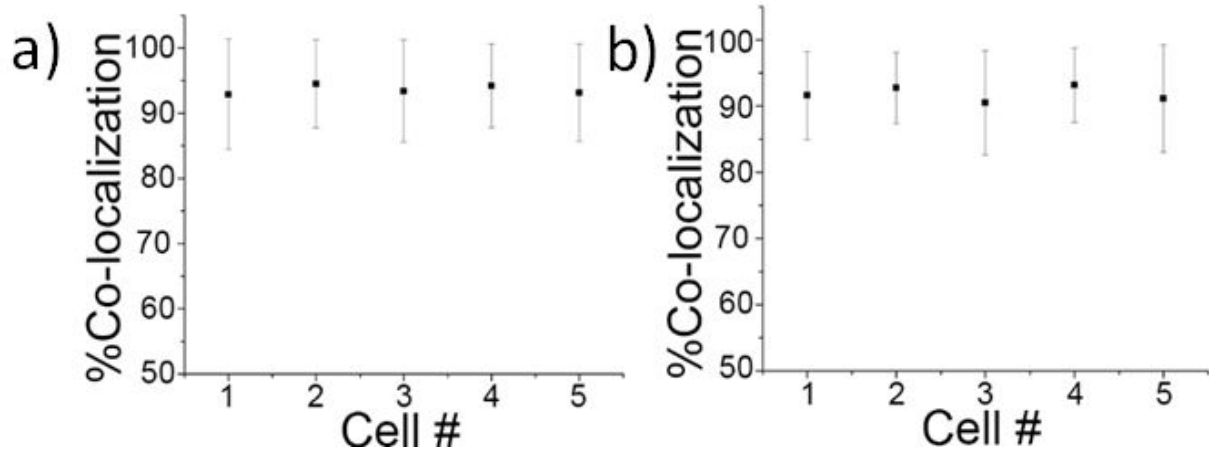


Figure 5. Quantification of the colocalization of antibodies and the membrane label. (a) Colocalization result of GFP in mitochondria and the membrane label (n=5), (b) Colocalization result of GFP in Endoplasmic Reticulum and the membrane label (n=5)

Iterative Membrane Expansion Microscopy (imExM)

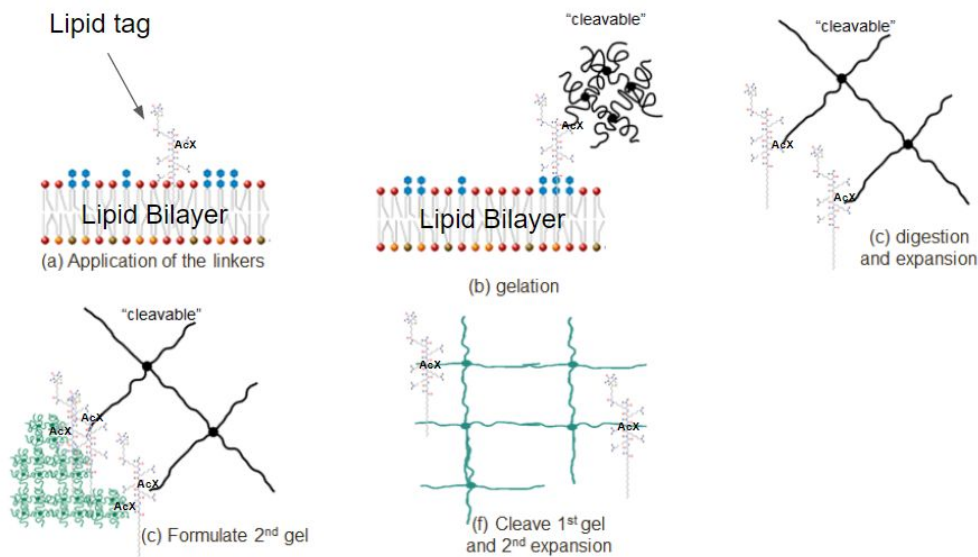


Figure 6. Concept of iterative membrane expansion microscopy. (a) label the membrane with pGk5b, (b) anchor the membrane probe to cleavable gel, (c) digestion

and expansion: mechanically homogenize the sample and expand the sample with water, (d) Formulate 2nd gel: formulate the second gel after re-embedding the first gel, (e) cleave the first and re-embedding gel, then expand the sample with water

Iterative expansion microscopy technologies can be applied after performing mExM to achieve even higher effective resolution (~25nm). We first label the tissue with pGk5b, and anchor the probe to a first cleavable and expandable gel. Then mechanically homogenize the sample by either enzymatic digestion or FR buffer. Once the sample is expanded fully with water, we re-embedded the sample to hold the expanded state¹⁴. However, due to the free radical polymerization initiator ammonium persulfate in the re-embedding solution, which contains a small amount of salt, the re-embedded samples will evenly shrink down, resulting in the expanded samples to have 3.5-4x expansion factor. We then incubate the samples with AcX again, so that amines that were not used during the first round mExM are activated to have gel anchorable sites. Next, we create non-cleavable and expandable gel inside of the sample. We then cleave the first gel by using appropriate cleavage solution (ie. sodium metaperiodate for DATD crosslinker, NaOH for DHEBA). Once the cleavage step is complete, we label the lipids with fluorescent streptavidin. Finally, we immerse the sample into water, and the gel expands. The final effective resolution is about ~25 nanometer under a conventional confocal microscope.

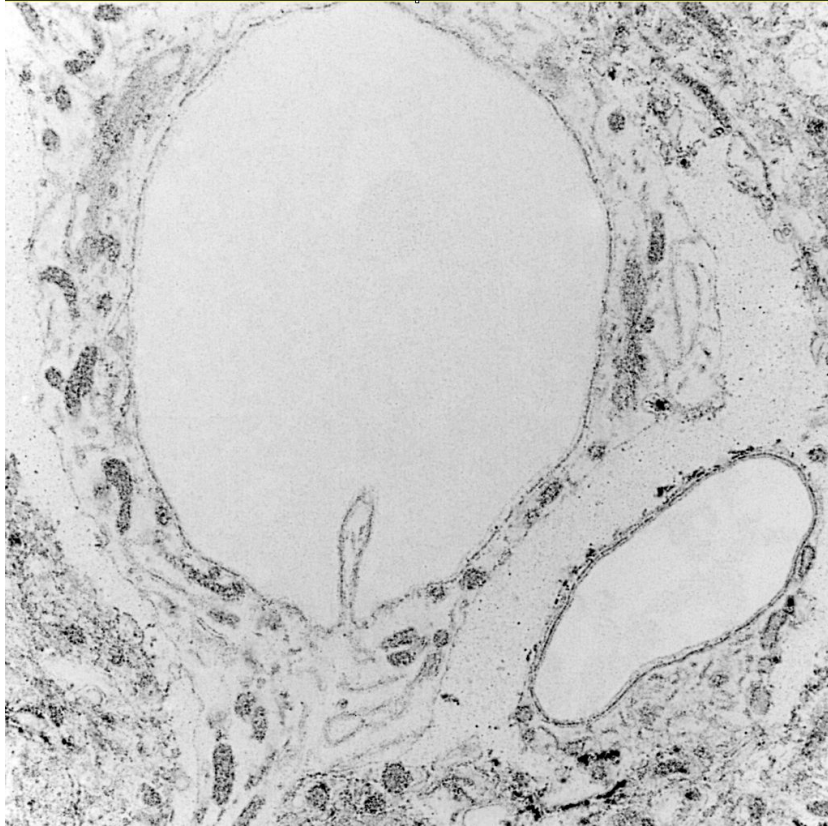


Figure 7. Iterative membrane expansion microscopy (imExM) of fixed mice brain tissue.

Although imExM allows ~25 nanometer resolution, we aimed to achieve even a higher expansion factor, to reveal even finer structure of neuronal pathways. To achieve this, we expand the sample another round after the double expansion. We used DHEBA crosslinker for the first gel, DATD crosslinker for the second gel, and BIS crosslinker for the third gel. The final resolution was about ~15 nanometer, which is the theoretical resolution limit of iExM due to the size of the linkers (ie. antibodies)¹⁴. However, we observed a significant reduction of signals-to-noise ratio (~ 2-3 folds) compared to the double expansion. To amplify the signal, we applied signal amplification technology called Tyramide Signal Amplification (TSA)³³, and the signal was boosted upto 2 folds.

However, the signals-to-noise ratio was still suboptimal. One possible reason for this is that there might not be enough free amines left in the membrane probe, so that the membrane probe did not anchor to the third gel. Thus, we treat the sample with wild-type streptavidin after the double expansion, introducing more free amines that could be activated by AcX. This showed significant improvement in signal-to-noise ratio but the final effective resolution was ~20-23 nanometers due to the diameter of wild-step streptavidin and biotinylated floufloures. Alternatively, we modified the first monomer solution to achieve a higher expansion factor for the first round, which will ultimately increase the final effective resolution after double expansion. We found that doubling the concentration of sodium acrylate concentration and reducing half of molarity concentration of DATD crosslinker from the original monomer solution allowed us to have ~6x expansion for the first round. With these solutions, we were able to reach ~17-18 effective nanometer resolution after the second round of expansion.

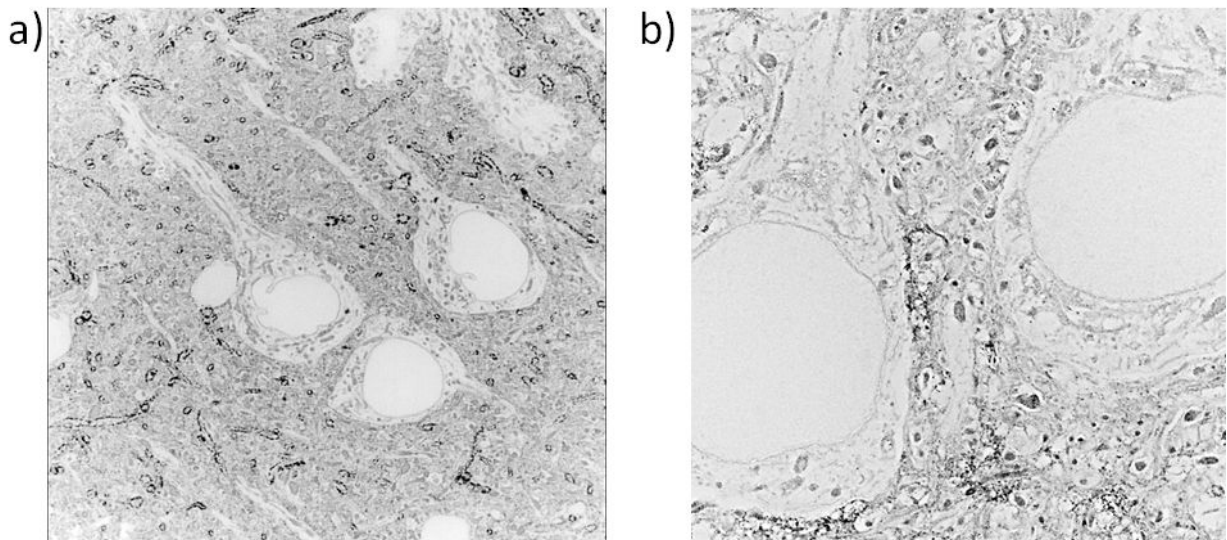


Figure 8. mExM and imExM with 8x monomer solution. (a) Membrane expansion microscopy with 6x expansion gel, (b) Iterative membrane expansion microscopy with 6x gel for the first round and 4.5x after the second round.

imExM with Immunohistochemistry

Similar to mExM with immunohistochemistry, imExM is also compatible with immunohistochemistry to visualize proteins of interest along with lipids. Once we anchor the lipids and proteins to the swellable polymer gel, we mechanically homogenize the sample with FR buffer, just like mExM with immunohistochemistry. We then stain the protein of interest with primary and secondary antibodies. After staining we expand and form re-embedded gel to maintain the expanded state and then incubate the sample with AcX. We make a second swellable gel and transfer the information from the first gel to the second gel via free-radical polymerization. Now we cleave the gel with cleavage solution, and immerse the gel into the water for full expansion. This will make the sample expand ~18-20 times. Here, the fluorophores have to be chosen carefully, as it degrades during the free-radical polymerizations¹³. We have screened through commercially available fluorophores and found that Cy3, Cy 5.5, Atto 565, Atto 561, Atto 488, Alexa Flour 488 and Alexa Flour 546 are robust during the polymerization steps in ExM procedure. As shown in Figure 9, we successfully identified NUP98, VAMP2, PSD 95 along with visualizing lipids. Although more efforts must be needed to further optimize the final outcome (in terms of signal-to-noise ratio, and non-specific binding of

antibodies), the current result clearly shows that the imExM is the tool to visualize ultrastructure of cells along with protein of interest at ~20 nanometer resolution.

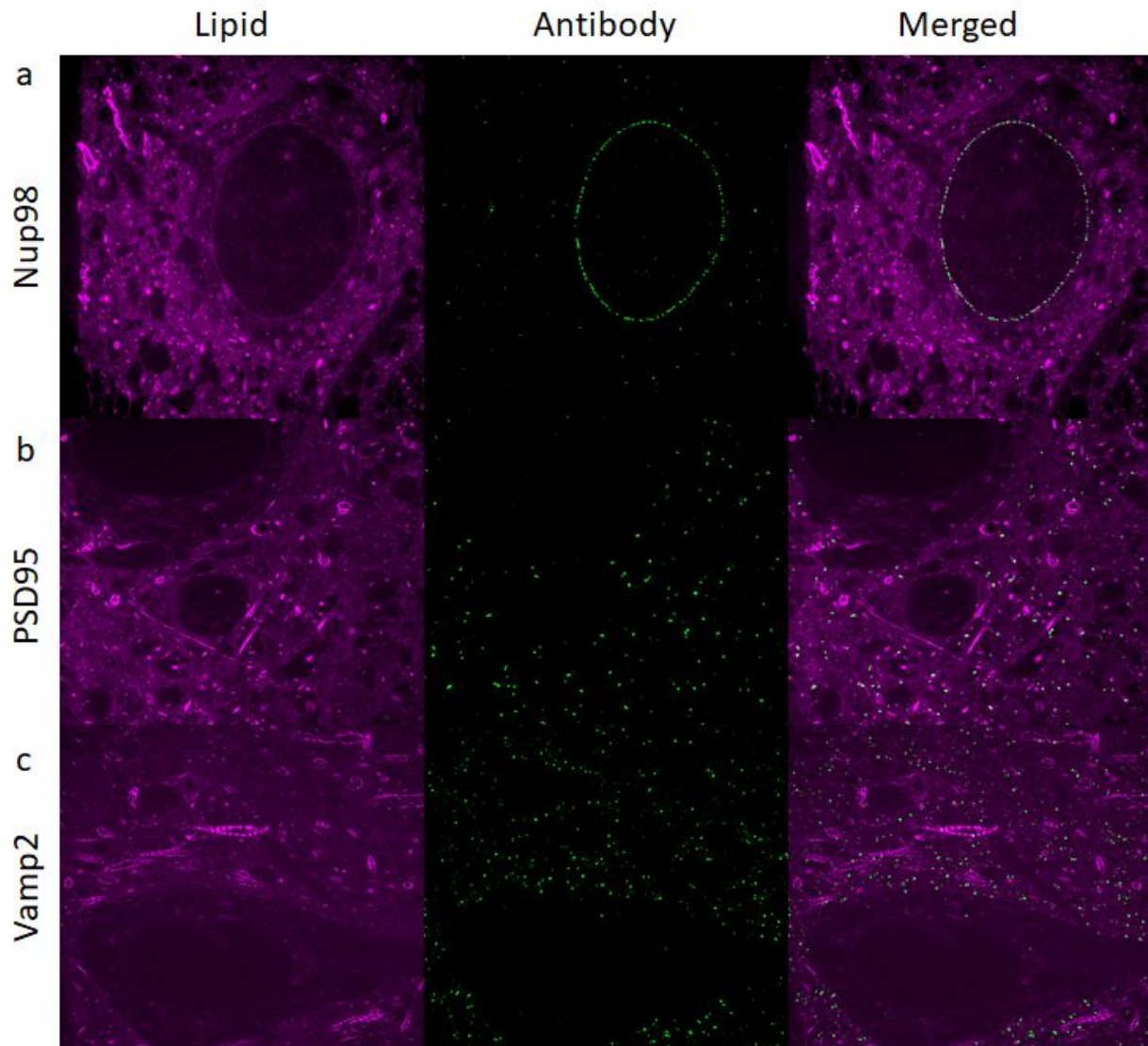


Figure 9. imExM with immunohistochemistry. The first column of a-c shows lipids (magenta), the second column shows proteins of interest, and the third column shows merged images of lipids and antibodies. (a) is for Nup98 with lipids, (b) is PSD 95 with lipids and (c) is Vamp2 with lipids.

Conclusion

This thesis presents a new membrane intercalating probe that enables the imaging of cellular membranes in the context of a lipid-optimized form of expansion microscopy. A post-expansion antibody labeling method was developed in order to allow the co-visualizing proteins of interest and membranes in ExM. In addition, an iterative form of mExM which enables resolutions of ~20 nm was proposed. The technology allows observation of structures of lipid membrane and their associated proteins at nanoscale resolution using conventional confocal microscopes.

Methods/Materials

Mouse tissue slice preparation

Isoflurane in oxygen was used to anesthetize mice. Perfusion was performed with 1x PBS at 4°C to clear the blood, then we perfused with 20mL of ice-cold fixative solution (4% PFA, 0.1% GLUT in 1xPBS). After the perfusion, the brain is extracted and stored in the same ice-cold fixative for 12 hours. Finally, the brain is sliced by using a vibratom (Leica VT1000s) to obtain 100 µm. The slices are stored at 4°C with 1xPBS.

Lipid Label Synthesis and Lipid Solution

The lipid tag (pGk5b) were synthesized by a private company (Anaspec). The tag is lyophilized to solid powder (purity of the tag is above 95%), and it is stored in -20°C until use. We dissolve the tag into 50% DMSO and 50% ultrapure water to obtain a 10mM stock solution for mExM and imExM.

Membrane Expansion Microscopy (MxM)

Step1: Lipid labelling and anchoring

1. The fixed tissue slices are first treated with the lipid solution for overnight at 4°C.
2. Extract the tissue from the lipid solution, and incubate the tissue in the AcX solution (10 mg/ml AcX/DMSO stock solution, 1:100 in PBS).

Step2: Pre-gelation and gelation

3. Incubate the tissue in the monomer solution (1x PBS, 2.5% (w/w) acrylamide, 7.5% (w/w) sodium acrylate, 0.2% (w/v) tetramethylethylenediamine (TEMED),

0.01% (w/w) 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (H-TEMPO), 0.05% (w/w) ammonium persulfate (APS), 2M NaCl, and 0.5% (w/w) (+)-N,N'-Diallyltartramide (DATD)) for 30 minutes at 4°C.

4. With fresh new monomer solution, place the tissue on the coverglass, and place it between #1 coverglass.
5. Incubate in the chamber at 37°C for two hours.

Step3: Digestion

6. Use a razor blade to trim the gel, and use a paintbrush to gently extract the tissue from the coverglass.
7. Make a digestion solution by diluting proteinase K of 8 units/mL 1:100 in digestion buffer (50 mM Tris pH 8, 1 mM EDTA, 0.5% Triton-X100, 1 M NaCl stock solution)
8. Immerse the gelled tissue into the digestion solution for overnight at room temperature with gentle shaking.

Step4: Lipid staining

9. Once the digestion is complete, wash the sample with 1xPBS for four times (30 minutes each) at room temperature.
10. Label the lipids with fluorescent streptavidin (1:100 in 1xPBS)

Step5: Expansion

11. Wash the sample with 1xPBS for four times (30 minutes each) at room temperature.

12. Immerse the sample into water for four times (30 minutes each) at room temperature.

MxM with Immunohistochemistry

Step1: Lipid labelling and anchoring

1. The fixed tissue slices are first treated with the lipid solution for overnight at 4 °C.
2. Extract the tissue from the lipid solution, and incubate the tissue in the AcX solution (10 mg/ml AcX/DMSO stock solution, 1:100 in PBS).

Step2: Pre-gelation and gelation

3. Incubate the tissue in the monomer solution (1x PBS, 2.5% (w/w) acrylamide, 7.5% (w/w) sodium acrylate, 0.2% (w/v) tetramethylethylenediamine (TEMED), 0.01% (w/w) 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (H-TEMPO), 0.05% (w/w) ammonium persulfate (APS), 2M NaCl, and 0.5% (w/w) (+)-N,N'-Diallyltartramide (DATD)) for 30 minutes at 4 °C.
4. With fresh new monomer solution, place the tissue on the coverglass, and place it between #1 coverglass.
5. Incubate in the chamber at 37 °C for two hours.

Step3: Digestion using FR buffer

6. Use a razor blade to trim the gel, and use a paintbrush to gently extract the tissue from the coverglass.
7. Make a FR digestion solution by diluting proteinase K of 8 units/mL 1:100 in FR digestion buffer (0.5% PEG20000, 100mM DTT, 4% SDS, in 100mM Tris pH8)

8. Incubate the tissue in the FR digestion solution and boil it 30 minutes at 100°C and then held for 2 hours at 80°C

Step4: Antibody staining

9. Once the digestion is complete, wash the sample with 1xPBS for four times (30 minutes each) at room temperature.
10. Block the sample with MAX block Blocking Medium (Active Motif, catalog no. 15252) for at least 4 hours at room temperature.
11. Stain the protein of interest with primary antibody (concentration of 10 µg/mL in blocking buffer) overnight at 4°C.
12. Then, wash the sample with MAX wash Washing Medium (Active Motif, catalog no. 15254) at room temperature for 4 times (30 minutes each)
13. Incubate the sample in the secondary antibody solution (concentration of 10 µg/mL in the blocking buffer) for 12 hours at 4°C.
14. Wash the sample with MAX wash Washing Medium (Active Motif, catalog no. 15254) at room temperature for 4 times (30 minutes each)

Step5: Lipid staining

15. Label the lipids with fluorescent streptavidin (1:100 in 1xPBS)

Step5: Expansion

16. Wash the sample with 1xPBS for four times (30 minutes each) at room temperature.

Confocal and Light-Sheet Imaging

Andor spinning disk (CSU-W1 Tokogawa) confocal system on a Nikon Eclipse Ti-E inverted microscope body with 40x 1.15 NA water-immersion lens was used to take all of mExM and imExM images.

Expansion Factor measurement

The expansion factor and isotropicity of mExM and imExM was calculated by using the deformation vector fields with images from pre- and post-expanded samples.^{11,12,13}

Contribution

The membrane expansion microscopy and iterative membrane expansion microscopy project has been carried out with other members from the Synthetic Neurobiology lab. My specific contributions are 1) research design and experiment execution, including mice tissue preparation, membrane expansion microscopy and iterative expansion microscopy, 3) microscopy imaging, and 4) computational analysis, including image processing and statistical analysis.

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