Protein-retention expansion microscopy for visualizing subcellular organelles in fixed brain tissue

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ABSTRACT

Background: Protein expansion microscopy (proExM) is a powerful technique that crosslinks proteins to a swellable hydrogel to physically expand and optically clear biological samples. The resulting increased resolution (~70 nm) and physical separation of labeled proteins make it an attractive tool for studying the localization of subcellular organelles in densely packed tissues, such as the brain. However, the digestion and expansion process greatly reduce fluorescence signals making it necessary to optimize ExM conditions per sample for specific end goals.

New method: Here we compare the staining and digestion conditions of existing proExM workflows to identify the optimal protocol for visualizing subcellular organelles (mitochondria and the Golgi apparatus) within reporter-labeled neurons in fixed mouse brain tissue.

Results: We found that immunostaining before proExM and using a proteinase K based digestion for 8 h consistently resulted in robust fluorescence retention for immunolabeled subcellular organelles and genetically-encoded reporters.

Comparison with existing methods: With these methods, we more accurately quantified mitochondria size and number and better visualized Golgi ultrastructure in individual CA2 neurons in the mouse hippocampus.

Conclusions: This organelle optimized proExM protocol will be broadly useful for investigators interested in visualizing the spatial distribution of immunolabeled subcellular organelles in various reporter mouse lines, reducing effort, time and resources on the optimization process.

1. Introduction

Protein retention expansion microscopy (proExM) is a powerful tool that crosslinks proteins to a swellable hydrogel to optically clear and physically expand tissues up to ~4-fold in x, y, and z dimensions (Chozinski et al., 2016; Tillberg et al., 2016), and in some cases up to 10-fold (Truckenbrodt et al., 2018, 2019). Because expansion physically separates crosslinked moieties, this technology is particularly useful for visualizing subcellular structures in intact tissues that are densely packed, such as the brain. However, one consequence of tissue expansion is a decrease in the fluorescence intensity of labeled proteins primarily due to the digestion induced quenching of fluorescent proteins and the dilution of fluorescence signal per unit volume. Various ExM protocols have described different methods for improving fluorescence retention, primarily by modifying fixation, crosslinking, and/or digestion conditions to preserve protein epitopes (Tillberg et al., 2016; Asano et al., 2018; Ku et al., 2016; Park et al., 2019; Gambarotto et al., 2019; Katoh et al., 2020; Shi et al., 2019). One common ExM protocol uses a strong protease-based digestion (protease K (Tillberg et al., 2016)), but other gentler proteases have also been used (LysC (Tillberg et al., 2016; Asano et al., 2018)), as well as a combination of heat and detergents in place of proteases (e.g., autoclave in an alkaline buffer...
Immunostaining can also be done before or after ExM to boost fluorescence (Tillberg et al., 2016; Asano et al., 2018; Ku et al., 2016; Park et al., 2019)).

Immunostaining can be enhanced by digestion protocols. For digestion with proteinase K, we digested the tissue for 8 hours to optimize visualization of organelles in genetically-labeled neurons. IHC can be conducted prior to or following expansion. Digestion can be performed with proteinase K or autoclave-mediated disruption. For digestion with proteinase K, the gels were digested over various time points to determine optimal conditions.

To determine the optimal proExM protocol for visualizing immunolabeled subcellular organelles in expanded brain tissue, we started by varying the order of IHC and proExM under different digestion conditions. Protease digestion can decrease fluorescence intensity by impacting antibody fluorescence and/or target antigen availability. If IHC is done before ExM, fluorescently-labeled secondary antibodies could be quenched by subsequent protease activity, and if IHC is done after ExM, the target antigens could be disrupted by prior protease activity and thus unavailable for immunolabeling, both of which result in diminished fluorescence signal. Thus, the order of IHC, either before (IHC-pre) or after (IHC-post) ExM could greatly affect the quality of resulting signal. Protease-free digestion protocols (e.g., detergent plus heat created in an autoclave liquid cycle) have also been shown to effectively digest hydrogels, and avoid protease-dependent depletion of fluorescence intensity (Asano et al., 2018). However, the fluorescence intensity and quality of immunostaining resulting from protease-free digestion protocols have not been systematically compared to that of protease-based digestion protocols. In Fig. 2, we directly compared COX4-mitochondria labeling in expanded hippocampal CA2 neurons from 40 µm brain sections that underwent IHC-pre or IHC-post-ExM with either overnight proteinase K digestion (Tillberg et al., 2016; Asano et al., 2018) or mild protease-free autoclave digestion (Asano et al., 2018). Expanded hydrogels resulting from the four conditions were imaged with identical confocal acquisition parameters and quantitatively evaluated based on COX4 and reporter fluorescence retention. COX4 immunostaining on unexpanded sections requires antigen retrieval (boiling) prior to IHC, thus an antigen retrieval step was included for all COX4 samples except the mild digest IHC-post, since the mild-digest performs a similar function as antigen retrieval.

We detected COX4-labeled mitochondria using both proteinase K and autoclave digestion ExM protocols (Fig. 2), however proteinase K digested hydrogels performed better with IHC-pre (Fig. 2Bii) and autoclave-digested hydrogels performed better with IHC-post (Fig. 2Ci). In regards to reporter labeling, RFP fluorescence only fared well when IHC was done prior to ExM (IHC-pre), regardless of digestion method (Fig. 2Ai and Bi). Thus, for epitopes that require antigen retrieval and/or reporter labeling, IHC-pre-ExM is the preferred method of choice.

Note that antigen retrieval diminishes reporter labeling and explains the difference in RFP intensity between autoclave (Fig. 2Aii) and proteinase K (Fig. 2Bii) digested hydrogels. If only mitochondria labeling is required, the autoclave digestion protocol performs well with IHC-post (Fig. 2Ci). To provide a quantitative measure for each combination of methods tested in Fig. 2A–D, we scored the COX4 and reporter images on a scale from 0 to 100 using either the integrated density fluorescence (COX4 channel) or the mean fluorescence (reporter channel) as a proxy metric for the quality of antibody labeling (Fig. 2E). A higher score reflects greater fluorescence signal in the respective channel, as illustrated in Fig. 2J. Although these scores do not fully capture the differences seen in labeling quality between the different conditions, they do support our observation that the IHC-pre with proteinase K condition (ProK-Pre) is the best compromise to preserve both mitochondria and the reporter labeling. The IHC-pre mild condition also performed well (Mild-Pre). However, if the priority is preservation of COX4 label, the ProK-Post condition retained the most robust staining for COX4.
Beyond visualization, the organelle optimized ExM protocol would ideally allow for the accurate segmentation and quantification of the number or structure of subcellular organelles. Thus, to compare the morphology of mitochondria in unexpanded and expanded brain tissue using the organelle optimized protocol (IHC-pre with Proteinase K), we quantified the number and size of mitochondria per labeled CA2 neuron. Compared with unexpanded COX4-labeled mitochondria, expanded COX4-labeled mitochondria were on average slightly greater in number per cell (ExM: 117.5 ± 5.6, No ExM: 95.8 ± 4.4) and smaller in size (mitochondria size: ExM 0.41 ± 0.07, No ExM 1.3 ± 0.02) after correcting for expansion (Fig. 2GH). To account for potential anisotropic expansion of cell somas, we compared the ratio of nuclear area (measured via DAPI) to the cell soma area (measured via reporter labeling) and found them to be similar on average with and without ExM (ratio: ExM 0.357 ± 3.5E-4, No ExM 0.347 ± 9.8E-3; nuclear area: ExM 1383.1 ± 93.5, No ExM 154.5 ± 7.8; soma area: ExM 3869.5 ± 259.2, No ExM 443.3 ± 11.8), indicating that the decrease in percent cytoplasmic area of mitochondria (Fig. 2D) is due to better individually resolved expanded mitochondria and not due to differences in expansion. Indeed, using the organelle optimized ExM protocol (ProK-IHC), we can readily observe individual mitochondrion within reporter-labeled dendrites (Fig. 2KL).

We next compared the order of IHC and proteinase K or autoclave digestion conditions with GOLGA5-immunolabeling of Golgi apparatus (Fig. 3), which does not require antigen retrieval. Consistent with the COX4 results, GOLGA5-labeled Golgi were detected with either proteinase K (Fig. 3Ai) or autoclave digestion (Fig. 3Bi). Compared with Golgi in unexpanded sections (Fig. 3D), Golgi in expanded sections from both digestion protocols were well resolved and revealed complex Golgi structure, albeit with differences in fluorescence retention (Fig. 3Aii vs. Bii). In contrast to the COX4 results, GOLGA5-labeling fared well in both IHC-pre-ExM digestion conditions, likely due to robust GOLGA5-labeling in unboiled sections. Similar to COX4, GOLGA5-labeling post-ExM with proteinase K digestion was unsuccessful (Fig. 3C). IHC-post-ExM with autoclave digestion was not tested. GFP reporter labeling also fared well with IHC-pre and autoclave digestion, albeit at lower fluorescence intensities than proteinase K digestion (Fig. 3Aiii vs Biii). Thus, GFP and RFP fluorescence retention are comparable when immuno-labeled prior to ExM, and they retain more fluorescence with proteinase K digestion compared with autoclave digestion, unless antigen retrieval is used. The GOLGA5 signal and reporter signal were quantitatively scored as described above (Fig. 3E). Similar to mitochondria, the pre-IHC with proteinase K condition best preserves both Golgi structure, despite clear GOLGA5 labeled Golgi localized in CA2 dendrites (Fig. 3F), despite clear GOLGA5 labeled Golgi in CA2 cell bodies (Fig. 3FG).

**Fig. 2.** ProExM pipeline comparison for visualizing mitochondria. (A) 20X Max-projection image of a section immunostained for COX4 (i) and RFP (iii) pre-ExM with autoclave digestion. Images in panels A-D were acquired with identical imaging parameters to directly compare conditions. Brightness and contrast were adjusted to the same extent across the entire image. (B) 20X Max-projection image of a boiled section immunostained for COX4 (i) and RFP (ii) pre-ExM with overnight proteinase K digestion. (C) 20X Max-projection image of a section immunostained for COX4 (i) and RFP (ii) post-ExM with autoclave digestion. D) 20X Max-projection image of a boiled section immunostained for COX4 (i) and RFP (ii) post-ExM with overnight proteinase K digestion E) 40X Max-projection image from a subset of Z sections from a boiled, unexpanded section immunostained for COX4 and RFP. Inset is a single Z section from the indicated representative cell (white asterisk) with mitochondria outlined as quantified in G-I. Imaging parameters were optimized per condition in E-F. (F) 20X Max-projection image from a subset of Z sections from a boiled section immunostained for COX4 and GFP pre-ExM with overnight proteinase K digestion as in B. (G) The average number of mitochondria per cell in unexpanded (E) and expanded (F) sections. (H) Mitochondria area per cell (corrected for expansion factor). Line reflects median and whiskers are twice the inner quartile range. I) The average percent cytoplasm area (soma area - nuclear area) containing mitochondria. Note that ExM better resolves densely packed mitochondria, the pre-IHC with proteinase K condition best preserves both Golgi and the reporter labeling. Using the organelle optimal ExM conditions (ProK-pre-IHC), we found no evidence of GOLGA5-labeled Golgi localized in CA2 dendrites (Fig. 3F), despite clear GOLGA5 labeled Golgi in CA2 cell bodies (Fig. 3FG).
2.2. 8-hour proteinase K digestion achieves the most expansion while preserving the most fluorescence

ExM digestion conditions can impact fluorescence retention (Chozinski et al., 2016) and how much the tissue expands in water, or the expansion factor (Park et al., 2019). Sufficient digestion (also referred to as homogenization or hydrolysis in some protocols) is required to prevent sample distortion during expansion but can also quench fluorescence labeling and sacrifice hydrogel integrity. Thus, for optimal digestion conditions, there is a balancing act between obtaining the largest expansion factor (without sacrificing the integrity of the tissue) and preserving the most fluorescence. Digestion is highly dependent on several conditions, including time, temperature, pH, buffer composition and enzyme quality (Chozinski et al., 2016; Asano et al., 2018; Truckenbrodt et al., 2019). To determine the effect of digestion time on the fluorescence intensity, we performed a time course of enzymatic digestion with proteinase K on 40-micron sections from Amigo2-EGFP mouse brains. Hydrogels were digested for 2, 4, 8 or 16 h with proteinase K (8 U/mL, 50 mM Tris, 0.9 mM EDTA, 8 mM Triton X-100, 0.8 M NaCl, pH: 8.0) at room temperature. Because GFP fluorescence after digestion for 8 or more hours was too faint to compare across conditions, we immunostained for GFP prior to ExM, based on the results in Figs. 2 and 3. The resulting histological scores comparing tested conditions in panels A-C. (F-G) Tile scan and high magnification image of GOLGA5 staining in CA2 neurons after immunostaining pre-ExM with overnight proteinase K digestion (same conditions as B). Optimal imaging parameters were used for F and G. Note the lack of GOLGA5 signal in CA2 dendrites except that from glial cells (asterisk). Scale bars: 50 μm (AI, DI), 10 μm (AII, DII), 200 μm (F) and 25 μm (G). Scale bars have not been adjusted for expansion factor.

Fig. 3. ProExM pipeline comparison for visualizing Golgi. (A) 20X Max-projection image of a section immunostained for GOLGA5 (i) and GFP (iii) pre-ExM with autoclave digestion. (ii) Insets are zoomed images of the dotted box in (i). Images in panels A-C were acquired with identical imaging parameters to directly compare conditions. Brightness and contrast were adjusted to the same extent across the entire image. (B) 20X Max-projection image of a section immunostained for GOLGA5 (i) and GFP (iii) pre-ExM with overnight proteinase K digestion. (C) 20X Max-projection image of a section immunostained for GOLGA5 (i) and GFP (iii) post-ExM with overnight proteinase K digestion. (D) 40X Max-projection of a subset of Z sections from an unexpanded section immunostained for GOLGA5. (E) Quantitative histological scores comparing tested conditions in panels A-C. (F-G) Tile scan and high magnification image of GOLGA5 staining in CA2 neurons after immunostaining pre-ExM with overnight proteinase K digestion (same conditions as B). Optimal imaging parameters were used for F and G. Note the lack of GOLGA5 signal in CA2 dendrites except that from glial cells (asterisk). Scale bars: 50 μm (AI, DI), 10 μm (AII, DII), 200 μm (F) and 25 μm (G). Scale bars have not been adjusted for expansion factor.
Fig. 4. The effect of proteinase K digestion time on fluorescence intensity and expansion factor. (A) Representative single z-section images of unexpanded (i) and expanded GFP+ CA2 neurons after 2-hour (ii), 4-hour (iii), 8-hour (iv), or overnight (v) digestion with proteinase K. Image acquisition parameters were identical for each condition, except for the unexpanded condition. (B) GFP fluorescence intensity measured in the cell soma after the different digestion times. Fluorescence was corrected for background and log_2 transformed. Line represents the median. Digestion time had a significant effect on GFP fluorescence (one-way ANOVA; F-stat: 20.96, p-value: 8.17E-14, 32 ± 4 cells per time point from two animals, 159 total cells). (C) Expansion factor of the expanded cell somas in B. Expansion factors were calculated using cell soma areas relative to the average unexpanded cell soma area. Line represents the median. Digestion time had a significant effect on expansion factor (one-way ANOVA; F-stat: 21.07, p-value: 4.26E-11). (D) Matrix of p-values visualizing the results of pairwise Tukey’s post hoc tests comparing mean fluorescence (upper diagonal) or expansion factor (lower diagonal) at each digestion time point. Matrix is color coded by p-value (red = significant; grey = not significant; α = 0.05). E) Effect of digestion time on fluorescence intensity (cyan; left axis) and expansion factor (orange; right axis). Plot shows the mean and 95% confidence intervals from B and C. (G) Unexpanded (i) and overnight expanded (ii) max-projection 10X confocal images of GFP+ CA2 cells. Images are the same as in A(i) and (v). Imaging parameters were optimized separately to obtain the best image of both. (F) Unexpanded (i) and overnight expanded (ii) max-projection 10X confocal images of GFP+ CA2 cells. Images are the same as in A(i) and (v). Imaging parameters were optimized separately to obtain the best image of both. Scale bars = (A, F) 50 µm in pre-expansion dimensions.

Table 1
Digest time course. Table showing mean expansion factor, mean cell area and fluorescence intensities (FI) for each digestion time point and the unexpanded control. Also included is the average background fluorescence intensity (“BG FI”), the normalized log_2 transformed fluorescence (“Log2 Norm FI”), the dilution corrected fluorescence (“Area Adj FI”), the dilution corrected fluorescence normalized to the 2 h time point (“Area Adj FI Norm”), and the number of cells (“N Cells”) for each digestion condition.

| Digest Time | Exp Factor | Cell Area | Mean Fl | BG Fl  | Log2 Norm Fl | Area Adj Fl | Area Adj Fl Norm | N Cells |
|-------------|------------|-----------|---------|--------|--------------|-------------|------------------|---------|
| no ExM      | 1.0 (± 0.02) | 294 (± 8.8) | 69.0 (± 5.39) | 7.24 (± 0.63) | 5.795 (± 0.12) | 0.24 | 13.59 | 31      |
| 2 h         | 2.6 (± 0.08) | 2026 (± 121.8) | 32.91 (± 1.93) | 3.56 (± 0.4) | 4.78 (± 0.10) | 0.018 | 1.00 | 29      |
| 4 h         | 2.6 (± 0.06) | 2061 (± 87.1) | 42.79 (± 4.77) | 4.21 (± 0.24) | 5.024 (± 0.14) | 0.022 | 1.26 | 33      |
| 8 h         | 3.0 (± 0.08) | 2821 (± 147.5) | 32.43 (± 2.43) | 2.34 (± 0.01) | 4.742 (± 0.13) | 0.013 | 0.74 | 35      |
| overnight   | 3.2 (± 0.05) | 3177 (± 106.1) | 22.84 (± 2.72) | 2.28 (± 0.03) | 4.025 (± 0.18) | 0.008 | 0.429 | 31      |
that the average micro expansion factors increased as digestion time increased (one-way ANOVA; F-stat: 21.07, p-value: 4.26E-11, Fig. 4CD), with the exception of between the 2- and 4-hr digestions in which there was no significant difference. In general, these results demonstrate an inverse relationship between fluorescence intensity and expansion factor (Fig. 4E). Interestingly, we did not detect significant decreases in fluorescence intensities from pairwise comparisons between the 8-hr digestion and the 2- or 4-hr digestions (p = 0.90 and 0.55, respectively, Tukey’s post hoc test, Fig. 4D), despite significant increases in micro expansion factors between the same comparisons (p = 0.001 for each). These data indicate that 8-hr digestion retains the most fluorescence for a sizable micro expansion factor (~3) that is not significantly different from the overnight micro expansion factor (p = 0.18). However, we note that the fluorescence intensities reported here are corrected for background, and shorter digestion times have greater fluorescence background signal compared with longer digestion times, likely due to decreased light scattering of clearer, more digested samples (see Table 1).

When we compared the average micro expansion factor to the expansion factor of the whole tissue section (macro expansion factor), we found the macro expansion factor to be consistently greater than the micro expansion factor (Fig. 4F). As digestion time increased, the macro expansion factor remained remarkably consistent even as the micro expansion factor increased. Regardless of digestion time, we were able to successfully acquire robust fluorescent images at each time point, including overnight (Fig. 4G), as long as immunostaining was performed prior to ExM.

### 2.3. Subcellular structures in the tissue are minimally distorted after expansion with proExM

To confirm the organelle optimized proExM protocol with 8 h proteinase K digestion reliably maintains macro and micro tissue structure, we measured the macro expansion of the whole tissue section (Fig. 5AB) and the micro expansion of individual cells from 5 hydrogels from 5 animals (Fig. 5C). The average macro expansion factor was 3.88 and the average micro expansion was 3.33. The average micro expansion factor was considerably lower than the macro expansion factor in the first two animals in Fig. 5C (G060 and G157). These animals were part of the digestion time course experiment in Fig. 4, and the cells measured pre- and post-expansion in this experiment were from adjacent sections while the other three animals in the plot were the same tissue pre and post-ExM. This could explain the variation in micro-expansion factor in the first two animals. Importantly, the cells that were measured pre- and post-expansion represent the same population of CA2 pyramidal neurons within the same animal for all 5 specimens in Fig. 5C. The variation in micro expansion factor is most likely due to batch or sample differences as opposed to measuring different cell populations. Example tile images of the same tissue before and after expansion are shown in Fig. 5DE. To quantify the amount of distortion, we performed a root mean squares (RMS) analysis on three sets of GOLGA5 images of the same field of view before and after proExM, as described by Chozinski et al. (2016) (Fig. 5F). Although this type of RMS analysis has been done before using mitochondria (Tillberg et al., 2016), it is rarely done on tissue samples due to the difficulty of finding the exact same cell or field of view before and after expansion (Bütter et al., 2020). Example confocal images of GOLGA5 in the same cells before and after ExM are shown in Fig. 5G (pre-ExM) and Fig. 5H (post-ExM). To get the RMS error, the post-ExM image was registered to the pre-ExM image in two steps, with a rigid and then a non-rigid B-spline registration in Elastix. A vector field map was generated (Fig. 5I) and RMS was calculated with code provided by Chozinski et. al. (see Methods). Over a length of 10 µm, the average RMS error across the three animals was 0.2 µm, which is a 2% error. This is in line with previous publications (Chen et al., 2015) and demonstrates little distortion between the pre-ExM images and the post-ExM images.

### 2.4. Fine details of the Golgi apparatus are better resolved after ExM

To quantify the enhanced resolution of Golgi ultrastructure detected with the organelle optimized proExM protocol, we measured the signal to noise ratio and the peak width (full width at half max) from the same 6 GOLGAS-labeled cells pre-ExM (Fig. 5G) and post-ExM (Fig. 5H). Fig. 5J shows a representative plot profile for the lines shown in Fig. 5G and H. The organelle optimized proExM protocol increased the average signal to noise ratio by approximately 2 fold (Fig. 5K). The driving factor behind this increase in signal to noise ratio was a decrease in noise. Before expansion, the noise was 25.6% of the signal, and after expansion the noise was reduced to 12.1% of the signal. The full width at half max was measured for the highest signal peak of each line (black bars in Fig. 5J) and then corrected for expansion (see methods). As shown in the bar plot in Fig. 5L, the width at half max decreased after expansion, indicating sharper and more defined signal peaks. The reduction in peak width after expansion is consistent with a reduction in the FWHM.

### 2.5. Dendritic spines are best resolved in expanded GFP or RFP-immunolabeled tissue

In addition to resolving subcellular organelles such as mitochondria and Golgi, the ability to resolve fine dendritic structures such as spines allows us to address questions about function and plasticity at synapses. Thus, we set out to determine whether the organelle optimized proExM protocol preserves dendritic spine labeling in EGFP and tdTomato reporter mice. Table 3 qualitatively compares spines in unexpanded and expanded tissue, with or without immunolabeling for the reporter protein, and with or without antigen retrieval by boiling or citrate. Expanded samples were immunostained pre-ExM (if at all) and digested overnight with proteinase K, as described in the methods. To boost the signal of the reporter protein, we tested two antibodies against each EGFP and tdTomato reporters (anti-GFP and anti-RFP, respectively). The ability to resolve dendritic spines in each condition was qualitatively assessed by multiple investigators (not blinded to condition), and indicated by the number of ± in the table (from + to ++++++). A greater number of + indicates better discrimination of spines and.

“-” indicates dendritic spines could not be resolved for a given condition. Some conditions in the table have yet to be tested as indicated where applicable.

Fig. 6 shows representative images of dendritic spines for each of the four conditions tested. To better visualize the spines, the expanded images did not include the full z-depth of the tissue. We saw the best resolution of spines in expanded tdTomato- tissue that was stained with the rabbit anti-RFP without any antigen retrieval (Fig. 6A; “Am2-icre; tdTomato/RFP-rabbit” in Table 3). We noted that the chicken RFP antibody did not label spines as well as the rabbit RFP antibody (“Am2-icre;tdTomato/RFP-chicken”, Table 3), indicating that not all reporter antibodies are equal when it comes to labeling spines. In general, spines were easier to resolve with proExM in tdTomato reporter mice (top panel) compared to EGFP reporter mice (bottom panel). The reason is likely multifactorial: a combination of a better performing RFP antibody, a difference in fluorescence retention between tdTomato and EGFP after proExM (Tillberg et al., 2016), and a difference in fluorescence retention between their secondary antibodies (Alexa546 and Alexa488, respectively) after proExM (Tillberg et al., 2016). We find CA2 spines are difficult to visualize in either mouse strain without prior immunolabeling. Thus, we do not believe mouse strain differences account for the differences in spine labeling after proExM. Compared to unexpanded tissue with the same IHC conditions, the proExM protocol qualitatively increases the resolution of spines by increasing their physical size and separation from nearby dendritic branches and reducing background fluorescence and/or light scattering (Richardson and Lichtman, 2015), which collectively boost one’s ability to trace individual dendrites and the spines and organelles therein. Indeed, we found it easier to consistently visualize spines in expanded images.
compared with unexpanded images acquired at similar magnification (20X/1.0NA expanded versus 40X/1.4NA unexpanded).

3. Discussion

ProExM is a powerful tool that increases the resolution of conventional fluorescence microscopy to ~70 nm and can be performed with tools available in a standard molecular biology laboratory (Chen et al., 2015; Wassie et al., 2019). Because a fully expanded hydrogel is mostly water, the optically clear sample is well suited to resolve densely packed organelles and tissues. However, the digestion and expansion process greatly reduces fluorescence retention, making it necessary to optimize ExM conditions per sample for specific end goals. There are alternative expansion strategies that employ higher expansion factors, utilize different labeling strategies, advanced microscopy setups, or a combination of these to achieve nanoscale resolution (Gabarotto et al., 2019; Kang et al., 2020), our goal was to achieve robust and reproducible expansion immunolabeling of organelles in fixed tissue without the need for the available microscopy system, not the antibody linkage-error. Here we described a 4X proExM workflow optimized for resolving subcellular organelles (mitochondria and the Golgi apparatus) and spines in fixed mouse brain tissue. Although U-ExM is commonly used to achieve nano-scale resolution in culture (Gabarotto et al., 2019; Kang et al., 2020), we tested a protocol that achieved higher expansion factors nor did we pursue post-expansion labeling as our achievable resolution was limited by the available microscopy system, not the antibody linkage-error.

### Table 2
Antibodies and conditions. Table showing primary and secondary antibody conditions used for expansion microscopy.

| Primary Antibody | Host | Clonality | Supplier | Catalogue # | Dilution | Final Conc. (µg/mL) | Antigen retrieval | Incubation Time |
|------------------|------|-----------|----------|-------------|----------|---------------------|------------------|-----------------|
| GFP              | Chicken | Polyclonal IgY | Abcam | ab13970 | 1:500 | 20.0  | Not Required | 72 + hrs at RT |
| GFP Booster Atto 488 | Alpaca | Monoclonal VH | ChromoTek | gbA488-100 | 1:200 | 2.5 | Not Required | 72 + hrs at RT |
| GFP              | Rabbit | Polyclonal IgG | Rockland | 600-401-379 | 1:500 | 2.4 | Not Required | 72 + hrs at RT |
| GFP              | Rabbit | Polyclonal IgY | Abcam | ab224040 | 1:500 | 0.2 | Not Required | 72 + hrs at RT |
| GFP Booster Atto 488 | Rabbit | Polyclonal IgG | Sigma Aldrich | E1031 | 1:200 | 0.6-1.3 | Citrate at 80 C | 72 + hrs at RT |
| GFP Booster Atto 488 | Rabbit | Polyclonal IgG | Sigma Aldrich | AV46358 | 1:250 | 2.0-4.0 | Citrate at 80 C | 72 + hrs at RT |
| GFP Booster Atto 488 | Rabbit | Polyclonal IgG | Sigma Aldrich | HPA016480 | 1:500 | 0.2 | 5 min at 100 C | 72 + hrs at RT |

### Table 3
Detection of reporter labeled spines.

| Tissue/ Antibody | ExM (protK) | IHC (pre-ExM) | Spines |
|------------------|-------------|---------------|--------|
| Am2-GFP          | Unexpanded  | no IHC        | +      |
| Am2-GFP/GFP-chicken | Unexpanded  | IHC           | ++     |
| Am2-GFP booster-alpaca | Unexpanded  | ag-IHC |         |
| Am2-icre;tdTomato | Unexpanded  | no IHC        | +      |
| Am2-icre;tdTomato/RFP-chicken | Unexpanded  | IHC           | ++     |
| Am2-icre;tdTomato/RFP-rabbit | Unexpanded  | ag-IHC |         |

- not detected; + rare SLM only; ++ OK; +++ Good; ++++ Excellent; nt Not tested
super-resolution microscopy. In this study, we reliably found that immunostaining before proExM (IHC-pre-ExM) and using a proteinase K based digestion for 8 h resulted in the best fluorescence signal to resolve subcellular organelles while maintaining sufficient reporter labeling to visualize spines and trace individual neurons. With these methods, we were able to more accurately quantify mitochondria size and number and better visualize Golgi ultrastructure in reconstructed CA2 cell bodies in the hippocampus.

Several groups have optimized expansion protocols to visualize subcellular organelles across different sample types, including various cell lines (Chozinski et al., 2016; Park et al., 2019; Shi et al., 2019; Kunz et al., 2020; Büttner et al., 2020; Pesce et al., 2019; Sun et al., 2020), rat liver (Pernal et al., 2020), clinical specimens (Zhao et al., 2017), fungi (Gambaretto et al., 2019; Götz et al., 2020), songbird (Düring et al., 2019) and drosophila (Jiang et al., 2018; Cahoon et al., 2017). Others have used ExM to visualize subcellular structures, including mitochondria (Chozinski et al., 2016; Tillberg et al., 2016; Gao et al., 2019; Fecher et al., 2019; Karagiannis et al., 2019) and/or spines (Tillberg et al., 2016; Düring et al., 2019; Gao et al., 2019) in brain tissue, but few have systematically analyzed how fluorescence intensities and expansion factors compare across protocols or with unexpanded measurements. This is critically important as several groups have noted discrepancies in micro versus macro expansion factors in other sample types (Katoh et al., 2020; Büttner et al., 2020; Pernal et al., 2020; Martinez et al., 2020; Scheible and Tinnefeld, 2018; Vanheusden et al., 2020), including dissimilarities in expansion factors of different subcellular organelles (Büttner et al., 2020) or of subcellular organelles across neighboring cells and tissues (Pernal et al., 2020). Others, however, have reported minimal to no differences in micro vs macro expansion factors (Gambaretto et al., 2019; Götz et al., 2020). In our measurements, we found discrepancies between the micro and macro-expansion factors. On average, the 8-hour proteinase K digestion produced a micro expansion factor of 3.33 and a macro expansion factor of 3.88 (Fig. 4C). Interestingly, the macro expansion factor was relatively insensitive to digestion time past 2 h, while the micro factor continued to increase (Fig. 4F). The average micro expansion factors are lower than the commonly reported 4–4.5X macro expansion factor for proExM, which is consistent with other reports using micro expansion factor measurements (Büttner et al., 2020; Pesce et al., 2019) (but see also ref (Martínez et al., 2020)), reinforcing the notion that each sample type needs to be independently optimized and validated for ExM. Another study found discrepancies in cell soma vs nuclear expansion factors (Pernal et al., 2020), and there is some controversy in the field about whether membraneous organelles such as nuclei and mitochondria expand isometrically (Büttner et al., 2020). However, we showed that the ratio of nuclear area to cytoplasmic area remained constant after expansion with the optimized protocol, indicating isotropic expansion of the nucleus relative to the soma, perhaps due to our longer digestion times. In addition, our RMS analysis showed very little distortion of the Golgi before and after expansion (Fig. 5). Combined, this suggests that we do get isometric expansion of organelles with the organelle optimized proExM protocol.

In regards to labeling subcellular organelles in fixed brain sections, we were able to visualize expanded mitochondria with a COX4 antibody and expanded Golgi apparatus with a GOLGA5 antibody, using either the proteinase K digestion or the mild autoclave digestion. In our hands, the IHC-pre ExM with proteinase K digestion was the best compromise to preserve the fluorescence signal for both immunostained organelles and reporter proteins, particularly when antigen retrieval is not required. However, if the goal is only to visualize mitochondria, the mild autoclave digestion with IHC-post ExM also produced good COX4 staining as seen for other mitochondria immunostains, like TOMM202. However, this came at the expense of the signal in the reporter channel. While EGFP and tdTomato have been reported to have different percent fluorescence retention after ExM (Tillberg et al., 2016), they perform comparably when antibody-labeled prior to ExM, as recently reported in cultured cells (Min et al., 2020). They also retain more fluorescence with proteinase K digestion compared with autoclave digestion when antigen retrieval is not needed. Neither antibody performed well with IHC-post ExM and proteinase K digestion, likely due to digestion of target epitopes. It is important to note that conducting immunostaining prior to

![Ai. ExM RFP-labeled spines](image1)

![Ai. ExM RFP-labeled spines](image2)

![Bi. unexpanded RFP spines](image3)

![Bi. unexpanded RFP spines](image4)

![Ci. ExM GFP-labeled spines](image5)

![Ci. ExM GFP-labeled spines](image6)

![Di. unexpanded GFP-labeled spines](image7)

![Di. unexpanded GFP-labeled spines](image8)
proExM introduces small positional errors due to linking the fluorophores to the hydrogel. Immunostaining targets with primary and secondary antibodies imposes a linkage error of ~17.5 nm (Chang et al., 2017; Zwettler et al., 2020). This can cause a localization error between a protein of interest and its fluorophore in an expanded state, however, the relative distance of the fluorophore to the epitope stays unchanged. Alternatively, labeling post-ExM results in a relatively smaller antibody expansion factor (Gambarotto et al., 2019; Gambarotto et al., 2019; Zwettler et al., 2020; Kang et al., 2020). However, the expanded linkage-error imposed by pre-ExM labeling is still below the achievable resolution on a standard confocal microscope (Schermelh et al., 2019). Thus, post-ExM labeling would be more appropriate for super-resolution imaging setups.

The organelle optimized proExM protocol afforded better resolution for the quantification of subcellular organelles compared to unexpanded organelles. We quantified the number and size of expanded mitochondria and found that the expanded mitochondria were smaller and more numerous than unexpanded mitochondria. This presumably is indicative of tightly packed mitochondria in the unexpanded samples being lumped together that can be separately resolved with expansion. Although we saw no difference in the nucleus to soma ratio before and after expansion, it is possible that mitochondria have a different expansion factor than the cell bodies (Gambarotto et al., 2019; Bütter et al., 2020). The expansion factor that we used was based on the ratio of cell soma sizes before and after ExM. Thus, if the mitochondria expanded less than the cell somas, this could lower the resulting corrected mitochondrial sizes. In the data reported here, the expansion factor based on the average cell soma area was 2.95x, while the expansion factor based on the average mitochondrial area was slightly less, at 2.74x. This suggests that the smaller mitochondria size post-ExM (Fig. 2H) may be partially explained by differences in expansion. However, when we correct mitochondria size by the mitochondrial expansion factor, the expanded mitochondrial size was 0.47 µm² instead of 0.41 µm², which is still considerably smaller than the average unexpanded mitochondrial size of 1.3 µm². Thus, while unequal expansion may have contributed some to the decrease in mitochondrial size post-ExM, it seems that better segmentation of the mitochondria and a smaller optical PSF are likely the main contributing factors. The average expansion corrected mitochondria area we report (median: 0.26 µm², interquartile range: 0.11–0.55) is also in line with values reported in the literature using electron microscopy and super-resolution imaging techniques. Studies using transmission electron microscopy in CA1 reported an approximate average mitochondrial area of 0.15 µm² (Zheng et al., 2020) and 0.6 µm² (Battaglia et al., 2020). Additionally, our expansion corrected mitochondria area is similar to CYTC-labeled mitochondria observed in PV+ presynaptic boutons in CA1 of adult mice using dSTORM imaging (median: 0.287 µm², interquartile range: 0.158–0.368 (Cserep et al., 2018)). The fine details of the Golgi cisternae were also better resolved after expansion, whereas without expansion the Golgi were smoothened and much of the details lost. Expansion increased the signal to noise ratio of GOLGAS images by reducing the amount of noise (Fig. S5), and also reduced the width at half max of signal peaks (Fig. 5L), both of which indicate better resolution with the organelle optimized proExM protocol.

To determine the optimal digestion time for fluorescence retention in fixed brain sections, we performed a digestion time course and found the greatest drop in fluorescence after the 2-hr and overnight digestion. There were no significant drops in fluorescence between 2, 4 or 8 h of digestion (Fig. 1B). However, there was a significant increase in micro expansion factor between the 4- and 8-hr time points (Fig. 1C), indicating that the inverse relationship between expansion factor and fluorescence intensity is not entirely linear. Expansion factor begins to plateau after 8 h of digestion, and while there is a slight increase in expansion factor in the overnight condition it is not statistically significant. Therefore, under the conditions used here, a digestion time of 8 h is ideal for achieving a robust expansion (~3X) without further loss of fluorescence seen with overnight digestion. At all digestion time points, the expansion factor did not systematically vary by depth (data not shown), indicating that 2 h in protease K is sufficient for uniform penetration and isotropic expansion in the Z dimension of 40 µm brain sections, as previously reported for thicker sections and longer digestion times (Chen et al., 2015).

Because spines and synapses are common targets for subcellular organelles, we evaluated the optimized protocol for visualizing reporter-labeled dendritic spines. We found that the addition of IHC-pre ExM was necessary for the resolution of both EGFP+ and tdTomato+ spines. This was not surprising given the digestion of fluorescent proteins and expansion-induced fluorophore dilution incurred in the protocol. Dendritic spines were best resolved in IHC-pre ExM with protease K digestion with either GFP or RFP antibodies. The ability to label dendritic spines in ExM was antibody dependent, with some antibodies against the same reporter faring better than others under the same IHC conditions.

Our lab has begun applying the described proExM methods to answer open questions involving subcellular organelles in neurons. One such open question is whether there is Golgi present in dendrites, or if the Golgi is limited to neuronal cell bodies. It is known that local translation of RNA occurs in dendrites (Steward and Schuman, 2001); however, there is conflicting evidence of the existence of Golgi apparatus in the dendrites, which would normally process newly transcribed membrane bound proteins. Using combined GOLGAS-labeling of Golgi apparatus and reporter neuron labeling with the proExM protocol described here, we do not detect GOLGAS staining outside the cell soma or very proximal dendrites, consistent with reports that canonical Golgi markers are retained in the soma and not present in distal dendrites (Pierce et al., 2001; Karagiannis et al., 2019; Bowen et al., 2017; Kennedy and Ehlers, 2006).

3.1. Considerations for subcellular imaging of expanded samples

The benefits of expansion come at several costs, including diminished signal concentration, hydrogel mechanical integrity and movement, and increased imaging volume and time (Park et al., 2019). The loss of fluorescence is more pronounced when using intact tissue as opposed in culture because intact tissue requires longer digestion times. Here we comment on the proposed workarounds for these issues that have or have not worked well for subcellular imaging of expanded brain sections.

Expansion microscopy substantially increases the thickness of a sample, which limits its ability to be imaged with standard high magnification microscope objectives with limited working distances. Thus, expanded hydrogels generally require long working-distance objectives that inherently have lower NA values. It can be expected that the hydrogel thickness will be equivalent to 4-fold the depth of the gelation chamber. Further, keeping the tissue in plane during gel chamber assembly is difficult, often resulting in increased sample z-distance. Miniaturizing tissue thickness (40 µm versus 100 µm) and using a single coverslip for the gelation chamber helped minimize hydrogel thickness without sacrificing ability to reconstruct neurons. However, to retain sufficient fluorescence, digestion time needed to be decreased to 4 h when using a single coverslip versus 8 h for two coverslips as optimized here.

Loss of fluorescence due to the digestion of antibodies or epitopes and dilution of fluorescent molecules per unit volume can result in low contrast samples not suitable for high resolution imaging even with overexpressed fluorescent reporter proteins. We found that performing IHC beforehand and limiting the protease K digestion to 8 h largely negated this issue. When the fluorescence signal is insufficient, imaging in low concentrations of PBS (0.5X PBS instead of 0.001X PBS or water) substantially improves the contrast by increasing the concentration of fluorescent molecules per area. This will decrease the expansion factor,
but in our hands, even a 2–3-fold expansion in optically clear tissue produces better resolved images of subcellular structures than unexpanded tissue.

Hydrogel movement during imaging is another common issue. Because gels expand 4-fold in x, y and z compared with unexpanded brain sections, tile scans are required to reconstruct entire neurons even at 10x (see Fig. 2fii for a 10x single field of view of expanded neurons). Tile scanning increases the length of time required to image a gel and thus worsens gel shift. Gel shift was most noticeable while imaging on an upright microscope equipped with a water immersion lens, since the gel can easily shift when submerged. Gel shift was less noticeable when imaging either on an inverted scope or on an upright scope with air objectives (if the gel was dried for 30 min to adhere to the glass bottom plate), but these options negatively impact objective working distance or resolution, respectively. To minimize gel shift during upright imaging with a water dipping lens, we applied the following techniques to stabilize the gel in the imaging chamber (50 mm WellCo Well). Following full expansion in water, we surrounded the gel with 2% agarose in the imaging chamber. This noticeably reduced gel movement during acquisition of single images but shift was still detected during longer tile scans. We also tested re-embedding the gelated sample in an unexpandable gel and covalently linking it to the glass imaging dish (Tillberg et al., 2016). This completely eliminated movement of the gel and allowed us to take long tile scans on an upright or inverted microscope. However, this also seemed to dampen the fluorescence signal, which was irreversible. Also notable, re-embedding expanded gels reduces the size of the gel by approximately 30% (Chen et al., 2016). Additionally, we tested immobilizing expanded gels with Poly-L-lysine treated imaging chambers. This method appeared to be the most useful since there was no appreciable gel movement during imaging nor dampening of fluorescent signal.

4. Methods

4.1. Animals

Adult male and female Amigo2-EGFP (RRID:MMRRC 033018-UCD, bred for at least 10 generations onto C57BL/6 J background) or Amigo2-icreERT2;Rosa2TdTTomato transgenic mice were used. Amigo2-icreERT2;Rosa2TdTTomato mice were generated by crossing Amigo2-icreERT2 mice (Alexander et al., 2018) to Ai14 mice (Jax #007914). Amigo2-icreER;ROSA2TdTTomato mice were given 2 or 3 daily intraperitoneal injections of tamoxifen (Sigma T5648, 100 mg/kg freshly reconstituted in DMSO). Mice were group-housed under lab conditions and fed Rainbow mouse pellets (Harlan, MD) and water ad libitum. All procedures were approved by the Animal Care and Use Committee of Virginia Tech.

4.2. Immunofluorescence

Mice were anesthetized with 150 mg/kg fatal-plus solution (Vortech Pharmaceuticals) and perfused with ice-cold 4% paraformaldehyde. Amigo2-icreERT2;Rosa2TdTTomato mice were perfused one week post tamoxifen injections. Brains were dissected and post-fixed for at least 24 h before sectioning 40 µm thick sections in the horizontal plane on a vibratome (Leica VT1000S). Sections were to be stained with COX4 under mild digestion with Alkaline Buffer (alternative) with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

4.3. Protein expansion microscopy solution preparation

Solutions were prepared as described by Asano et al. 2018 (Asano et al., 2018). Anchoring stock solution prepared by mixing Acryloyl-x, SE (ThermoFisher A20770) in DMSO (1:100 w/v) was added to the gelation solution at the time of gelation. This solution was prepared by mixing Sodium Acrylate in nH2O (33% w/v, Sigma #408220), Acrylamide in nH2O (50% w/v, Sigma A9099), N, N-Methylenebisacrylamide in nH2O (2% w/v, Sigma M7279). Monomer solution was prepared by adding 2.25 mL of 33% SA solution (8.6% w/v), 0.5 mL of 50% Acrylamide solution (2.5% w/v), 0.75 mL of 2% N, N-Methylenebisacrylamide solution (0.15% w/v), 4 mL of 5 M NaCl (11.7% w/v), and 1 mL of 10X PBS. Inhibitor stock was prepared by dissolving 4-Hydroxy-TEMPO (0.5% w/v, Sigma 176141) in nH2O and initiator stock was made by dissolving Ammonium persulfate in nH2O (10% w/v, Sigma 248614). Accelerator solution was prepared by diluting TEMED in nH2O (10% v/v, Sigma T70024) immediately before use. All solutions except the TEMED accelerator were prepared before use and stored at -20°C.

4.4. Protein expansion microscopy

4X protein expansion microscopy (proExM) was carried out on horizontal mouse brain sections containing dorsal hippocampus as described in Asano et al. 2018 (Asano et al., 2018). Sections were incubated overnight in Acryloyl-x stock/PBS (1:100, ThermoFisher, A20770) at RT in the dark. Following incubation, the slices were washed twice with PBS for 15 min each at RT. The gelation solution was prepared by adding 384 µL of monomer solution, 8 µL 4-Hydroxy-TEMPO inhibitor (1:200 v/v, Sigma Aldrich, 176141), 8µL TEMED accelerator (10% v/v, Sigma Aldrich, T7024), and lastly 8µL of APS initiator (10% w/v, Sigma Aldrich, 248614) for each section. Sections were then incubated in the gelation solution for 30–45 min at 4°C in the dark. Gelated sections were placed on gelation chambers constructed from microscope slides with coverslips as spacers. Our gelation chambers produce gels with the thickness of two type No. 1.5 coverslips (~0.3 mm thick). The chambers were filled with gelation solution and allowed to incubate at 37°C for 2 h in a humidified container. Following gelation incubation, the gelation chamber was deconstructed to uncover the gelated brain section. To remove the gel from the chamber, digestion solution (50 mM Tris, 0.9 mM EDTA, 8 mM Triton X-100, 0.8 M NaCl, pH: 8.0) without protease K was applied and a coverslip was used to gently remove the sample. Digestion solution containing protease K (8 U/mL, New England Biolabs, P81075) was applied to gels and allowed to digest for 2–16 h (see Results) at room temperature. Upon completion of digestion, gels were stained with DAPI (Sigma Aldrich, D9542, 1:10,000 in PBS) for 10 min at room temperature with shaking. The gels were then washed twice for 10 min with nH2O to remove excess DAPI and fully expand the gel.

4.5. Mild digestion with Alkaline Buffer (alternative)

To better preserve epitopes for post-expansion labeling, a mild digestion technique was applied rather than Proteinase K-mediated digestion as described by Asano et al. (2018). First, samples were removed from the gelation chambers and immersed in an alkaline buffer (100 mM Tris base, 5% w/v Triton X-100, 1% SDS). Alkaline buffer was replaced and gels were autoclaved on a liquid cycle for 1 h at 121°C. Gels were then allowed to cool and subsequently washed in an alkaline buffer twice more. Next gels were either preserved in 1X PBS, expanded in H2O, or immunostained.
4.6. Stabilizing ExM gels with agarose

To prevent movement during imaging, gels were fully expanded in water or 0.001X PBS in WillCo wells (HBSB-5040) and reversibly immobilized by applying liquid 2% LE agarose (ThermoFisher, J32802) around and on top of the gel (in areas not containing tissue). Following application, the gel embedded with agarose was placed at 4 °C for at least 15 min to allow the agarose to fully solidify prior to imaging.

4.7. Stabilizing ExM gels with poly-L-lysine coated imaging dishes

Adhering expanded gels to Poly-L-lysine treated imaging dishes effectively reduced gel movement during long scans. First, glass bottom plates were rinsed with nanopure H2O twice for 5 min each. Next, the plates were washed with ethanol (100% v/v) and allowed to dry at room temperature. Plates were then incubated with Poly-L-lysine (Sigma Aldrich, P6407, 1% w/v in water) for 30 min at room temperature. Plates were washed with water twice and then allowed to dry before use. Following glass treatment, gel surfaces were dried and the expanded gels were transferred to the treated dish.

4.8. Re-embedding and linking gels to imaging dish

Re-embedding and covalently linking gels to a WillCo well allowed long-term imaging without gel shifting as described by Tillberg et al. (2016). First, the gel was completely expanded in water and then incubated in a non-expanding re-embedding solution (3% w/v acrylamide, 0.15% w/v N,N-methylenebisacrylamide, 0.05% w/v APS, 0.05% w/v TEMED, and 5 mM Tris). The gels were incubated with shaking at room temperature for 20 min. The gel was then transferred to a Bind-Silane treated WillCo Well plate and covered with a coverslip. Fresh re-embedding solution was then lightly applied surrounding the sample, which was then incubated at 37 °C for 1.5–2hrs without shaking. Once the re-embedding solution gelated, the coverslip was removed from the covalently linked gel and could be imaged.

4.9. Bind silane treatment of imaging dishes

Immediately before use, imaging dishes were treated with a bind silane solution as described by Tillberg et al. (2016). Before bind silane treatment, imaging dishes were briefly washed with nanopure H2O, 100% Ethanol, and then allowed to dry. Bind silane solution (80% v/v EtOH, 2% v/v acetic acid, 0.05% v/v Bind-Silane) was then applied with shaking for 5 min. The dish was then washed with 100% EtOH and allowed to dry before usage.

4.10. Image acquisition

Images were acquired on an upright Zeiss 710 or inverted Zeiss 700 confocal microscope equipped with a motorized stage, 488/561/633 laser lines, and 5X/0.16 NA, 10X/0.3NA, 20X/1.0 NA water immersion, 20X/0.4 NA air, or 63X/1.4NA lenses. Gels were expanded by washing with 0.001X PBS three times and by incubating overnight at RT in 0.001X PBS. Gels were immobilized in 50 mm glass bottom wells (WillCo Wells, HBSB-5040) by applying 2% agarose to the edges of the gels. Gels were then imaged in a fully expanded state in 0.001X PBS. A subset of images (Figs. 2KL, 3FG) were acquired on an Olympus SpinSR10 spinning disk confocal with a 25X silicone lens. Scale bars are not adjusted for expansion factor unless specifically stated.

4.11. Image processing

Czi files were imported into Fiji (v. 2.10/1.53c, NIH (Schindelin et al., 2012)), individual channel images were split and adjusted for brightness and contrast equivalently across conditions, then images were viewed with the volume viewer plugin (v. 2.01.2). If images showed considerable shift or too many neurons overlapped, a subset stack was created without the offending Z sections. Mode was set to max-projection and interpolation was set to tricubic smooth with z-aspect and sampling optimized per image (typically 0.5–2.0 for each parameter). Snapshots were taken in the XY plane at 1.0 and 2.0 scale in grayscale and 1D. The resulting snapshot images were imported into Photoshop (v. 21.2) and converted to 300 dpi. Any further brightness and contrast edits done in photoshop were minimal and applied equivalently to all comparable images in the figure.

4.12. Quantitative histological assessment of ExM protocols

To quantify the extent of organelle labeling and the brightness of reporter staining after expansion with the various ExM protocols tested in Figs. 2 and 3, we developed a scoring system similar to what was done by the Boyden lab (Yu et al., 2020, see Fig. 11). To measure the extent of COX4 staining (Fig. 2) for each of the conditions tested, a subset of cells were identified in the reporter channel and individual mitochondria in the COX4 channel were segmented using the nucleus counter plug-in in Fiji, similar to the analysis in Fig. 2E-I. The integrated density of the segmented mitochondria was measured for each cell analyzed. To assess GOLGA5 staining in Fig. 3, a similar subset of cells were identified and the integrated density was measured of the whole cell body ROIs. To measure the brightness of the reporter staining (GFP or RFP), the mean fluorescence was measured for the same subset of cells in the reporter channel. Then, a noise measurement was taken in both channels per condition by measuring the integrated density or mean fluorescence in an area without signal. The noise was subtracted from the values before they were normalized to the largest value in the set and multiplied by 100 to get a score between 1 and 100.

4.13. Analysis of expanded and unexpanded mitochondria

To determine if the proExM protocol affects our ability to resolve and quantify mitochondria, we analyzed the number and size of expanded mitochondria compared to unexpanded mitochondria. Tissue from an adult male EGFP reporter mouse was immunostained with COX4 to label mitochondria and expanded using our optimized ExM protocol with overnight digestion in proteinase K (see Expansion Microscopy methods). An adult male tdTomato reporter mouse was similarly processed and immunostained with COX4 but not expanded. Confocal images were taken of both samples for image analysis in Fiji. Note that the COX4 signal was imaged in different color channels for the expanded and unexpanded samples (546 nm vs 488 nm, respectively).

Confocal images were imported into Fiji and converted to 8-bit for the analysis. An intensity threshold was chosen separately for each image which best represented the signal in the original image. Using the reporter label as a guide, four cells were analyzed from the expanded sample and five cells from the unexpanded sample. Cells were chosen using the same criteria as for the digestion time course analysis. An ROI for each cell was drawn by fitting an oval to the cell body using the oval selection tool, rotating if necessary, and the signal outside of the cell’s ROI was removed for the analysis. The “nucleus counter” plug-in from the Fiji “ Cookbook” microscopy analysis collection was used to segment mitochondria within the ROI of each cell analyzed. The size threshold used for the expanded sample was adjusted for expansion factor, which was calculated using the ratio of the cell body diameters in the expanded images compared to the unexpanded images. The calculated expansion factor for the expanded images was 3x in the X and Y dimensions—an expansion factor of 9x in total area.

Once the mitochondria were properly segmented, Fiji’s “measure” feature was used to measure the number, area and intensity of each individual mitochondrial ROI. A custom Python code was written to calculate and plot the averages of the mitochondria number, size and total area for the expanded cells and the unexpanded cells. To account for the effect of expansion on size, the area of the expanded...
mitochondria were divided by expansion factor, which was calculated by getting the ratio of the average expanded cell soma area to the average unexpanded cell area. The mitochondrial coverage was calculated by dividing the total summed mitochondrial area in the cell by the area of the cytoplasm (soma area – nucleus area) and converting it to a percentage.

4.14. Analysis of expanded and unexpanded golgi

To compare the resolution of Golgi in the same cell before and after the optimized pro-ExM protocol, we did a plot profile analysis of the GOLGAS label in six cells imaged before and after ExM (Fig. 5GH). A line of the same length (150 pixels) was drawn through each cell, intentionally including areas without signal at either end and in the very center of the lines, for ease of measuring noise later. A plot profile was generated by Fiji for each line, which plots the intensity of every pixel along a line VS distance. The plot profile data was exported to Python, where the pre and post-ExM plot profiles for each cell were overlaid on the same plot for visual comparison. A signal to noise ratio was calculated for each plot profile. Noise was defined as the average of the pixel intensities of the first two, the middle two, and the last two pixels of the line. Golgi signal was defined as one standard deviation above the average intensity of the plot profile. The full width at max height of the tallest signal peak was also measured for each plot profile in pixels, and then converted to microns and corrected for expansion by normalizing the widths by the expansion factor. The expansion factor was calculated by dividing the length of the post-ExM plot profile line in microns to the pre-ExM plot profile line. The average signal to noise ratio and the full width at max height were then compared across the ExM and no ExM image to quantify the resolution increase seen after Pro-ExM.

4.15. Digestion time course experiment

To assess the effect of digestion time on tissue expansion and fluorescence retention, we ran a digestion time course experiment by varying the amount of time the ExM gels were in digestion solution—either 2 h, 4 h, 8 h or 16 h (overnight). The brains of two Amigo2-EGFP mice (one male and one female, 21–23 weeks old) were processed as above. During a pilot run, we noted that samples with digestion > 2 h lost the majority of EGFP fluorescence, making it impossible to acquire images with identical parameters for direct comparison. Therefore, to boost the EGFP signal, approximately ten sections per brain (two sections per condition per mouse) were first immunostained in a 24-well plate as described in the immunohistochemistry methods, with a primary chicken antibody against EGFP (Abcam, ab13970; 1:500 concentration) at RT for 3 days, followed by a secondary antibody (Invitrogen Alexa-488, A11039; 1:500 concentration) incubation for 48 h. As a control, a few sections were set aside after immunostaining to mount on slides without expanding.

Sections processed for ExM were anchored in Acryloyl-X overnight, washed twice with 1X PBS and incubated in gelation solution for 30 min at 4 C the following day. The gels were incubated in a humid environment at 37 C for 2 h to set, and then carefully removed from the chamber and placed in a digestion solution with 8 U/mL of proteinase K (New England Bio, Cat # P8107S) for the designated period of time. All gels in this experiment were digested on a shaker at room temperature. At the end of the digestion time, the digestion solution was replaced with 1X PBS several times to wash the gels. Gels were stored in 1X PBS in the dark at 4 C until imaging, at which point the PBS was replaced with nPH2O to fully expand the gels. Gels were imaged at 10X (EC-Plan-Neofluor lens; 10x; 0.3 NA; 5.2 mm WD) applying a z-step size of 10 µm on a Zeiss 710 confocal microscope with the same image acquisition parameters, as described in more detail under “image acquisition”.

The ExM images from the time course experiment were analyzed with the image processing program Fiji (v. 0.3.30, NIH). Before analysis, images were flattened across the Z dimension with the “Z Project” function to get an idea of how many GFP positive cells were present. This flattened image was only used for the selection of cells. Cells were excluded from analysis if they met any of the following criteria: (1) Incomplete cell (i.e. on the image border); (2) Any cell overlapping in Z with a cell already analyzed; (3) Cells with an ambiguous border or that were difficult to differentiate. Given these exclusion criteria, an average of 32 cells were analyzed for each time point across both animals. For some time points, cells were included from multiple hippocampal sections from the same animal.

For the fluorescence analysis, each cell body meeting the inclusion criteria was manually traced with the freehand selection tool in a single z section at the widest point of the cell. Once the cells were traced, the “measure” tool in Fiji was used to measure the mean intensity and the area within each individual cell ROI. To subtract background signal, one cell ROI was selected (at random) and moved to a location without EGFP signal and the background mean intensity was measured. The background mean intensity value was subtracted from the mean fluorescence intensity of each cell from the same image, resulting in a normalized mean fluorescence. The area of the cell body ROIs were used to calculate expansion factor by comparing it to the area of 37 unexpanded cell bodies, which was calculated to be 300 µm². To calculate the linear expansion factor for each cell, we used the below formula:

$$\text{Linear Expansion Factor} = \sqrt{\frac{\text{cell area}}{\text{avg non expanded cell area}}}$$

A total of 159 cells were included in the analyses (average of 32+/-.4 cells per time point from two brains). The mean fluorescence and expansion factor measurements were imported into Python (v 3.7, installed with the Anaconda distribution), where the average mean fluorescence and average linear expansion factor were calculated for each time point, including the unexpanded control (see Fig. 4). The mean fluorescence data was not normally distributed (Shapiro-Wilk test for normality; p < 0.0001), so the mean fluorescence data was log2 transformed and 11 cell means were removed as outliers, pre-defined as two standard deviations from the mean. These same outliers were also removed from the expansion factor data; however, because this data was normally distributed (Shapiro-Wilk: p = 0.235), it was not transformed. To correct for the effect of dilution on fluorescence, an area adjusted fluorescence was calculated by dividing the mean fluorescence (not log-transformed) by the average cell area for each condition, and this adjusted fluorescence was normalized to the 2-hour digestion condition (see Table 1). One-way ANOVAs were run using an ordinary least squares model with the StatsModels package (ols, ANOVA_lm, statsmodels v0.11.1) comparing fluorescence intensity and linear expansion factors across digestion time points. After overall significance was reached, pairwise post-hoc Tukey’s multiple comparison tests (pairwise_tukeyhsd, statsmodels v0.11.1) were performed to determine which digestion time points were significantly different (see Fig. 4D).

4.16. RMS analyses

Horizontal 40 µm EGFP+ sections were immunostained for GFP and GOLGAS as described above for the pre-IHC-ExM with 8 µm proteinase K digestion. The sections were transferred to a glass bottom plate under a drop of 1XPBS and imaged at 5x (0.16 NA) and 63x (1.4 NA). 63X images were taken at the anatomically identifiable CA1-C2 border. Sections were then washed in PBS and processed for ExM as described above. After gelation, resulting hydrogels were trimmed and the size of the tissue and gel were measured with a caliper. Tissue and gel sizes were also measured following digestion and after expansion in water. All caliper measurements were taken at the widest point of the section. Gels were post-stained with DAPI (1:5000 in water) for 30 min and transferred to glass-bottom plates. Gels were then washed in 0.001X PBS overnight. The next day, 10x tile scans of the hippocampus were acquired for each sample. Single 20x images at the CA1-C2 border were

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acquired to match the 63x pre-expansion images.

Images were imported into Fiji and processed to obtain matching ROIs from 63X pre-expansion and 20X post-expansion images. Using GFP labeling as landmarks, images were cropped to a square which included only overlapping regions in both pre- and post-expansion images (typically 50 × 50 µm in pre-expansion dimensions). Substacks were created from the cropped ROIs to include only the matching cells. Max-projected ROIs were converted to 16-bit grey-scale TIFF files. The pre-ExM image was then scaled in X-Y (with Fiji) to match the pixel dimensions of the post-ExM image, which is needed for proper image registration (Shamoun et al., 2013). The processed images were saved as RAW image files and the associated MHD metadata files were manually created for each image. The GOLGA5 channel was analyzed to get the RMS error. The post-ExM image (moving; Fig. 5H) was registered to the pre-ExM image (fixed; Fig. 5G) using Elastix (Klein et al., 2010), with a rigid and then a non-rigid registration as described previously (Chozinski et al., 2016; Chen et al., 2015). In the first step, a similarity registration was done to align the post-ExM image to the pre-ExM image without warping. The resulting image was then registered again to the pre-ExM image with the B-spline non-rigid registration. The results of the first rigid registration and the second B-spline registration were imported into the Wolfram analysis notebook provided by Chozinski et al. to generate a vector field filter of the distortion between the two images (Fig. 5I).

The transformix command was used in Elastix to apply the B-spline transform to a skeletonized image of the post-ExM image. To skeletonize the image, a median and gaussian filter were both applied and the image was binarized. With the output of transformix, the RMS error was then calculated for points along the skeletonized image in Wolfram. For plotting, length was binned per micron and cut off at 30 µm to match previously published RMS error data. The three biological replicates were averaged together (Fig. 5F).

4.17. Statistical analyses

Statistical analyses were done in python (v 3.7) with an alpha of 0.05 considered significant.

Declarations

Ethics approval and consent to participate

All animal use and procedures were approved by the Animal Care and Use Committee of Virginia Tech.

Availability of data and materials

Please contact the corresponding author for data requests.

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CRediT authorship contribution statement

Shannon Farris: Conceptualization. Logan A. Campbell, Katy E. Pannoni, Niesha A. Savory, Dinesh Lal, Shannon Farris: Methodology. Logan A. Campbell, Katy E. Pannoni, Niesha A. Savory, Shannon Farris: Formal analysis. Logan A. Campbell, Katy E. Pannoni, Niesha A. Savory, Dinesh Lal, Shannon Farris: Investigation. Logan A. Campbell, Katy E. Pannoni, Shannon Farris: Writing - original draft. Logan A. Campbell, Katy E. Pannoni, Shannon Farris: Writing - review & editing. Logan A. Campbell, Katy E. Pannoni, Shannon Farris: Visualization. Shannon Farris: Supervision. Shannon Farris: Funding acquisition.

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Conflict of interest

The authors declare that they have no competing interests.

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