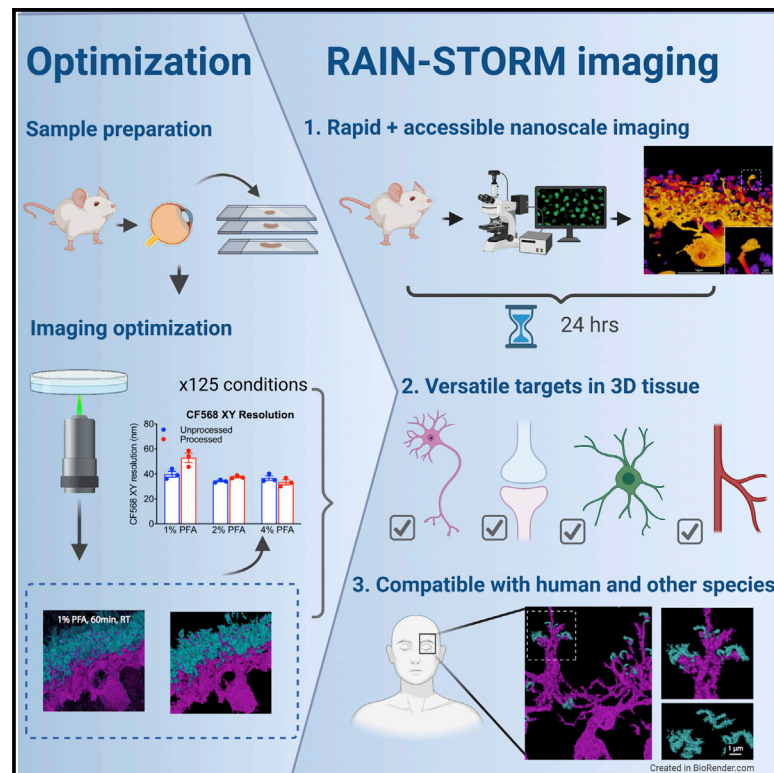


Rapid 3D-STORM imaging of diverse molecular targets in tissue

Graphical abstract



Authors

Nicholas E. Albrecht, Danye Jiang, Viktor Akhanov, Robert Hobson, Colenso M. Speer, Michael A. Robichaux, Melanie A. Samuel

Correspondence

msamuel@bcm.edu

In brief

Albrecht et al. develop RAIN-STORM, a nanoscopic imaging optimization method that improves the accessibility, throughput, and compatibility of current 3D nanoscopic tissue imaging. Moreover, the RAIN-STORM-optimized method is compatible with a wide range of tissue sources and a large number of molecular targets across a spectrum of biological structures.

Highlights

- Developed RAIN-STORM, a 3D nanoscopic imaging optimization approach
- RAIN-STORM is rapid, with a 1-day turnaround from sample to image
- RAIN-STORM is versatile, enabling 3D nanoscale imaging for numerous molecular targets
- Tissues from humans and other species reveal nanoscale neuron and synapse features



Article

Rapid 3D-STORM imaging of diverse molecular targets in tissue

Nicholas E. Albrecht,^{1,2} Danye Jiang,^{1,2} Viktor Akhanov,^{1,2} Robert Hobson,³ Colenso M. Speer,⁴ Michael A. Robichaux,⁵ and Melanie A. Samuel^{1,2,6,*}

¹Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030, USA

²Huffington Center on Aging, Baylor College of Medicine, Houston, TX 77030, USA

³Bruker Nano Surfaces Division, Salt Lake City, UT 84108, USA

⁴Department of Biology, University of Maryland, College Park, MD 20742, USA

⁵Departments of Ophthalmology and Biochemistry, West Virginia University, Morgantown, WV 26506, USA

⁶Lead contact

*Correspondence: msamuel@bcm.edu

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MOTIVATION The precise organization of fine-scale molecular architecture is critical for the nervous system and other biological functions and would benefit from improved nanoscopic imaging methods with enhanced accessibility, throughput, and native tissue compatibility. Here, we report RAIN-STORM, a rapid and scalable nanoscopic imaging optimization approach that improves three-dimensional nanoscale target visualization for multiple subcellular and intracellular targets within tissue at depth.

SUMMARY

Fine-scale molecular architecture is critical for nervous system and other biological functions. Methods to visualize these nanoscale structures would benefit from enhanced accessibility, throughput, and tissue compatibility. Here, we report RAIN-STORM, a rapid and scalable nanoscopic imaging optimization approach that improves three-dimensional visualization for subcellular targets in tissue at depth. RAIN-STORM uses conventional tissue samples and readily available reagents and is suitable for commercial instrumentation. To illustrate the efficacy of RAIN-STORM, we utilized the retina. We show that RAIN-STORM imaging is versatile and provide 3D nanoscopic data for over 20 synapse, neuron, glia, and vasculature targets. Sample preparation is also rapid, with a 1-day turnaround from tissue to image, and parameters are suitable for multiple tissue sources. Finally, we show that this method can be applied to clinical samples to reveal nanoscale features of human cells and synapses. RAIN-STORM thus paves the way for high-throughput studies of nanoscopic targets in tissue.

INTRODUCTION

The advent of single-molecule localization microscopy (SMLM) techniques has greatly increased the ability to resolve the location, density, and nanoscale spatial relationships of diverse molecules using fluorescence (Klein et al., 2014). Yet, large-scale adoption of these techniques for tissue analysis remains limited, in part because most SMLM approaches are challenging to apply to thick samples where aberrations and background fluorescence can limit imaging. Furthermore, relatively little side-by-side comparative data from which to build protocols for new applications or targets are available. As a result, most analyses of the molecular architecture within tissue continue to rely on immunofluorescence microscopy and fluorescent protein reporters. These powerful tools have shown that cellular and tissue functions intimately depend on small-scale arrangements of pro-

teins within subcellular compartments. However, the molecular composition and organization of many nanoscale biological structures are still unknown.

To help address this challenge, a number of bespoke SMLM solutions have been developed. These include in-house, specialized fluorescence microscopy and optical systems, customized analysis software, and advanced sample preparation techniques. For example, fixed fluorescently labeled cultured cells have been visualized in three-dimensional volumes acquired with a number of Z-positioning approaches, including astigmatism (Huang et al., 2008), biplane imaging (Juetter et al., 2008), engineered point spread functions (PSFs) (Pavani et al., 2009), and 4pi imaging (Bewersdorf et al., 2006). Fluorescent markers in tissue samples have been visualized in 3D using adaptive optics (Mlodzianoski et al., 2018), biplane imaging (Bewersdorf et al., 2006), self-interfering PSFs (Bon et al., 2018), light-sheet



approaches (Greiss et al., 2016), and ultrathin physical sample sectioning (Sigal et al., 2015). While these methods have greatly improved 3D SMLM capability in the field and led to numerous discoveries (Bowler et al., 2019; Chamma et al., 2016; Leterrier et al., 2015; Sigal et al., 2015; Suleiman et al., 2013; van den Dries et al., 2013), important challenges remain. The first is accessibility. Most 3D SMLM approaches rely on techniques, systems, and expertise that are available only to a handful of specialists. The second is simplicity. For instance, one useful approach is serial-section STORM (stochastic optical reconstruction microscopy), which requires ultrathin sectioning and sequential reconstruction (Sigal et al., 2015). However, the labor and imaging time for this technique make it best suited for deep interrogation of small sample numbers. The third is target compatibility within native tissue environments. Many current methods report imaging capabilities using only a small number of antibodies and lack a systematic comparison between relevant conditions for sample preparation and imaging (Chen et al., 2015; Ku et al., 2016; Tillberg et al., 2016; Mikhaylova et al., 2015; Monkemoller et al., 2015). As a result, only a few studies have used tissue samples for SMLM imaging (Baddeley et al., 2011; German et al., 2017; Nanguneri et al., 2012; Dani and Huang, 2010).

We set out to develop a nanoscopic imaging optimization method for central nervous system (CNS) tissue that would complement and combine the strengths of currently available methods for nanoscopic tissue imaging in general and for neural circuits specifically. Our criteria for this method were to improve the accessibility, throughput, and compatibility of 3D nanoscopic tissue imaging so that it can be readily applied to diverse tissue sources and multiple targets. Furthermore, we wished to understand how different conditions can be tuned to improve outcomes in a given biological specimen. Toward this goal, we present rapid imaging of tissues at the nanoscale (RAIN-STORM), an optimized method for the preparation and imaging of standard tissue samples to generate SMLM data at depth for a wide range of molecular targets using commercially available reagents and imaging systems. To achieve this goal, we first identified and then quantitatively tested 125 distinct tissue preparation, fixation, quenching, labeling, and imaging conditions. From these parameters, we identified a select set of 3D tissue imaging parameters based on resolution, antibody labeling density, and reduction of background fluorescence. We show that the resulting RAIN-STORM optimized method is rapid and versatile, enabling 3D nanoscopic sub-diffraction limited imaging with a 24-h turnaround. Furthermore, these imaging parameters are compatible with a wide range of tissue sources and a large number of molecular targets across a spectrum of biological structures. Finally, we show that the optimized set of parameters can be applied to clinically derived samples to study the nanoscale features of molecular targets in human tissue. RAIN-STORM optimization thus enables 3D nanoscopic imaging for a range of molecules, paving the way for high-throughput studies of nanoscale biological structures in intact tissue from diverse sources.

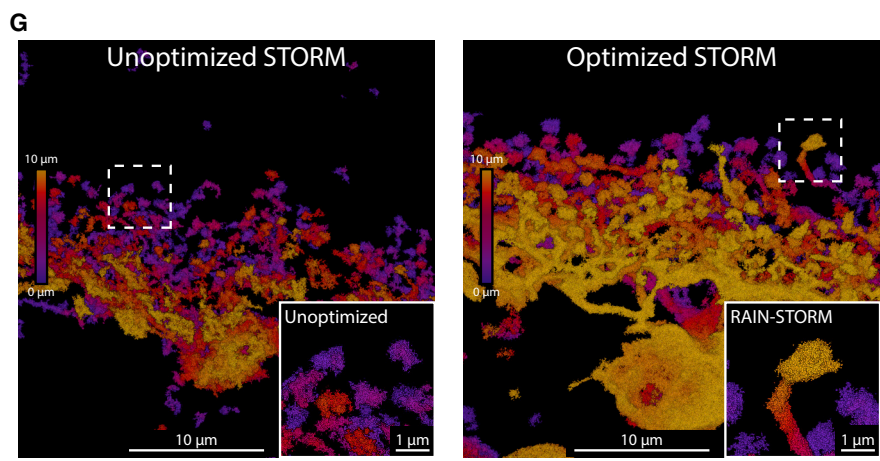
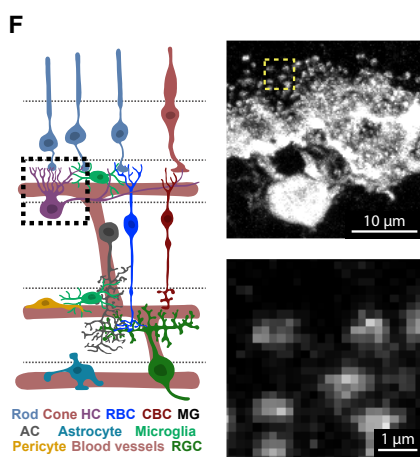
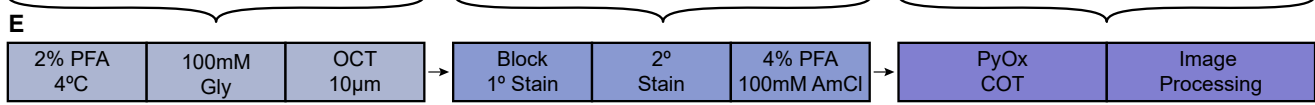
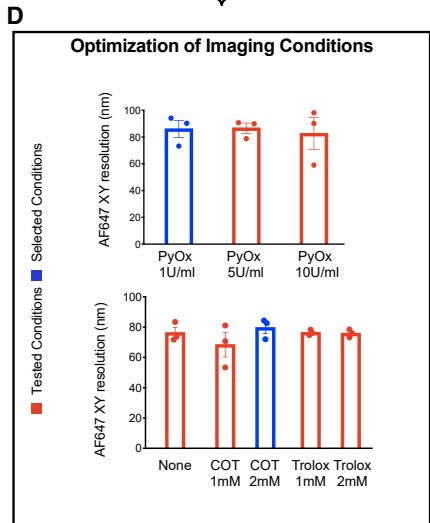
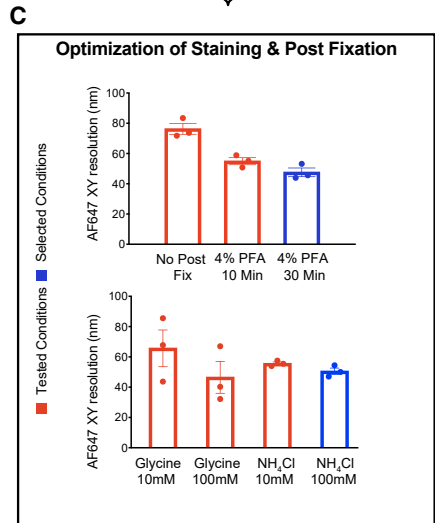
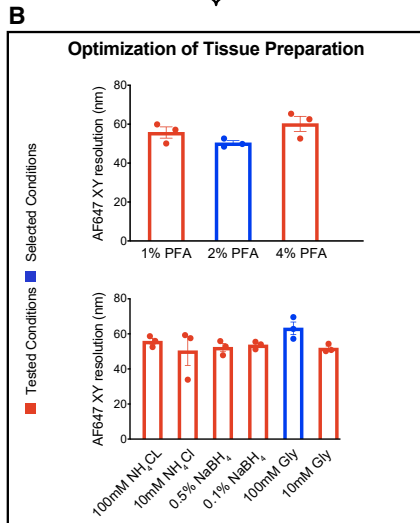
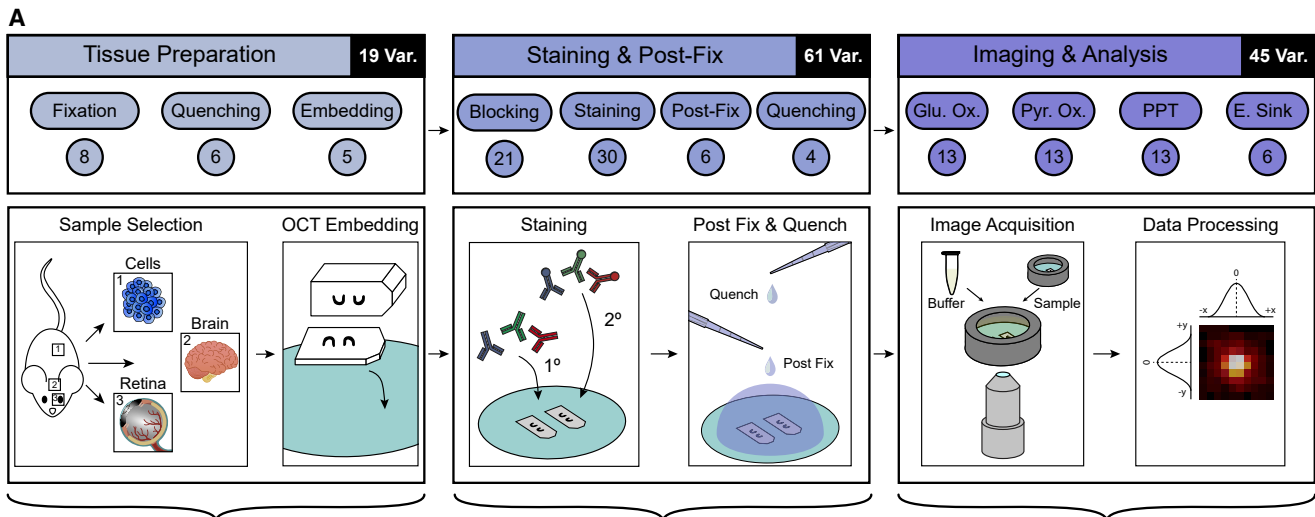
RESULTS

To improve the accessibility of STORM imaging we set out to develop an optimization method compatible with standard tissue

preparation and a turnkey SMLM imaging system. We chose to use mouse retina tissue because it has a highly defined laminar structure that provides endogenous fiducials for evaluating the labeling precision of multiple molecular targets (Sanes and Zipursky, 2010). For nanoscopic SMLM imaging, we utilized the Vutara 352 (Bruker, Billerica, MA). This system enables multi-color STORM imaging within a 40 × 40- μ m planar region of interest and achieves single-molecule imaging of individual emitters by recording the PSF in two imaging planes simultaneously (biplane imaging; Figure S1) rather than utilization of an astigmatic lens. Thus, the system is able to refine three-dimensional information more accurately through thick samples without objective-based distortion. Single-molecule localization in 3D is based on calibration data generated *a priori* from fiducial imaging.

To optimize our labeling and imaging parameters, we selected the calcium buffering protein calbindin (Calb1) and the synaptic protein PSD95 (postsynaptic density protein 95, also known as Dlg4). In the outer plexiform layer (OPL) of the mouse retina, calbindin specifically and densely fills the cell bodies and neurite terminals of retina horizontal neurons (Celio, 1990; Uesugi et al., 1992), whereas PSD95 is localized at the synaptic terminals in photoreceptor neurons (Hunt et al., 1996; Koulen et al., 1998). We reasoned that optimization with these targets would enable us to evaluate the efficacy of our method across two different cell structures and protein densities. Moreover, because calbindin and PSD95 are found in other CNS regions (Celio, 1990; Hunt et al., 1996), preparation methods compatible with these targets may extend to other neural tissues.

To begin RAIN-STORM optimization, we adapted a standard tissue preparation protocol for immunofluorescent labeling of mouse retina tissue that involves fixation, autofluorescence quenching, cryoprotection, embedding in freezing medium, tissue cryosectioning at 10 μ m, and mounting on coated glass slides (Albrecht et al., 2018). We selected this approach because these sample preparation techniques are widely available, do not require specialized reagents or labor-intensive handling, and result in staining ready slides within hours of tissue harvest. Within this framework, we identified eight tissue preparation, staining, and imaging steps where protocol variation could significantly alter the quality and degree of nanoscale labeling and background fluorescence. We reasoned that tuning these parameters from baseline parameters previously established for 3D-STORM imaging (e.g., tissue blocking with 5% NDS, 1% BSA, 0.1% saponin in PBS, tissue fixed at 4% paraformaldehyde [PFA] for 24 h, quenched with 0.1% NaBH₄ [German et al., 2017]) could markedly improve nanoscale image quality. Toward this goal, we identified a total of 125 test variations within these steps that included 19 primary fixation, primary quenching, and embedding conditions, 61 blocking, staining (Table S2), and secondary quenching conditions, and 45 imaging buffer variations (Figure 1A). We then systemically and quantitatively evaluated each variation for its effect on imaging outcomes while holding other parameters stable based on the following metrics: (1) the total number of localizations acquired, (2) epitope and morphological detail preservation, (3) the relative background fluorescence level (noise) observed as unclustered localizations or spurious antibody signal, and (4) the calculated resolution across



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planar (XY) and axial (XZ) image dimensions using Fourier ring correlation metrics for each channel of analysis (Nieuwenhuizen et al., 2013) (Figures 1B–1D; Table S2; Figure 2). Each of the 125 variations was tested independently in tissues from three individual animals resulting in a database of ~375 3D-STORM images from which quantitative metrics were evaluated. Processed reconstructions were generated with the OPTICS algorithm (ordering points to identify the clustering structure [Ankerst et al., 1999]) using a particle distance of 0.16 μm and a particle count per cluster of 25. For each condition, both processed and unprocessed images were evaluated. Representative images from these conditions are presented in Figure 2, while all quantitative metrics are presented in Table S2. The best performing condition within each parameter space was selected for inclusion in the final optimized protocol.

Final conditions for each step were selected through a combination of performance metrics. First, the resultant image and data had to display accurate antibody staining. Some tissue preparation conditions were noted to strongly distort staining patterns and, thus, no matter how well performing their other metrics (e.g., high resolution of poorly refined structures), they were not suitable conditions for biologically relevant imaging. Second, the number of localizations for each channel was assessed as either a gain or a loss from the basal test conditions. Gains in localizations were desirable both pre- and post-processing as these allow for better reconstructions of relevant biological structures and for more stringent post-processing if needed. Third, the number of localizations removed from the dataset was considered. Here, unclustered localizations removed during the processing steps were utilized to obtain an estimate for how much extraneous signal was introduced by a given condition compared with basal conditions.

The final metric we considered was improvements in the resolution for structures labeled with either of the STORM-compatible fluorophores used in this study, AF647 and CF568, at depth over the 10- μm tissue section thickness in the XY, XZ, and XZ dimensions. We found that resolution decreased relatively modestly as a function of the distance from the objective for all conditions due to the increased material thickness and resulting diffraction artifacts (Figure 3). For example, the XY resolution of 1- μm optical slices reconstructed at a Z-position near the objec-

tive following staining for calbindin in unoptimized conditions was 61.7 nm, whereas slices reconstructed in the middle (5 μm depth) and at the farthest point from the objective (10 μm depth) within the imaging volume had XY resolutions of 69.0 and 77.2 nm, respectively. We also found that axial resolutions at depth (R_{xz} , R_{yz}) were roughly equal to each other across preparation variations (Figure 3). To account for resolution differences across the imaging volume, we acquired image stacks encompassing the entire 10- μm tissue section depth for reconstruction and report the resolution for conditions discussed below using the average planar resolution computed from the entire depth of the image stack as a conservative estimate for the resolution of the whole 3D 10- μm sample volume (denoted R_{xy} 10 μm average). We now highlight a selection of the tested conditions and their effects on resolution and the other metrics to demonstrate their impact on imaging outcomes.

For tissue fixation, we tested PFA, glutaraldehyde (GA), or a combination of the two in conjunction with the quenching reagents NH_4Cl , glycine, or NaBH_4 . We found that combinations of fixatives that incorporated GA were poorly suited to high-fidelity imaging and generally resulted in a large increase in background localizations (Data S1; Table S2). For PFA-based fixation, we tested various PFA concentrations (1%, 2%, and 4%), durations (3, 60, and 120 min), and temperatures (25°C versus 4°C). From these tests, we found that autofluorescence could generally be lowered by reducing PFA concentrations and that a cold fixation (4°C) for 60 min using a 2% concentration of PFA yielded the best labeling density and imaging metrics (Table S2; Figure 2; Data S1). We also found that inclusion of a post-fixation step after antibody staining markedly improved resolution. For example, pre-clustering AF647 sample resolutions without post-fixation were typically 76.3 ± 3.6 nm but the inclusion of a 4% PFA post-fixation step for 30 min improved resolution by ~30 nm to 47.6 ± 2.9 nm (Table S2).

Antibody dilutions also significantly impacted image resolution and quality and, in some cases, gains in certain metrics came at a cost to other measures of image quality (Table S2). First, we found that targets benefitted from using increased primary antibody concentrations relative to standard histological preparations (e.g., anti-calbindin primary antibody is optimal at 1.0 $\mu\text{g}/\text{mL}$ in STORM compared with 0.1 $\mu\text{g}/\text{mL}$ in diffraction-limited

Figure 1. Nanoscale 3D imaging of neurons in tissue

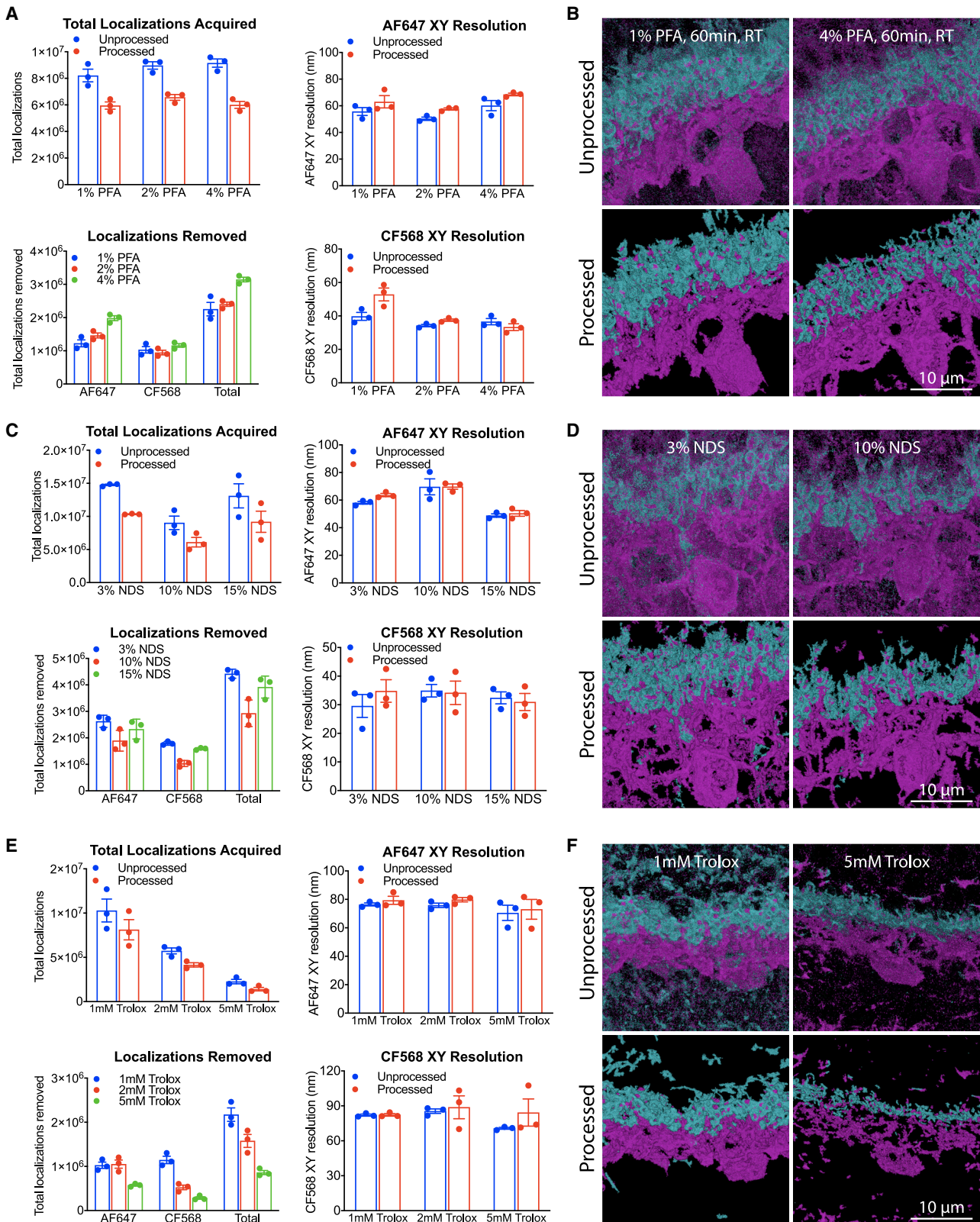
(A) Schematic of the RAIN-STORM optimization workflow. A total of 125 condition variations were tested consisting of 19 tissue preparation parameters (fixation, autofluorescence quenching, and embedding), 61 variations in staining and post-fixation (blocking buffer formulations, primary and secondary antibody staining, post-fixation conditions, and autofluorescence quenching), and 45 different conditions in imaging buffer formulations.

(B–D) Representative AF647 quantifications for tested parameters in one analysis plane for exemplar sample conditions in tissue preparation, staining, and imaging. Red bars indicate the tested conditions, and black bars denote the chosen condition from that set. For tissue preparation, 2% PFA and 100 mM glycine performed optimally (B), whereas for staining and post-fixation 4% PFA for 30 min followed by a quenching treatment of 100 mM NH_4Cl was ideal based on the resolution of the resulting imaging (C). Pyranose oxidase (1 U/mL of) and 2 mM of COT did not alter resolution but improved the number of localizations acquired (D). Data are represented as the mean \pm SEM. Please see Table S2 and Figure S4 for quantitative data from all tested parameters.

(E) The best performing parameters from each test set were selected for inclusion in the final RAIN-STORM optimized protocol.

(F) Retina schematic showing the location and structure of horizontal cells (HC, boxed region) relative to other retinal cell types (RBC, rod bipolar cell; CBC, cone bipolar cell; MG, Muller glia; AC, amacrine cell; RGC, retinal ganglion cell). In confocal imaging, horizontal cell synapses are poorly resolved following calbindin staining (inset).

(G) Unoptimized STORM image of a horizontal cell labeled with calbindin. Putative synapses are indistinct with little defining morphology or contiguous structure (unoptimized inset). In optimized RAIN-STORM conditions, both horizontal cell synapses and their connecting structures are well labeled and contiguous (optimized inset), demonstrating clear structural detail across the neuronal arbor. Images are representative from $N = 3$ animals. All confocal images are intensity-based representations of fluorescence, whereas STORM-based images show reconstructions formed from individual localizations. STORM images are color coded by depth (purple, 0 μm , to yellow, 10 μm). Scale bars, 10 and 1 μm .



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fluorescence microscopy). Second, we also found that increasing the concentration of secondary antibodies improved the overall image quality as it resulted in denser structural filling but that this could come at a cost to image resolution. For example, using higher secondary antibody concentrations resulted in somewhat poorer resolution for AF647-labeled calbindin (R_{xy} 10 μm average for 5.0 $\mu\text{g}/\text{mL}$ = 63.4 ± 3.3 nm, 0.5 $\mu\text{g}/\text{mL}$ = 44.0 ± 1.0 nm, and 0.1 $\mu\text{g}/\text{mL}$ = 38.4 ± 1.7 nm), but lower secondary concentrations reduced the amount of data collected, with commensurately less filling of the structures of interest (localizations_{xy} for 5.0 $\mu\text{g}/\text{mL}$ = $6.07 \times 10^6 \pm 0.490 \times 10^6$, 0.5 $\mu\text{g}/\text{mL}$ = $3.74 \times 10^6 \pm 0.239 \times 10^6$, and 0.1 $\mu\text{g}/\text{mL}$ = $1.19 \times 10^6 \pm 0.038 \times 10^6$). In this case, imaging results suggest that the increased localization and structural filling obtained with higher secondary antibody concentrations outweighed the resolution gains of lower concentrations. Of note, resolution metrics for CF568-labeled PSD95 structures did not vary as dramatically across secondary antibody concentrations (R_{xy} 10 μm average for 5.0 $\mu\text{g}/\text{mL}$ = 29.0 ± 1.1 nm, 0.5 $\mu\text{g}/\text{mL}$ = 35.5 ± 2.0 nm, and 0.1 $\mu\text{g}/\text{mL}$ = 29.8 ± 2.2 nm) despite a similar impact on localizations (localizations_{xy} for 5.0 $\mu\text{g}/\text{mL}$ = $6.30 \times 10^6 \pm 0.347 \times 10^6$, 0.5 $\mu\text{g}/\text{mL}$ = $3.43 \times 10^6 \pm 0.254 \times 10^6$, and 0.1 $\mu\text{g}/\text{mL}$ = $0.656 \times 10^6 \pm 0.079 \times 10^6$). From these variations, we thus selected 0.5 $\mu\text{g}/\text{mL}$ for general use during testing of other conditions to accurately assess each condition's impact on the sample and image quality but chose 5.0 $\mu\text{g}/\text{mL}$ as the desired concentration of secondary antibodies for the final protocol.

Finally, we undertook an examination of imaging buffer conditions and tested the effects of (1) oxygen-scavenging enzymes, (2) catalase concentration, (3) the ratio of thiols (β -mercaptoethanol [BME] versus ethanolamine [MEA]), and (4) triplet-state quenching (cyclooctatetraene [COT]). Since the imaging buffer is intended to promote blinking behavior and limit photobleaching of fluorophores, we reasoned that the demands of imaging complex, 3D biological tissue structures might differ from those of more commonly imaged cell cultures. Results from these tests are summarized in Table S2. We found that the use of pyranose oxidase was preferable to glucose oxidase as it enabled longer imaging with less observable photobleaching, and we empirically determined that the addition of 2 mM COT led to an increase in the total number of localizations for both AF647 and CF568 channels (Table S2; Figure 2). In addition, we found that the total acquired localizations for each channel were affected by the relative levels of BME and MEA in pyranose oxidase-based buffers. In the case of MEA, AF647 localizations were decreased with increasing concentrations (localizations_{xy} for

0 mM = $6.77 \times 10^6 \pm 0.499 \times 10^6$ versus 40 mM = $4.21 \times 10^6 \pm 0.437 \times 10^6$), yet resolution remained relatively constant (R_{xy} 10 μm average for 0 mM = 85.3 ± 1.4 nm versus 10 mM = 77.7 ± 2.6 nm) as long as at least some amount of MEA was present (R_{xy} 10 μm average for 40 mM = 74.9 ± 1.7 nm). This trend was similar for the CF568 channel, although fewer localizations were acquired (localizations for 0 mM = $2.07 \times 10^6 \pm 0.267 \times 10^6$, 10 mM = $1.29 \times 10^6 \pm 0.326 \times 10^6$, and 40 mM = $2.27 \times 10^6 \pm 0.221 \times 10^6$). From these data, we determined that MEA is necessary for AF647 resolution gains and that CF568 performs better when MEA is at higher concentrations. Therefore, we selected a concentration of 40 mM MEA as preferred both in terms of resolution and acquired localizations across both fluorophore channels. Using a similar method, the concentration of catalase and BME were tested to arrive at final optimized concentrations (140 mM BME and 100 U/mL catalase).

Following analysis of each reagent and their variations, the final sample preparation and imaging method were chosen based on the underlying quantitative metrics using the best combination and tradeoff between localizations acquired, resolution improvements to both imaging channels, overall image quality, and antibody fidelity. Our data highlight the importance of considering factors in addition to resolution, as it is possible to have highly resolved "noise" in poorly prepared tissue that yields images of little biological relevance. For example, with post-fixation quenching, a slight decrease in resolution was accepted as the necessary tradeoff for improving overall image quality and localizations acquired. Our final sample preparation workflow is comprised of readily accessible reagents that include a moderate initial fixation (2% PFA for 60 min at 4°C), primary quenching of autofluorescence (100 mM glycine for 60 min at 4°C), primary and secondary staining using an optimized serum-based buffer (5% NDS, 0.3% Triton X-100), post-fixation of secondary antibodies (4% PFA for 30 min at 4°C), and a second round of quenching (100 mM NH_4Cl for 30 min).

To illustrate the improvement achieved through RAIN-STORM optimization we reconstructed retinal horizontal cells in 3D. We found a marked increase in the resolution of nanoscopic horizontal cell features (Figure 1G). For example, in optimized RAIN-STORM imaging, we were able to observe specific and contiguous well-labeled neurites arising from the cell body, which terminated in a distinct postsynaptic invagination (Figure 1G; Videos S1 and S2). These synaptic terminals were easily resolved and structurally separated from their neighbors, demonstrating markedly improved spatial resolution (R_{xy} 10 μm average = 44.0 ± 1.0 nm

Figure 2. Modifying sample conditions improves visual quality and image metrics

(A and B) Representative primary data metrics (A) and corresponding unprocessed and processed images (B) are shown for variations in primary fixation concentrations. A small increase in total localizations acquired was observed with increasing concentrations of PFA, although the gain in localizations was offset by background localizations at these concentrations. Based on these parameters, 2% PFA was selected for optimized imaging.

(C and D) Representative primary data metrics (C) and corresponding unprocessed and processed images (D) are shown for variations in blocking buffer serum concentrations. In general, the best image metrics were obtained for mid-level serum concentrations (e.g., 3%–5% NDS), whereas the lower (e.g., 1% NDS) and higher (e.g., >10%) serum concentrations resulted in either increased filtered localizations or decreased resolution overall and poorer image quality.

(E and F) Representative primary data metrics (E) and corresponding unprocessed and processed images (F) are shown for variations in imaging buffer formulations using Trolox. Increasing the Trolox concentration reduced the total amount of data collected, resulting in poorer image quality relative to low Trolox concentrations (e.g., 1 mM). Images are representative of those acquired from $N = 3$ animals. Calbindin, magenta; PSD95, cyan. Scale bars, 10 μm . Data are represented as the mean \pm SEM. Both unprocessed (blue) and processed (red, OPTICS algorithm) values are shown for each dataset. For graphs of "localizations removed," color differences indicate a concentration change in a given reagent.

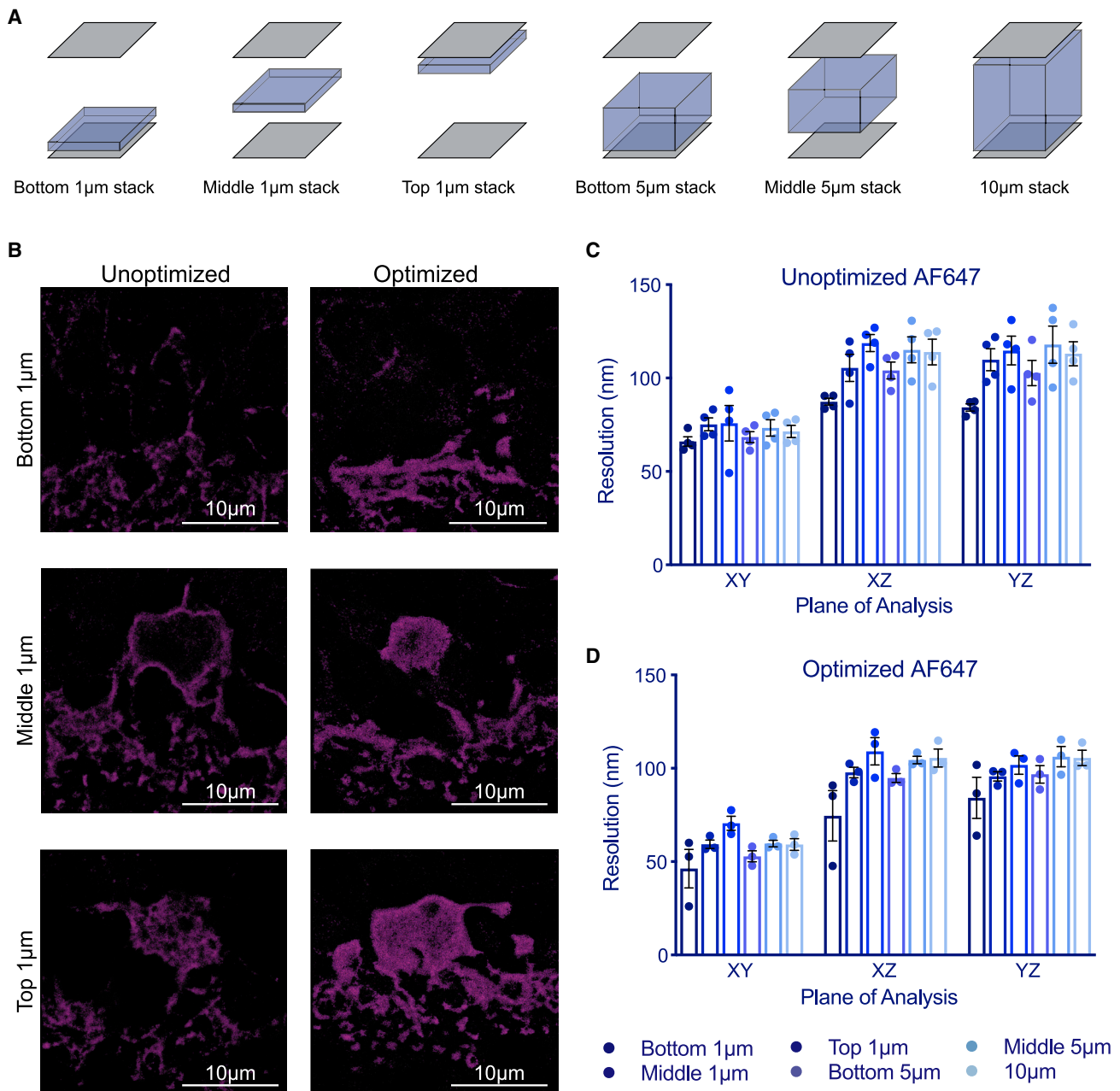


Figure 3. RAIN-STORM resolution as a function of sample depth

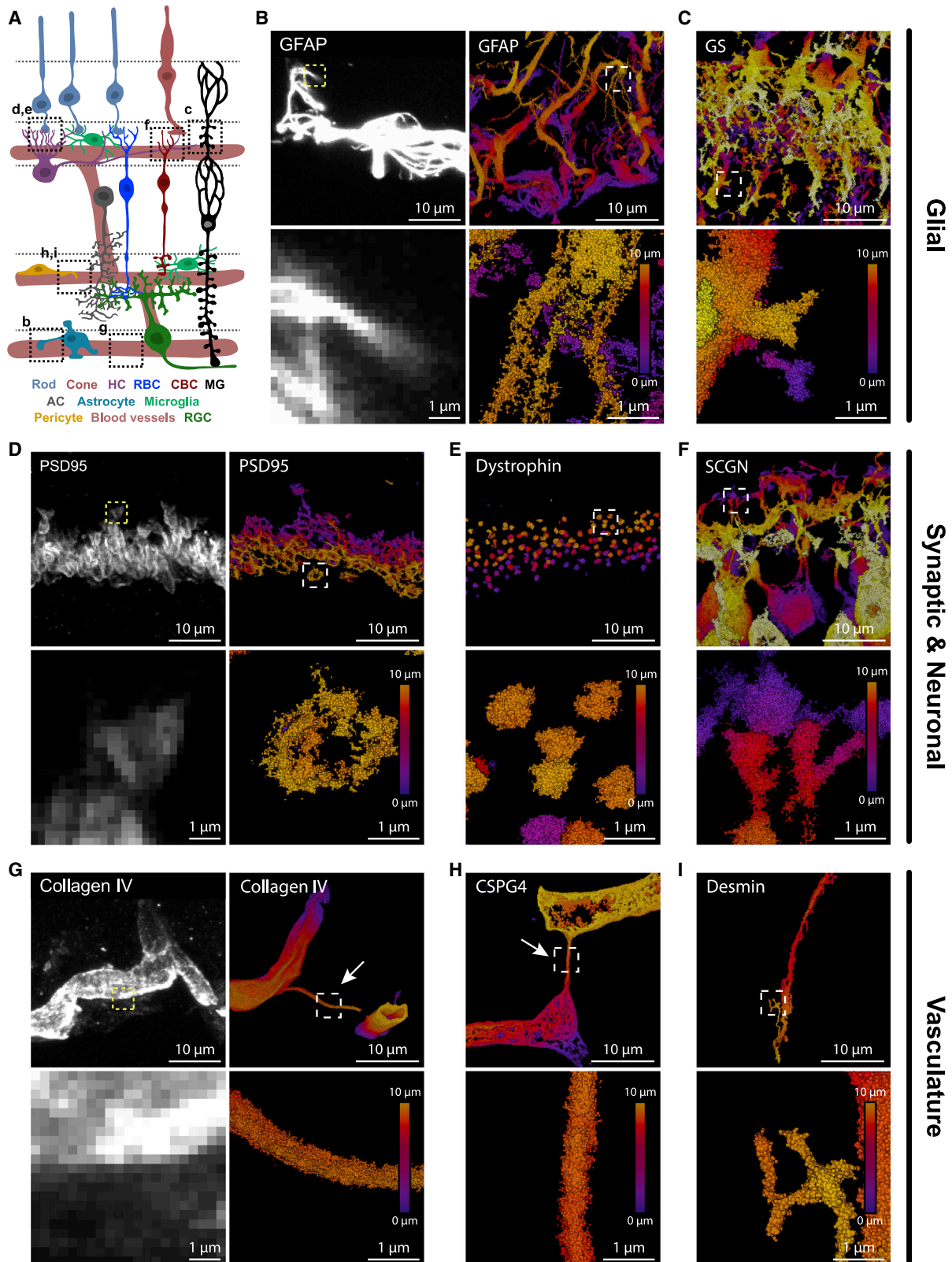
(A) Schematic of the image depths used to calculate sample resolution. The resolution was compared in: (1) single-micron slices at the bottom of the image (nearest the objective), the middle of the image stack, or the top of the image; (2) 5- μm slices from the bottom of the image to the midpoint; and (3) the entirety of the 10- μm stack.

(B) Example images of bottom, middle, and top single-micron slices in optimized and unoptimized RAIN-STORM conditions following staining with calbindin. Scale bars, 10 μm .

(C and D) Sample planar (XY dimension) and axial resolutions (XZ and YZ dimensions) in AF647 following staining for calbindin are measured in unoptimized RAIN-STORM conditions (C) and optimized conditions (D) across different imaging depths schematized in (A). Planar resolutions are improved in the optimized condition compared with the unoptimized condition across all imaging depths, whereas axial resolutions do not significantly differ. $N = 3$. Data are represented as the mean \pm SEM.

for calbindin labeled with AF647, compared with $\sim 70\text{--}90$ nm for unoptimized STORM conditions and $\sim 200\text{--}250$ nm resolution for excellent confocal microscopy). These data suggest RAIN-

STORM is well suited for 3D super-resolution imaging of molecular targets within tissue volumes. We also note that lower resolutions were achieved when focusing on a sub-volume of interest, with



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single plane XY resolution as low as 33.3 ± 1.1 and 21.7 ± 0.9 nm for AF647 and CF568, respectively.

RAIN-STORM optimized parameters also readily extend to a diverse array of molecular targets that span diverse structures and cell types and were robust in other commercial STORM imaging systems. To assess this, we took advantage of the wide spectrum of specific antibody markers available in the retina (Sanes and Zipursky, 2010) and tested the robustness of optimized methods across 21 validated molecular targets (Table S1; Figures 4, and S3). These included diverse cell structures, types, and molecular targets, such as synapse proteins (Bassoon, Bsn; RIBEYE, Ctbp2; Connexin 43, Gja1; dystrophin, Dmd; Piccolo, Pclo; PSD95, Dlg4), vasculature markers (CD31, Pcam1; collagen IV, Col4A1; desmin, Des; CSPG4, Cspg4), glial proteins (Iba1, Aif1; GFAP, Gfap; GS, Glul), excitatory interneuron markers (PKC α , Prkca; SCGN, Scgn), presynaptic photoreceptor targets (CAR, Arr3), and intracellular proteins and structures (Tau, Mapt; Tomm20, mitochondria) (Figures 4 and S3). We found that approximately 90% of the commercially available antibodies tested were compatible with optimized methods and could be used to successfully obtain SMLM images of individual targets at depth within intact tissue slices (Figure 4; Videos S2, S3, and S4). Furthermore, in all cases, labeling of the proper cell type or structure at the proper location was observed relative to the well-documented confocal staining patterns for these targets (Figures 4A and S3). In addition, our results resolve novel nanoscale structural features and molecular distributions. For example, we observed that astrocyte endfeet form fine (~100–200 nm) filamentous structures and what appears to be a mesh of fibers interdigitating the ganglion cell layer (Figure 4B; Video S4). Alongside these, we show that other glial cell types, such as Müller glia, display many fine protrusions thought to encapsulate nearby vessels and synapses (Figure 4C) (Bringmann et al., 2013; Vecino et al., 2016). To determine whether RAIN-STORM optimized conditions could be readily extended to other commercial STORM systems, we tested our method on the Nikon N-STORM system. We found that the RAIN-STORM imaging protocol was robust in this system and resolved nanoscale features of both synaptic and neural targets in mouse retina cryosections from both

2D- and 3D-STORM (astigmatism) acquisitions with good reconstruction metrics in both cases (Figure S4). Notably, the z-scanning procedure that we used for RAIN-STORM reconstructions improved the achievable z section depth in the N-STORM system by at least 10-fold over that using astigmatism from a single focal plane, a method previously reported to reconstruct a z section depth of $<1 \mu\text{m}$ (Robichaux et al., 2019) (Figure S4).

RAIN-STORM optimized imaging was also robust across a variety of synaptic and neuronal targets (Figures 4D–4F and S3). For example, with RAIN-STORM, we resolved the morphology of individual PSD95-labeled synapses and showed that the sub-synaptic space within rod terminals could be visualized (Figure 4D). We also resolved individual dystrophin puncta in the outer retina synapse layer, which are thought to interact with actin filaments to enable contact formation between photoreceptors and ON-bipolar cells (Schmitz and Drenckhahn, 1997) (Figure 4E). In parallel, we tested a range of neural markers, including the cone bipolar cell marker secretagogin (SCGN) (Puthussery et al., 2010) (Figure 4F). Here, the fine arbors and neurites of bipolar cells are seen as distinct and separate from their neighbors, enabling the study of how individual neurite tips may be organized with individual presynaptic terminals.

Finally, we expanded our target analysis to the retina vasculature. Using antibodies to CSPG4, a pericyte marker (Ivanova et al., 2021), and collagen IV, a structural component of the vasculature (Sarthy, 1993; Ishizaki et al., 1993), we observed vascular associated interpericyte tunneling nanotubes (IP-TNTs) (Figure 4G; Video S3). These ~500-nm diameter structures enable pericyte-driven neurovascular coupling (Alarcon-Martinez et al., 2020) but their molecular composition is largely unknown (Video S3). Our imaging results suggest that collagen IV may be a primary structural component of IP-TNTs (Figure 4H). We also stained for desmin, a filament protein that labels select pericytes and vascular-associated smooth muscle cells (Mendes-Jorge et al., 2012), and observed small filamentous structures arising from these cells (Figure 4I). These results suggest that RAIN-STORM optimization is effective for imaging many diverse targets and can be used to discover unknown nanoscopic cellular structures with high fidelity.

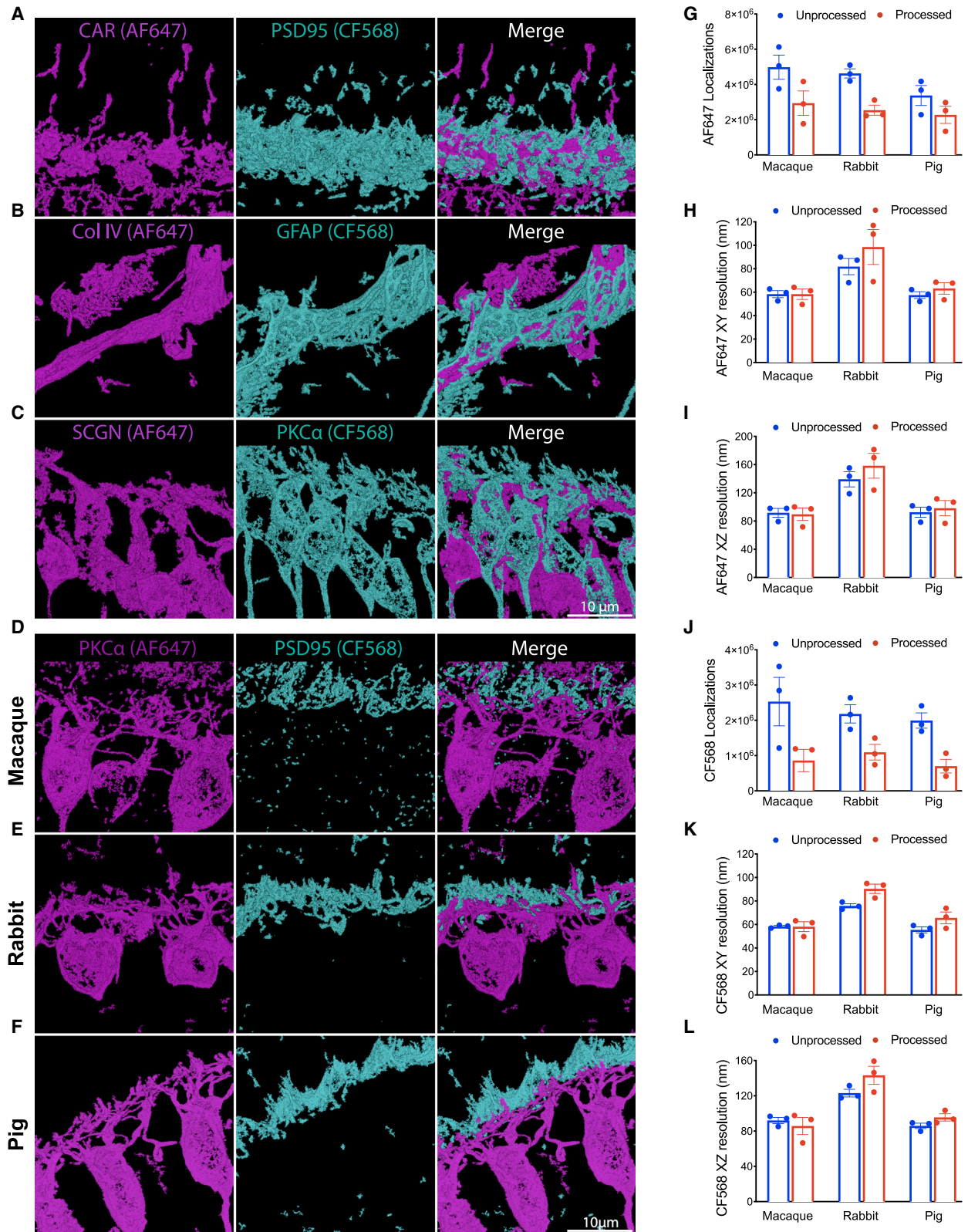
Figure 4. RAIN-STORM delivers robust imaging for a diverse array of molecular targets

(A) Retina schematic showing the diverse cell types and structural features of interest for this figure. Lettered inset boxes indicate the cell type or structure of interest displayed in the corresponding confocal and STORM images. RBC, rod bipolar cell; CBC, cone bipolar cell; MG, Muller glia; AC, amacrine cell; RGC, retinal ganglion cell; HC, horizontal cell.

(B and C) RAIN-STORM imaging of glial proteins. Using GFAP (B), a marker for astrocytes, RAIN-STORM (right column) offers markedly improved detail of nanoscale structures over confocal microscopy (left column). These include visualization of small filament-like protrusions from the astrocytes. RAIN-STORM imaging of glutamine synthetase (GS) (C), a marker for Müller glia, also shows the small protrusions arising from these cells.

(D–F) RAIN-STORM imaging of diverse synaptic and neuronal proteins. Following staining with PSD95 (D), a marker for photoreceptor synaptic terminals, individual synapse terminals can be observed more clearly using RAIN-STORM (right column) relative to confocal (left column). For example, the interior space of the magnified terminal is now visible. RAIN-STORM imaging of dystrophin (E), a synaptic structural protein in the outer plexiform layer shows that individual puncta for each synapse can be clearly observed and isolated. RAIN-STORM imaging of SCGN (F), a marker for excitatory cone bipolar cells, shows individual postsynaptic invaginating terminals.

(G–I) RAIN-STORM imaging of diverse vasculature associated proteins. Following staining with collagen IV (G), a marker for select blood vessels, clear structural detail can be observed in RAIN-STORM images (right column) relative to diffraction-limited confocal images (left column). These include defined vessel walls, interpericyte tunneling nanotubes (IP-TNTs, boxed region), and the hollow interior region of the microvasculature. RAIN-STORM imaging of CSPG4 (H), a marker for vascular pericytes, also shows IP-TNTs connecting two blood vessels. RAIN-STORM imaging of desmin (I) a filament protein that marks subsets of pericytes and vascular-associated smooth muscle cells shows fine protrusions arising from these cells (boxed region). Images are representative from N = 3 animals. All confocal images are intensity-based representations of fluorescence, whereas STORM-based images show reconstructions from individual localizations. STORM images are color-coded by depth (purple, 0 μm , to yellow, 10 μm). Scale bars, 10 and 1 μm .



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RAIN-STORM is also useful for multicolor imaging, is robust across multiple intracellular targets and genetic models, and is useful for tissue from diverse species (Figure 5). For multicolor imaging, we used our optimized method in multiple target combinations. These included a neuron subtype marker together with synaptic antibodies, staining for two distinct postsynaptic neuron types, and co-staining for vascular markers and astrocytes (Figures 5A–5C). Using these combinations, we were able to resolve contact sites, overlapping and non-overlapping cellular structures, and fine cellular interactions. For example, astrocytes and blood vessel co-staining revealed fine astrocytic filaments enshrouding neighboring vessels (Figure 5B). RAIN-STORM optimized imaging was also compatible with tissue from diverse species, revealing both conserved and unique nanoscopic features in mouse, rabbit, macaque, and pig tissue (Figures 5D–5L). For instance, co-staining for rod bipolar cell dendrites and the presynaptic terminals they invaginate showed that, despite similar functions and molecular identities, rod bipolar cells have incredibly diverse dendritic structures across species (Figures 5D–5F). Rabbit rod bipolar cell bodies were rounder and less elongated relative to pig, macaque, and mouse, with arbors that branched closer to the cell body. Notably, PSD95 labeling was confined to the outer layer of rod bipolar dendrites in all samples, suggesting that photoreceptor terminal staining is consistent across species.

We next used RAIN-STORM to visualize and quantify individual ribbon synapses in the retina OPL (Figure 6A). This region has two advantages. First, the architecture and composition of outer retina synapses have been well characterized, allowing comparisons between our method and metrics reported using other approaches. Second, because each photoreceptor forms connections at one distal location, the relationship between the structure of both pre- and postsynaptic neurons relative to their connectivity can be directly examined. To resolve both murine ribbon synapses and their postsynaptic partners, we applied our optimized method using antibodies against the synapse scaffolding protein RIBEYE (Moser et al., 2020) together with the postsynaptic bipolar marker PKC α (Figures 6A–6C) and used CF568- and AF647-conjugated secondary antibodies to serially visualize both targets. We reconstructed individual RIBEYE labeled ribbons and assessed their 2D-projected length and 2D-projected area. RIBEYE showed a rich variety of ribbon morphologies, including many that appeared in a horseshoe shape and others that appear flatter, consistent with the more elongated contacts of basally located rods (Li et al., 2016). Measurements taken of individual ribbons (n = 844) across four adult

mice showed an average ribbon length of $1.92 \pm 0.30 \mu\text{m}$ and a 2D projected area of $0.52 \pm 0.13 \mu\text{m}^2$ (Figures 6D–6F, Video S5). Notably, these values are comparable with those obtained using structured illumination microscopy (1.25 ± 0.05 to $1.95 \pm 0.07 \mu\text{m}$ in length) (Dembla et al., 2020), validating the specificity and accuracy of our optimized methods for measuring cellular structures. This comparison also demonstrates that our tissue preparation and imaging optimization do not distort the structures being measured.

Finally, we found that our RAIN-STORM optimized protocol is compatible with human tissue. Ribbon synapses are also present in the human outer retina (Moser et al., 2020), but their relative size and organization have not been well mapped. Eyes from two adult human donors (a 43-year-old female and a 58-year-old male) were processed for RAIN-STORM with similar parameters to those in mice and stained with antibodies against RIBEYE and PKC α . As above, images were acquired using sequential imaging for each of the two channels. Reconstruction and quantification of human RIBEYE-labeled synapses showed an average length of $1.81 \pm 0.03 \mu\text{m}$. Human ribbons also displayed a similar variety in shape and morphology as those found in mice (Figures 6G–6I, Video S6). Of note, we did observe fewer synapses in the human samples relative to the mouse, although this may be due to the inherent delay in human postmortem sample collection rather than a biologically relevant difference.

DISCUSSION

In this paper, we introduce RAIN-STORM, a rapid and scalable nanoscopic imaging optimization approach that enables 3D target visualization for multiple subcellular and intracellular fluorescent targets within the tissue at depth. We took advantage of the well organized but structurally complex retina circuit to demonstrate that RAIN-STORM optimization can resolve nanoscale features for a wide range of cell types and structures. This enabled us to validate known nanoscale structures as well as map novel cellular features of neurons, glia, and vasculature. In addition, we visualized and quantified hundreds of single human and mouse synapses across multiple individuals. The acquisition of this dataset was facilitated by the high-throughput nature of RAIN-STORM methods, and we show that this approach is practical for analyzing specimens from multiple samples and species. Finally, because RAIN-STORM was developed to be compatible with a commercial imaging system and standard tissue processing, this approach is open to a range of researchers, applications, and clinical samples.

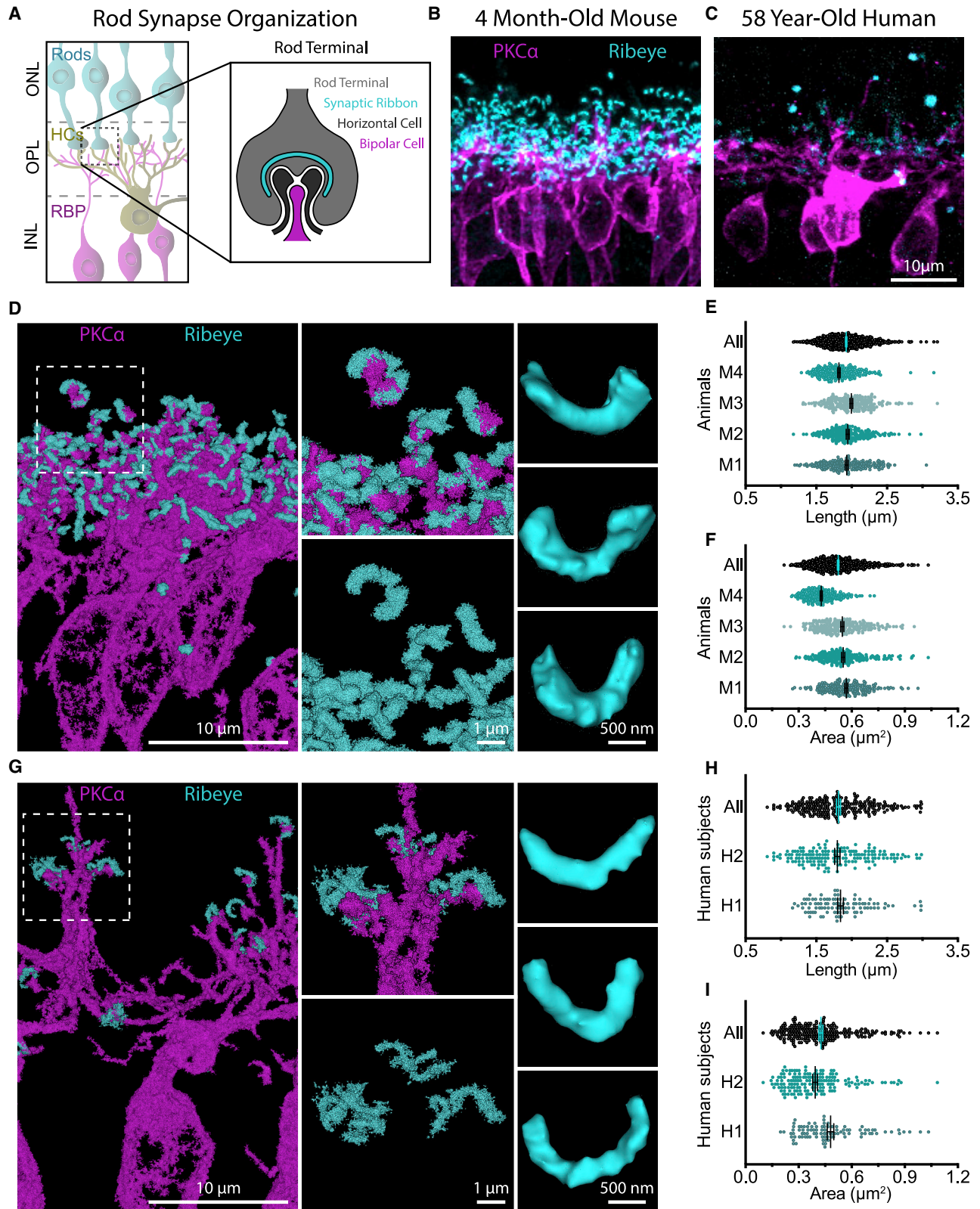
Figure 5. RAIN-STORM enables dual-channel super resolution imaging among diverse species

(A–C) RAIN-STORM imaging of diverse sets of two independent molecular targets, including (A) cones (CAR, magenta, AF647) and synaptic terminals (PSD95, cyan, CF568), (B) vasculature (collagen IVa, magenta, AF647) and astrocyte (GFAP, cyan, CF568) interactions, and (C) cone bipolar cells (SCGN, magenta, AF647) and rod bipolar cells (PKC α , cyan, CF568).

(D–F) RAIN-STORM imaging can be applied to tissue derived from diverse species. Retinas from (D) macaque (N = 2 animals), (E) rabbit (N = 2 animals), and (F) pig (N = 3 animals) were labeled with antibodies to rod bipolar cells (PKC α , magenta, AF647) and rod photoreceptor terminals (PSD95, cyan, CF568). Representative RAIN-STORM images from these species are shown.

(G–I) The number of AF647 localizations (G) and resolution for the XY (H) and XZ imaging planes (I) for PKC α AF647 images in (D–F). Pre- (blue bars) and post-processing (red bars) values for each dataset are shown.

(J–L) The number of AF647 localizations (J) and resolution for the XY (K) and XZ imaging planes (L) for PSD95 CF568 images in (D–F). Pre- (blue bars) and post-processing (red bars) values for each dataset are shown. These data show comparable localization and image resolution to that obtained using mouse tissue for these antibody combinations. Scale bars, 10 and 1 μm . Data are represented as the mean \pm SEM.



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How does RAIN-STORM optimization compare with other efforts aimed at 3D nanoscopic fluorescent imaging of CNS tissue? Dani et al. (2010), achieved 2 to 3 μm imaging depth in brain tissue sections using astigmatism and was able to localize a number of pre- and postsynaptic proteins with excellent resolution using photoswitchable dye pairs (14 nm in a single XY plane and 35 nm in the z direction). Similarly, approaches suitable for synapse imaging at 20–30 nm lateral and 50–70 nm axial resolution at a depth of 0.6–4 μm have also been reported (Herrmannsdorfer et al., 2017). However, the former approach used photoswitchable dye pairs, increasing the level of technical difficulty for new users. In addition, in both cases, the restricted imaging depth precluded examination of whole-cell structures we demonstrate here. Thus, RAIN-STORM methods are complementary to these approaches but offer the advantage of a large set of comparative quantitative data for different imaging parameters and a step-by-step guide to adopting user-friendly sample preparations to acquire nanoscopic data for a host of targets at depths not reported previously.

With these advances, optimized 3D tissue RAIN-STORM imaging has the capacity to be as routine as confocal imaging in many laboratories. This contrasts with many other 3D-STORM imaging approaches that, while excellent (Xu et al., 2015; Huang et al., 2008; Nehme et al., 2020; Punge et al., 2008), rely on custom-built microscopes and require optics expertise and resources that are unavailable to most researchers. Furthermore, unlike serial array tomography electron microscopy (Micheva and Smith, 2007), serial section SMLM (Nanguneri et al., 2012), or related approaches (Li et al., 2016), RAIN-STORM does not require ultrathin sectioning, successive section imaging, alignment, or reconstruction. This further simplifies the application of our method and enables comparative data to be generated quickly both for optimization and application. For instance, in our study of human and mouse outer retina synapses, each dataset consisted of hundreds of synapses and was acquired with a 24-h turnaround from sample collection to image-ready tissue, followed by approximately 8 h of imaging time per species. RAIN-STORM optimization is thus uniquely suitable for large sample numbers and the acquisition and quantitative analysis of extensive datasets.

While improvements in accessibility, speed, and target compatibility were major motivations for this work, several parameters were uncovered that could be useful in other nanoscopic imaging applications. Toward this end, we report quanti-

tative imaging metrics for all 125 tested parameters as a resource for the community and validated our approach using two independent commercial STORM systems. For some applications, we anticipate that the final optimized method we provide will be immediately useful, whereas for others additional tissue- or target-specific testing using the steps we outline may improve outcomes. Among the parameters we tested, some unexpected advantages of various conditions were uncovered. For example, including a post-fixation step after primary and secondary antibody staining markedly improved resolution by \sim 10–40 nm. We also found that the ratio of BME to MEA could be used to tune the relative frequency of localizations between the AF647 and CF568 imaging channels. This enabled us to arrive at concentrations that provide a balanced blinking behavior between both channels such that the acquired localizations in one channel are not heavily favored at a cost to the other. Taken together with our other results, these data demonstrate the importance of titrating concentrations for optimal image quality (Figure S2).

Results of this study also shed light on the importance of considering parameters in addition to resolution when selecting conditions for nanoscopic imaging. In short, resolution, although important, is not a sole indicator of performance for a given condition. It is possible to generate images with highly resolved noise that yield good resolution metrics but offer little biological insight. These artifacts could be the result of antibodies binding non-specifically within the tissue or autofluorescent signal, both of which can contribute to the signal observed during acquisition. To account for this, we prioritized the preservation of biological structures and tuning data acquisition and post-processing to remove small localization clusters (noise or “background”) that were not associated with major structures. Our effort was greatly aided by the well-mapped structure of the retina, which provided a backdrop against which we could judge the reliability of imaging parameters to select optimal conditions for inclusion in our final method.

RAIN-STORM optimization also offers opportunities for the discovery of novel nanoscopic structures or molecular components. We found that a diverse array of targets could be readily visualized across a range of cell types and subcellular structures, with \sim 90% of tested commercial antibodies showing good labeling and resolution. This broad target compatibility enabled us to examine novel aspects of neural, glial, and vasculature architecture. For example, we visualized IP-TNTs and showed that

Figure 6. RAIN-STORM resolves structural and molecular features of synapses

(A) Schematic of retina outer plexiform synapse organization. Rod photoreceptor terminals (cyan) are presynaptic to invaginating postsynaptic horizontal cells (gray) and bipolar cells (magenta).

(B and C) Diffraction-limited imaging of murine (B) and human (C) retina outer plexiform synapses. Presynaptic photoreceptor terminals are labeled with RIBEYE (cyan), whereas postsynaptic bipolar cells are labeled with PKC α (magenta).

(D) Dual-color RAIN-STORM imaging of murine rod bipolar cells (PKC α) and ribbon synapses (RIBEYE) allows individual quantification of ribbons and shows a rich variety of morphologies.

(E and F) Individual outer retina synapses (n = 844) were reconstructed from adult mice (N = 4), and the largest 2D projected length (E) ($1.92 \pm 0.30 \mu\text{m}$) and total area (F) ($0.52 \pm 0.13 \mu\text{m}^2$) were quantified for each synapse.

(G) RAIN-STORM imaging of human rod bipolar cells (PKC α , magenta) and ribbon synapses (RIBEYE, cyan) resolves interactions between pre- and postsynaptic neurons.

(H and I) Individual outer retina synapses (n = 263) were reconstructed from human adult donors aged 43–58 years (N = 2), and the largest 2D projected length (H) ($1.81 \pm 0.03 \mu\text{m}$) and total area (I) ($0.43 \pm 0.01 \mu\text{m}^2$) were quantified for each synapse. Scale bars, 10 μm , 1 μm , and 500 nm. Data are represented as the mean \pm SEM.

they appeared to contain a solid collagen IV core, which may provide stability to these small structures as they engage in neurovascular coupling (Alarcon-Martinez et al., 2020). In addition, we documented ~100–200-nm filaments arising from astrocytes in the ganglion cell layer. Various roles for retinal astrocytes have been described, but these data suggest that they may also physically interact with and perhaps modulate adult retinal neurons (Arizono et al., 2020; O’Sullivan et al., 2017; Tao and Zhang, 2016).

Finally, as with other SMLM methods, the relative density of a given protein and the specificity of available antibodies are key factors in determining good targets for RAIN-STORM optimization. Because single molecules are visualized with this approach, the labeling density of a given target can report on the location and area of individual substructures. In addition, the relative density of a given protein and the availability of high-quality antibodies can make some targets more useful than others. For example, PKC α densely and specifically labels rod bipolar cells and reliably reveals the entirety of the bipolar cellular architecture at the nanoscale, whereas Iba1, a microglia marker, appears less dense. Notably, RAIN-STORM performed consistently well across molecular targets and species, suggesting that these parameters and their variations should provide a good baseline for imaging additional targets and tissues not tested here.

In sum, RAIN-STORM enables precise and data-driven imaging resulting in improved 3D nanoscopic tissue visualization approaches that reveal important aspects of neural circuit organization. In addition, because the optimized RAIN-STORM methods presented here are readily compatible with a wide array of molecular targets and tissue sources, this protocol may prove useful in a number of other applications. These include studies of human clinical specimens, which will enable direct comparisons between human disease and animal models at the nanoscale.

Limitations of the study

In this study we focused on the retina for optimization, so ideal imaging conditions for other types of tissue may require additional parameter testing. However, the methodology we present can be used for protocol optimization for different applications.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
 - Lead contact
 - Materials availability
 - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
 - Coverslip preparation
 - Tissue preparation
 - Antibody staining
 - Imaging buffer
 - Imaging and image processing

- QUANTIFICATION AND STATISTICAL ANALYSIS
 - Image resolution quantification

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.crmeth.2022.100253>.

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AUTHOR CONTRIBUTIONS

M.A.S. and N.E.A. conceived and designed the experiments. N.E.A. and V.A. performed RAIN-STORM imaging and analysis. D.J. performed the confocal imaging and synapse quantifications. R.H. and C.M.S. helped develop and optimize the imaging protocol. M.A.R. performed N-STORM imaging and analyzed the data. N.E.A., D.J., and V.A. designed and made the figures. M.A.S., D.J., and N.E.A. wrote the paper with input from C.M.S. and M.A.R.

DECLARATION OF INTERESTS

R.H. is an employee of Bruker Nano Surfaces.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Calbindin D-28K	Swant	Cat# CB38a, RRID: AB_10000340
CD31	BD Biosciences	Cat# BDB5500274, RRID: AB_393571
Cone arrestin	EMD Millipore	Cat# AB15282, RRID: AB_11210270
Collagen IV	EMD Millipore	Cat# AB769, RRID: AB_92262
Connexin 43	Sigma-Aldrich	Cat#C6219, RRID: AB_476857
Desmin	Thermo Fisher Scientific	Cat# MA513259, RRID: AB_11000611
Dystrophin	Abcam	Cat# ab15277, RRID: AB_301813
Glutamine synthetase (GS)	BD Biosciences	Cat# 610517, RRID: AB_397879
GFAP	Sigma-Aldrich	Cat# G3893, RRID: AB_477010
Iba1	Abcam	Cat# ab5076, RRID: AB_2224402
Islet1	R&D System	Cat# AF1837, RRID: AB_2126324
NG2	Abcam	Cat# ab129051, RRID: AB_2877152
PKC α	Abcam	Cat# ab31, RRID: AB_303507
PSD95	Abcam	Cat# ab12093, RRID: AB_298846
RIBEYE	Synaptic Systems	Cat#192103, RRID: AB_2086775
Secretagogin (SCGN)	BioVendor	Cat# RD181120100, RRID: AB_2034060
Tau	Proteintech	Cat#66499-1-1g, RRID: AB_2881863
α -Tubulin	Sigma-Aldrich	Cat#T5168-.2ML, RRID: AB_477579
Tomm20	Abcam	Cat# ab78547, RRID: AB_2043078
Tyrosine hydroxylase (TH)	EMD Millipore	Cat# AB1542, RRID: AB_90755
Vesicular glutamate transporter 1 (VGlut1)	Abcam	Cat# ab77822, RRID: AB_2187677
Vesicular glutamate transporter 3 (VGlut3)	Millipore	Cat#AB5421, RRID: AB_2187832
Biological samples		
Macaque eyes	Baylor College of Medicine Center for Comparative Medicine veterinary	N/A
Rabbit eyes	Baylor College of Medicine Center for Comparative Medicine veterinary	N/A
Pig eyes	Baylor College of Medicine Center for Comparative Medicine veterinary	N/A
Human eyes	Lions Eye Bank of Texas	
Chemicals, peptides, and recombinant proteins		
Sodium hydroxide	Fisher Scientific	1310-73-2
Ethanol	Decon Labs	2716
Gelatin A	Sigma	G2500
Chromium (III) potassium sulfate	Sigma	243361
Glutaraldehyde	Electron Microscopy Sciences	16019
Paraformaldehyde	Electron Microscopy Sciences	15710
Ammonium chloride	Sigma	A9434
Glycine	Sigma	G8898
Sodium borohydride	Sigma	452882
OCT compound	Sakura	4583
Normal donkey serum	Jackson ImmunoResearch	017-000-121
Triton-X	EMD Millipore	TX1568-1
Saponin	Sigma	47036-50G-F

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pyranose oxidase	Sigma	P4234-250UN
Glucose oxidase	Sigma	G2133-50KU
Protocatechuate 3,4-dioxygenase	Sigma	P8279-25UN
Catalase	Sigma	C1345-10G
Cysteamine hydrochloride	Chem-Impex International	02839
2-mercaptoethanol	Sigma	M6250-250ML
Protocatechuic acid	Sigma	37580-25G-F
Cyclooctatetraene	Sigma	138924-1G
Trolox	Sigma	238813-1G
Experimental models: Organisms/strains		
C57BL/6J mice	Jackson Laboratory	000664
Software and algorithms		
Vutara Software	Bruker	https://www.bruker.com/en/products-and-solutions/fluorescence-microscopy/super-resolution-microscopes/srx-software.html
GraphPad Prism Version 8.4.3 (471)	GraphPad software	https://www.graphpad.com
Other		
#1.5H 22mm round glass coverslips	Neuvitro	GG-25-1.5H
Cell chamber for microscopy	Thermo Fisher Scientific	A7816

RESOURCE AVAILABILITY

Lead contact

Information and requests for resources and reagents can be directed to the lead contact, Melanie Samuel (msamuel@bcm.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the [Lead contact](#) upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [Lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

For mouse tissue, retinas were collected from four 6 to 8-week-old C57BL/6J animals. Experiments were carried out in male and female mice in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH under protocols approved by the BCM Institutional Animal Care and Use Committee. Macaque (N = 2), rabbit (N = 2), and pig (N = 3) retinal tissue were obtained via the Baylor College of Medicine Center for Comparative Medicine from unrelated surgical procedures. Human donor eyes (N = 2) were obtained in collaboration with the Lions Eye Bank of Texas at Baylor College of Medicine. Informed consent was acquired from all patients and/or participating family members in accordance with EBAA and FDA regulatory standards. Subjects were a 43-year-old female and a 58-year-old male and had no documented history of eye disease.

METHOD DETAILS

Coverslip preparation

All samples were mounted and prepared on #1.5H 22mm round glass coverslips. To prepare the coverslips, small batches were placed in beakers and submerged in 5M sodium hydroxide and sonicated for 30 min. Coverslips were subsequently rinsed with deionized water and sonicated for 30 min, three times, using fresh deionized water each time. Coverslips were then sonicated in serial dilutions of ethanol (70%, 90%, 100%) for 30 min each. Once the last wash was complete, slides were removed from the solution and allowed to dry. Coverslips were then dipped in a solution of 0.5% (m/v) Gelatin A and 7mM Chromium Potassium Sulfate in PBS.

Excess solution was allowed to drain prior to placing the coverslips upright to air dry. Coverslips were stored for up to one month at room temperature once fully dry.

Tissue preparation

Details for tissue preparation and staining methods are provided for the optimized RAIN-STORM protocol. Tested variations on this protocol are listed in [Table S2](#). Briefly, tested parameters were analyzed in parallel and considered as independent variables from one another. Tested variations for each parameter included both 25 and 4°C fixations, PFA concentration gradients, and fixation duration. Glutaraldehyde preparations were also tested alone (0.3% GA) or in combination with PFA (2% PFA, 0.3% GA) prepared in phosphate buffered saline (PBS). Quenching conditions included ammonium chloride (10mM, 100mM), glycine (10mM, 100mM) or sodium borohydride (0.1%, 0.5% w/v). For the final selected tissue preparation condition, mouse eyes were enucleated and placed in 2% PFA for 1 h at 4°C, and then subsequently rinsed in 100mM glycine solution for 1 h at 4°C. Samples were then washed in PBS for 30 min and stored in PBS until processed for the next steps.

Following fixation, eyecups were dissected, and the cornea and lens were removed from the optic cup. Samples were then allowed to fully equilibrate in 30% sucrose at 25°C until the tissue sank (~45–60 min). Tissue was serially washed by hand in Optimal Cutting Temperature (OCT) compound to remove excess sucrose and subsequently placed into molds filled with OCT compound (Sakura, Torrance, CA). Embedded tissue was then frozen by submersion into methyl butane chilled on dry ice. Human eyes were prepared using our final selected fixation condition of 2% PFA at 4°C but were fixed for 4 h given the increase in tissue thickness and size of the optic cup. All other conditions were identical to those detailed above. All frozen blocks were then stored at –80°C until ready for use. For analysis and quantification, tissue was sectioned at 10µm and mounted on prepared coverslips.

Antibody staining

For quantification and final images, slides were incubated in the optimized blocking solution (5% normal donkey serum and 0.3% Triton X-100 in PBS) for 1 h and then with primary antibodies diluted in blocking solution for a minimum of 12 h at 4°C. Other tested conditions include variations in concentrations of the blocking buffer components (serum, Triton-X100, or Saponin). Components of the blocking buffer were tested on an individual basis, keeping all other components of the buffer at baseline conditions (NDS 3%, Triton-X100 0.3%) and included: Triton-X100 (0.1%, 0.3%, 0.5%, 1.0%, 2.0%) or Saponin (0.1%, 0.3%, 0.5%, 1.0%, 2.0%), and Normal Donkey Serum (1%, 3%, 5%, 10%, 15%), made to the final concentration using PBS.

After blocking and staining, samples were washed with PBS three times for 20 min and then incubated with commercial dye-conjugated secondary antibodies diluted in the blocking solution being tested for 1 h at room temperature. Slides were next washed with PBS three times for 20 min prior to applying a 4% PFA solution as a postfix for 30 min. Slides were washed with PBS three times for 20 min each, 100mM NH₄Cl was applied for 30min, and slides were again washed with PBS three times for 20 min each. Samples were stored in PBS until imaging.

Imaging buffer

Imaging buffer for use in STORM was prepared using stock solutions of one of three oxygen scavenging enzymes: pyranose oxidase, glucose oxidase, or protocatechuate 3,4-dioxygenase. Each oxygen-scavenging enzyme was used to name the associated buffer formulation and any of its derived variations. For Nikon N-STORM experiments, 10 mM of sodium sulfate was used in place of oxygen-scavenging enzymes, based on a previous report ([Hartwich et al., 2018](#)). Tested imaging buffers also contained bovine-derived catalase, cysteamine hydrochloride, and 2-mercaptoethanol. When PCD was used, its substrate protocatechuic acid was also included.

One of the four components of a given buffer formulation was varied in concentration while the others remained constant. This testing of each component was carried out for a single type of enzyme (e.g., pyranose oxidase, glucose oxidase, or PCD), and repeated for each enzymatic type, yielding three different buffer types, each with four components tested in a cross-comparative manner. Once this was performed for each buffer-enzyme combination, the final selected buffer was tested with either cyclooctatetraene or Trolox in varying concentrations (1, 2, or 5mM) as an additive known to affect photostability of the fluorophores and blinking of the fluorophores ([Olivier et al., 2013](#); [Rasnik et al., 2006](#)). Stock solution aliquots were kept frozen at –80°C until just prior to use. Aliquots were thawed at room temperature and added to a freshly prepared solution of 30% (m/v) glucose in PBS. Once all components for a given buffer were added together to the base solution, the imaging buffer was allowed to equilibrate for 20 min and subsequently used.

Imaging and image processing

Image acquisition was performed on a Bruker Vutara 352 (Bruker, Billerica, MA) using a water objective (UPLSAPO60XW). Stained samples were mounted in a collared well, and 1mL of imaging buffer was added on top of the sample, submersing it in an open well configuration. All images for quantification purposes were acquired in the following standard manner unless otherwise stated: the sample was interrogated at 200nm axial steps using a framerate of 67 Hz for AF647 and 40Hz for CF568 across the full 10µm thickness of tissue. Thus, in a 10µm volume, 51 “steps” were imaged and 250 frames were acquired per step. The entire 51 steps were imaged three sequential times in this manner, yielding a total of 38,250 frames (51*250*3) for a single channel per sample. Between each cycle, a timed 600-s pause was included to allow for the imaging buffer to re-equilibrate and recover. 3D nanoscopic imaging volumes were thus 40µm by 40µm by 10µm (16,000 µm³).

For the 640, 561 and 405nm excitation lasers, laser powers of ~ 98 – 110 mW (6125 – 6875 W/cm²), ~ 90 – 100 mW (5625 – 6250 W/cm²), and ~ 2 – 6 mW (125 – 375 W/cm²), were measured at the sample using a $40\mu\text{m}$ -by- $40\mu\text{m}$ field of view. Data were then clustered to determine associated and contiguous structures within the image using the Ordering Points to Identify the Clustering Structure (OPTICS) algorithm (Ankerst et al., 1999) encoded with the Vutara software. To analyze images, a general particle distance of $0.16\ \mu\text{m}$ and a particle count per cluster of 25 was used for all channels on all images. The particle distance refers to the maximum allowed distance that a particle can be from another particle in order to be included in a given cluster while the particle count reflects the minimum required number of particles required to form a cluster. These general parameters were selected as the most inclusive (least stringent) for filtering out data with the goal of preventing the removal of “real” data from structures that are imaged. Of note, depending on the application, stricter clustering can be performed, but this may not be suitable for all biological targets of interest. Any localizations that failed to fall into clusters as determined by these parameters was removed from the image and considered unstructured data. Once filtering and clustering were complete, an FRC analysis was performed (Banterle et al., 2013; Saxton and Baumeister, 1982) to determine the global aggregate resolution of the sample in the XY and XZ dimensions for each target imaged using three repeat measurements for curve smoothing.

For Nikon N-STORM experiments, image acquisition was performed on an N-STORM 5.0 (Nikon Instruments Inc.) with a CFI SR HP Apo TIRF 100x oil objective (NA 1.49). $10\ \mu\text{m}$ retinal cryosections were prepared and immunostained as described above and then attached onto empty glass-bottom dishes (MatTek 35 mm dish, No. 1.5 coverslip). Sections were covered with 1 mL of imaging buffer and an additional No. 1.5 coverslip. For 2D-STORM on the N-STORM, acquisitions were performed at a single focal plane, while for 3D-STORM acquisitions, a cylindrical lens was added to the light path for astigmatism Z-position fitting based on a fluorescent bead calibration. For both modes, 40,000 frames were acquired from 561 to 647 nm laser channels sequentially at ~ 33 frames per second for a $40\ \mu\text{m}^2$ imaging area. STORM analysis details for these experiments are described in the legend for Figure S4.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image resolution quantification

Image resolution was measured using a Fourier Ring Correlation analysis within Vutara software. Statistical analysis was performed using the GraphPad Prism software. For image resolution quantifications, three images were used per condition tested, and three repeat measurements per image were taken for curve smoothing. Data is reported as the mean \pm SEM.

Cell Reports Methods, Volume 2

Supplemental information

**Rapid 3D-STORM imaging
of diverse molecular targets in tissue**

Nicholas E. Albrecht, Danye Jiang, Viktor Akhanov, Robert Hobson, Colenso M. Speer, Michael A. Robichaux, and Melanie A. Samuel

Figure S1. Optical Diagram of Vutara, Related to Figure 1.

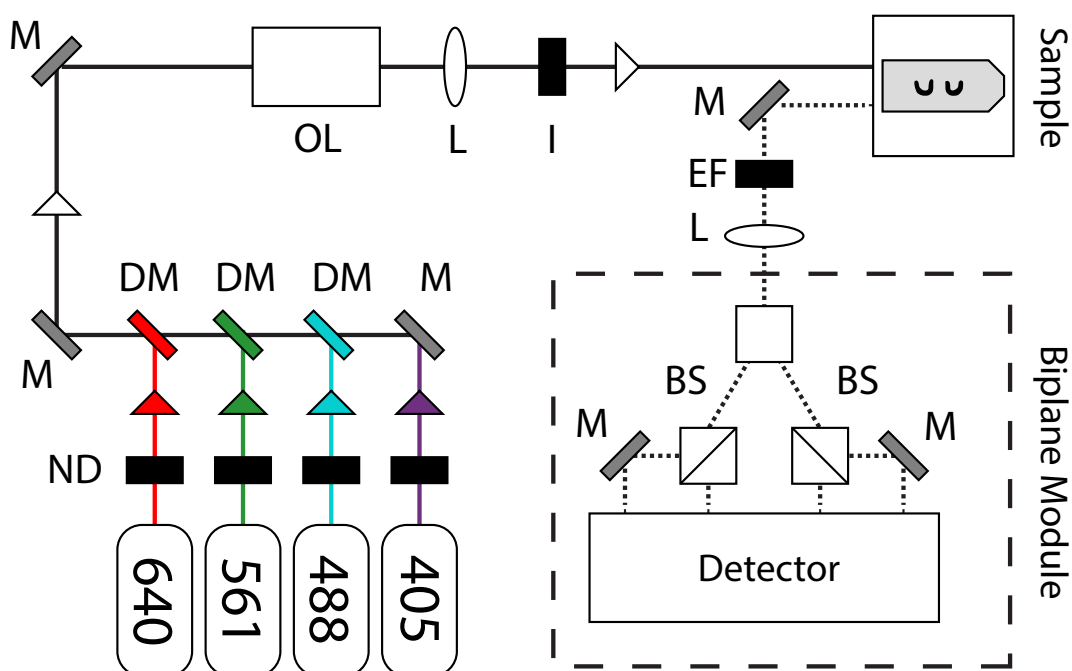


Figure S1. Optical Diagram of Vutara, related to Figure 1.

Layout and optical design of the Bruker Vutara SRX352, which allows 3D STORM imaging and PSF localization via a biplane module in place of a cylindrical lens. M: Mirror, DM: Dichroic mirror, ND: Neutral density filter, L: lens, BS: Beam splitter, I: iris/aperture, OL: Objective lens, EF: Emission filter.

Figure S2. RAIN-STORM resolution and localizations as a function of secondary labeling density, Related to Figure 1.

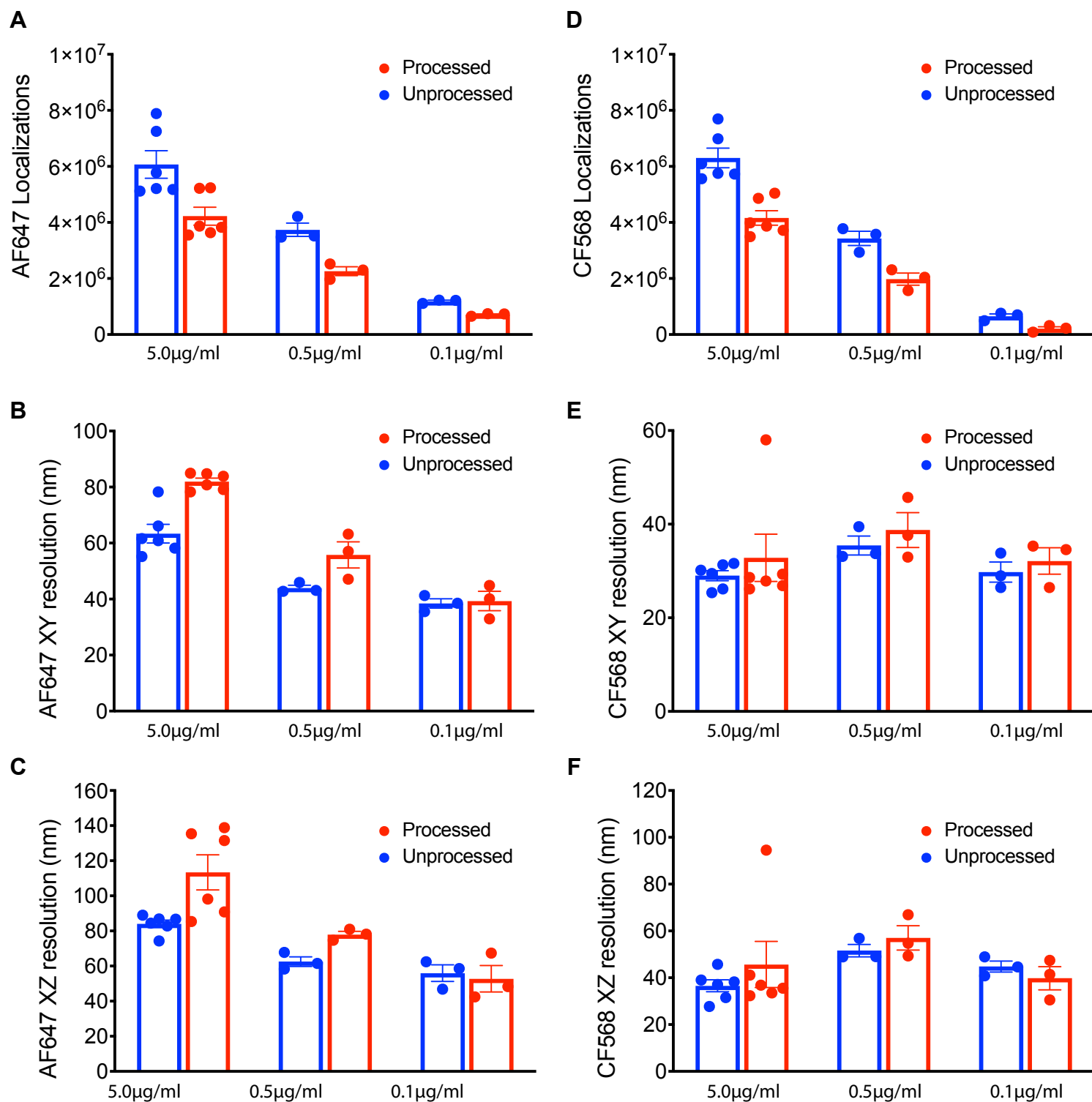
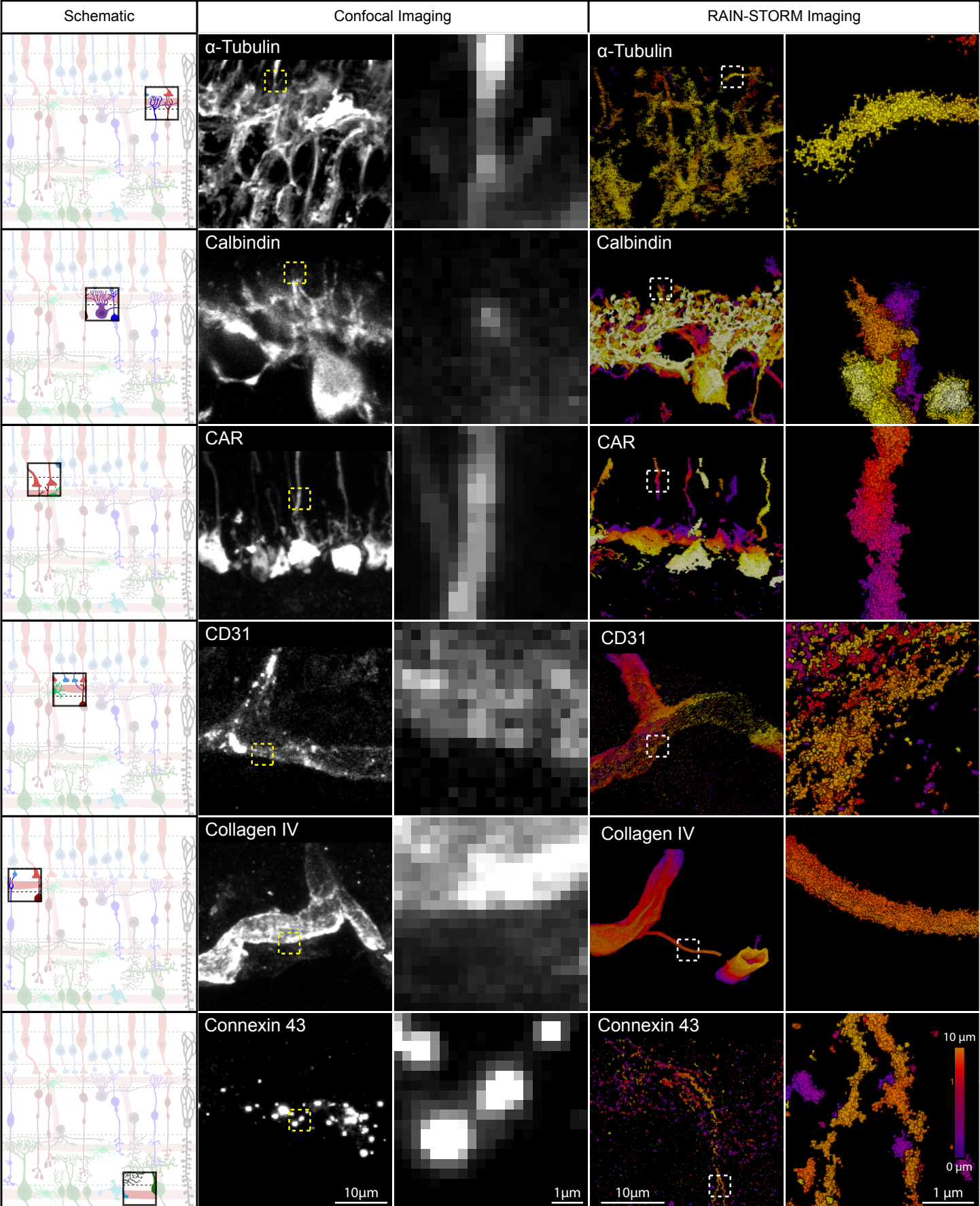
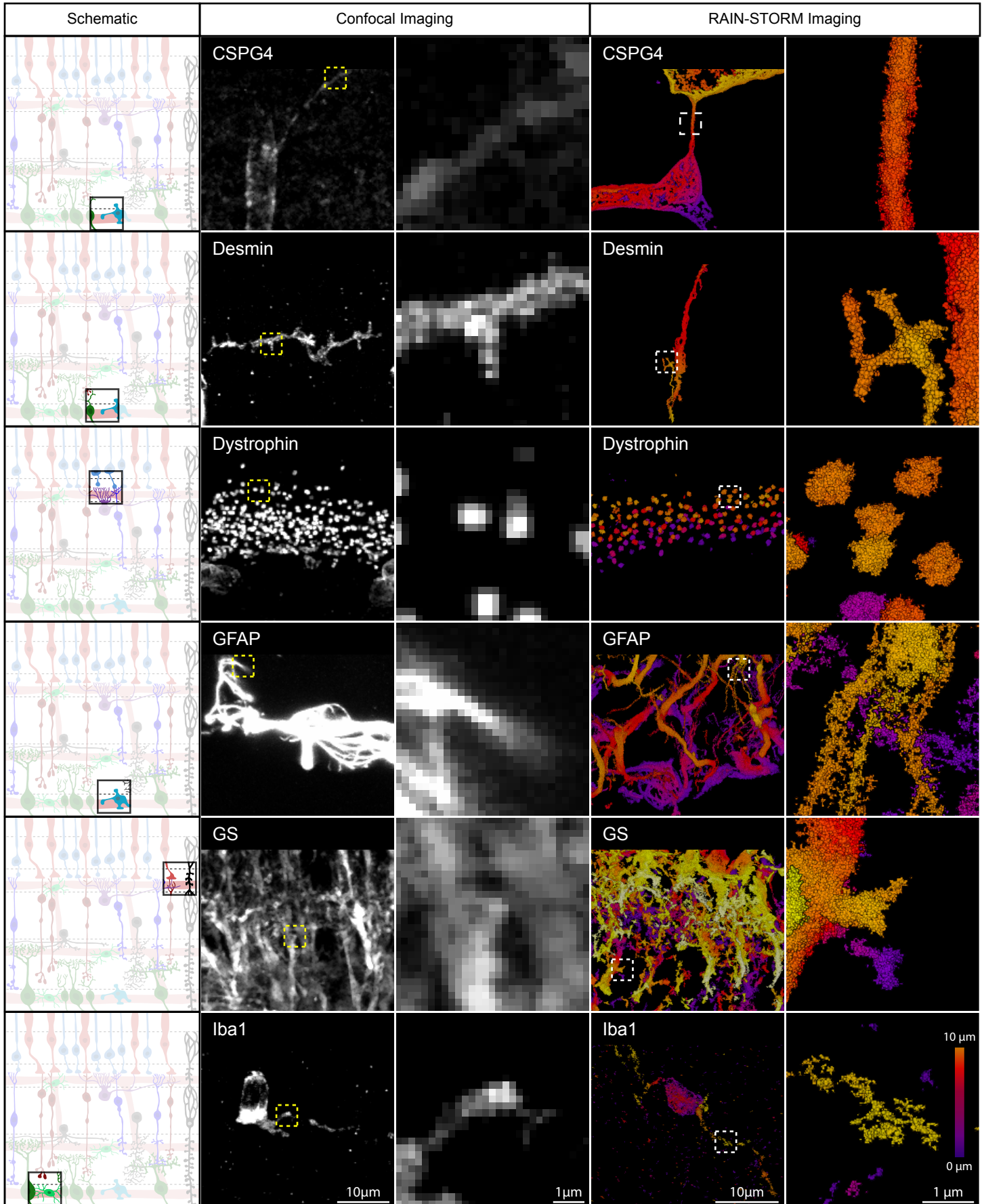


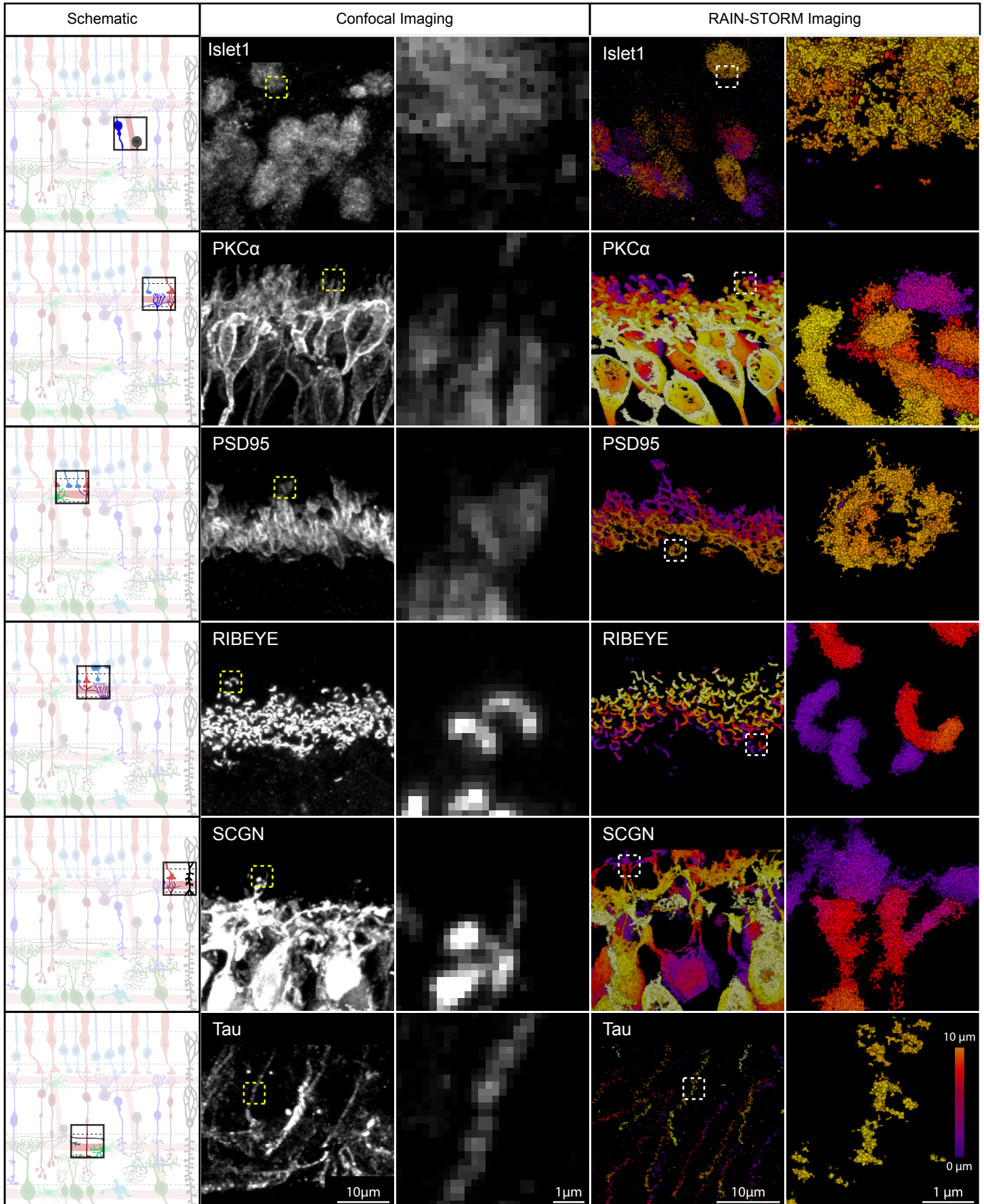
Figure S2. RAIN-STORM resolution and localization as a function of secondary labeling density, related to Figure 1.

A. Total localizations acquired when the concentration of the secondary antibody (AF647) is changed while the primary antibody concentration (Calbindin) is kept constant. Total acquired localizations decrease as secondary antibody concentration is reduced, showing $6.07 \times 10^6 \pm 0.490 \times 10^6$ localizations at $5.0 \mu\text{g/ml}$, $3.73 \times 10^6 \pm 0.239 \times 10^6$ at $0.5 \mu\text{g/ml}$, and $1.19 \times 10^6 \pm 0.038 \times 10^6$ at $0.1 \mu\text{g/ml}$. **B-C.** The associated XY (**B**) and XZ (**C**) resolutions as secondary antibody concentration is changed. At $5.0 \mu\text{g/ml}$, $R_{xy} = 63.4 \pm 3.3 \text{nm}$ and $R_{xz} = 84.0 \pm 2.1 \text{nm}$, at $0.5 \mu\text{g/ml}$, $R_{xy} = 44.0 \pm 1.0 \text{nm}$ and $R_{xz} = 62.6 \pm 2.8 \text{nm}$, while at $0.1 \mu\text{g/ml}$, $R_{xy} = 38.4 \pm 1.7 \text{nm}$ and $R_{xz} = 56.0 \pm 4.7 \text{nm}$. **D.** Total localizations acquired when the concentration of the secondary antibody (CF568) is changed while the primary antibody concentration (PSD95) is kept constant. As for AF647, total acquired localizations decrease as secondary antibody concentration is reduced showing $6.30 \times 10^6 \pm 0.347 \times 10^6$ localizations at $5.0 \mu\text{g/ml}$, $3.43 \times 10^6 \pm 0.254 \times 10^6$ at $0.5 \mu\text{g/ml}$, and $0.656 \times 10^6 \pm 0.079 \times 10^6$ at $0.1 \mu\text{g/ml}$. **E-F.** The associated XY (**E**) and XZ (**F**) resolutions as secondary antibody concentration is changed. At $5.0 \mu\text{g/ml}$, $R_{xy} = 29.0 \pm 1.1 \text{nm}$ and $R_{xz} = 36.6 \pm 2.6 \text{nm}$, at $0.5 \mu\text{g/ml}$, $R_{xy} = 35.5 \pm 2.0 \text{nm}$ and $R_{xz} = 51.6 \pm 2.6 \text{nm}$, while at $0.1 \mu\text{g/ml}$, $R_{xy} = 29.8 \pm 2.2 \text{nm}$ and $R_{xz} = 44.8 \pm 2.3 \text{nm}$. $N = 3$. Data are represented as the mean \pm the s.e.m.

Figure S3. Protein target validation and RAIN-STORM imaging of diverse molecular targets, Related to Figure 4.







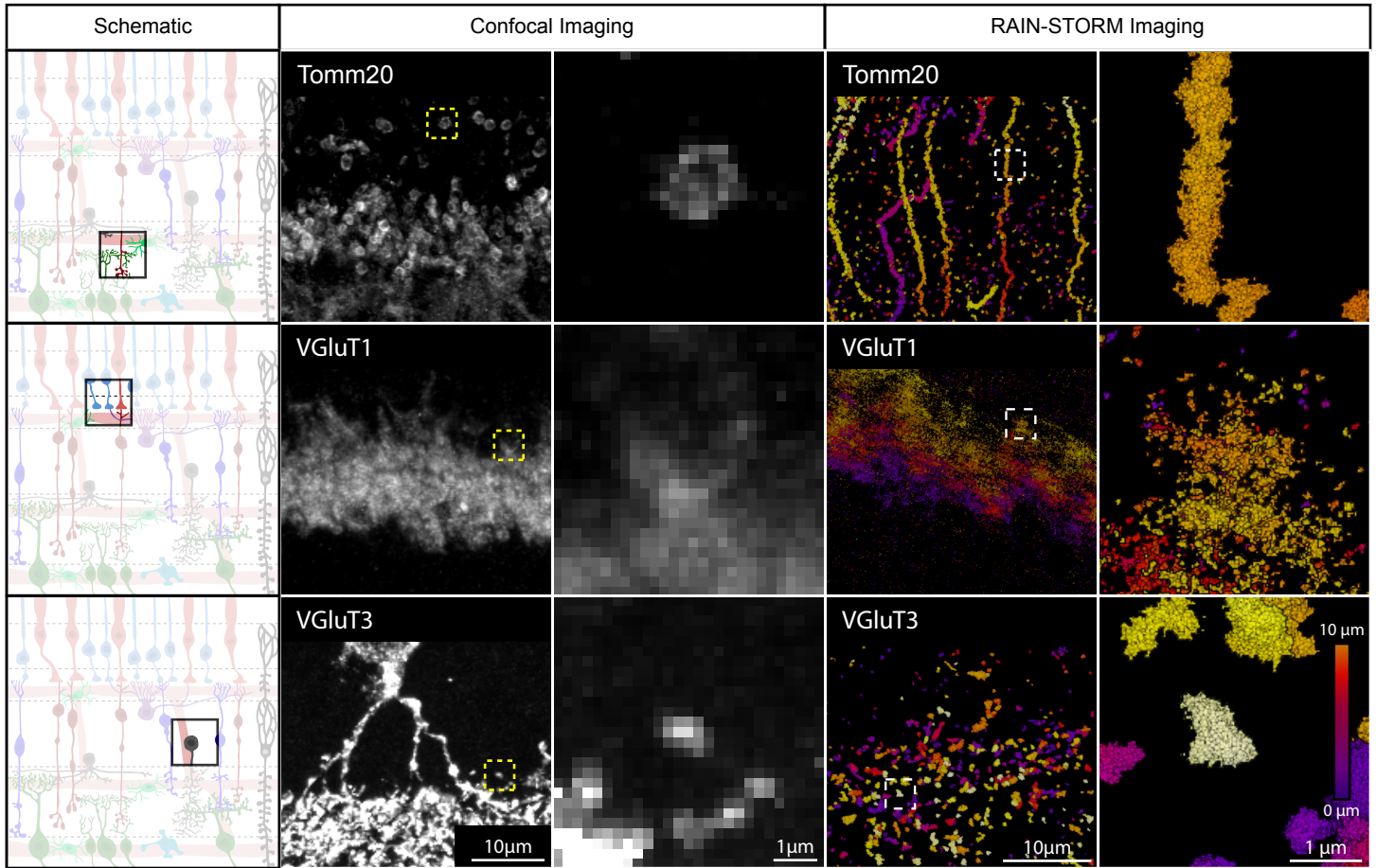


Figure S3. Protein target validation and RAIN-STORM imaging of diverse molecular targets, related to Figure 4.

A representative retina schematic for each target is shown in which the region of interest is boxed (left column). Cellular structure and labeling patterns from the fluorescence confocal based images were obtained (middle columns) and used as a baseline with which to compare the reliability and robustness of RAIN-STORM imaging for various targets. Boxed regions in confocal images are magnified in images to the right. For each target, tissue was then prepared using our optimized RAIN-STORM protocol (right most columns). Representative images for each antibody are shown, followed by a magnified view of the same general structure (boxed region) shown in the fluorescence image. RAIN-STORM optimized imaging was robust across these diverse targets and antibodies, demonstrating the combability of this protocol with a wide variety of proteins. Images are representative from N = 3 animals. All confocal images are intensity-based representations of fluorescence while STORM-based images show reconstructions formed from individual localizations. STORM images are color-coded by depth (purple, to yellow, 10 μ m). Scale bars = 10 and 1 μ m.

Figure S4. RAIN-STORM delivers robust imaging in the Nikon N-STORM system, Related to Figure 1.

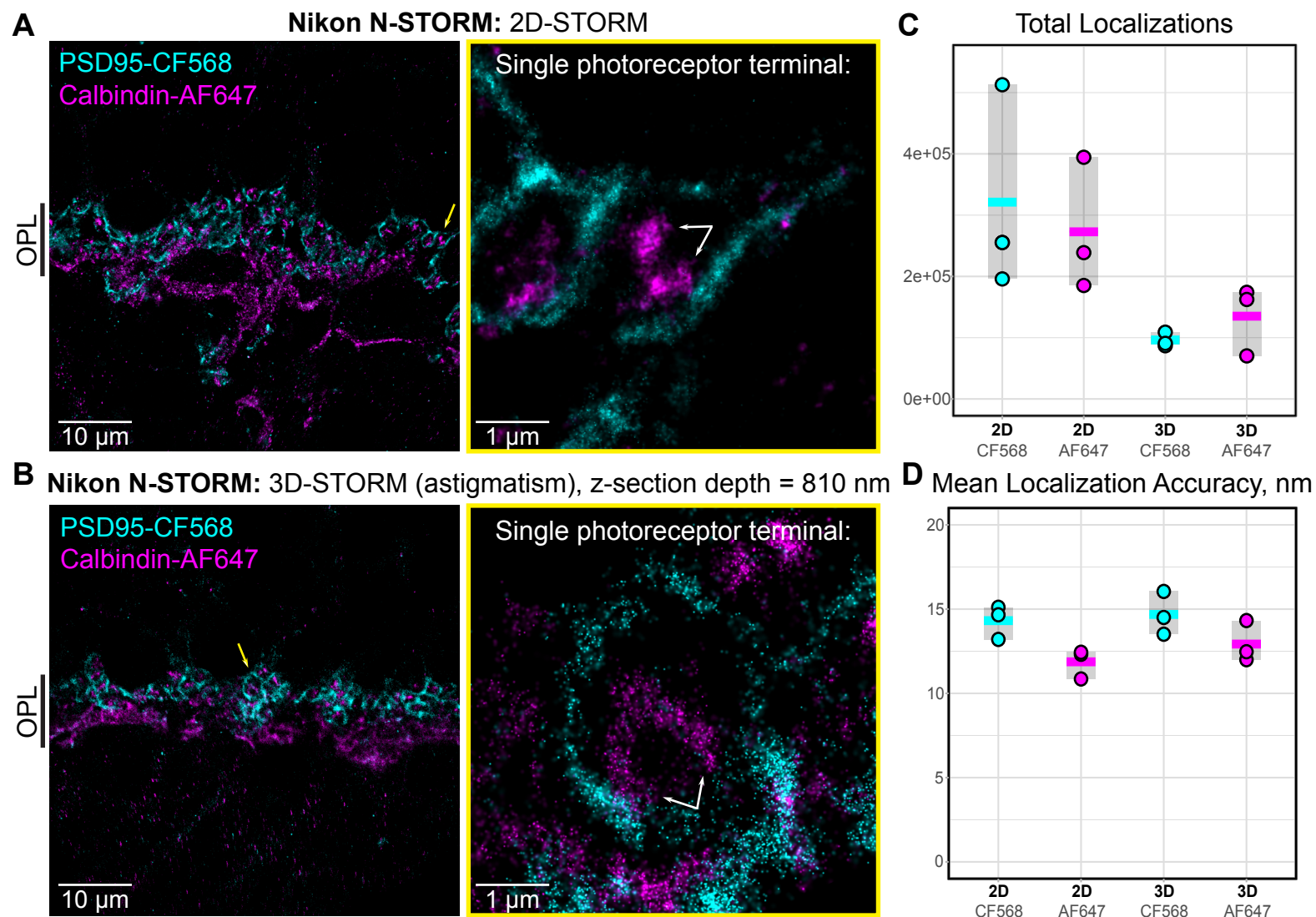


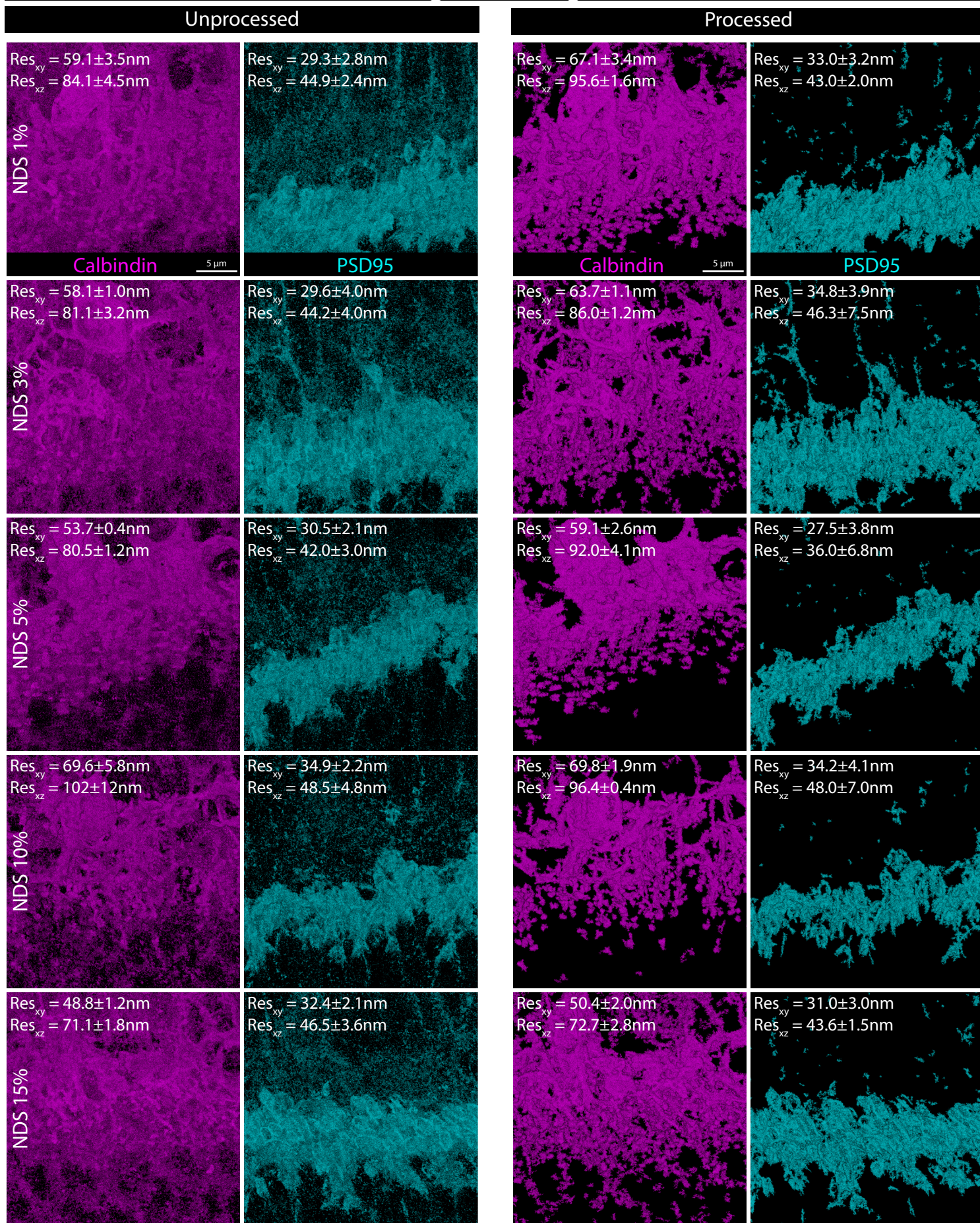
Figure S4: RAIN-STORM delivers robust imaging in the Nikon N-STORM system.

Mouse retina cryosections were prepared using our optimized RAIN-STORM protocol. Horizontal cells in the outer plexiform layer (OPL) were immunolabeled with Calbindin + AF647 secondary labeling (magenta), and pre-synaptic photoreceptor terminals (spherules and pedicles) were labeled with PSD-95 + CF568 secondary labeling (cyan). STORM acquisition and analyses were performed on a Nikon N-STORM system. **A.** A 2D-STORM example reconstruction is shown with an adjacent magnified example of an individual photoreceptor terminal (yellow arrow) containing distinct Calbindin+ horizontal cell processes (white arrows). Analysis settings for 2D-STORM data were based on (Robichaux et al., 2019) and were as follows: Minimum PSF height: 1,000, Maximum PSF height: 65,636, Minimum PSF Width: 200 nm, Maximum PSF Width: 400 nm, Initial Fit Width: 300 nm, Max Axial Ratio: 1.15, Max Displacement: 1 pixel. **B.** A 3D-STORM example reconstruction after Z-position astigmatism fitting is shown with another example magnified photoreceptor terminal (yellow arrow) encircling horizontal cell processes (white arrows). More permissive 3D-STORM analysis settings were used to enable astigmatism fitting: Minimum PSF height: 1,000, Maximum PSF height: 65,000, Minimum PSF Width: 200 nm, Maximum PSF Width: 700 nm, Initial Fit Width: 300 nm, Max Axial Ratio: 2.5, Max Displacement: 1 pixel. The z-sectioning depth of 3D-STORM reconstructions was 810 nm. All data points in the STORM reconstructions were visualized as “Gaussians” based on individual brightness and localization accuracy values. **C.** Total localizations and **D.** mean localization accuracy values are plotted as circles for both channels from 3 replicate 2D- and 3D-STORM acquisitions demonstrating reproducibility and high-quality fitting. Gray bars indicate the range, and

horizontal lines indicate the mean values. Graphs were generated using PlotsOfData (Postma and Goedhart, 2019).

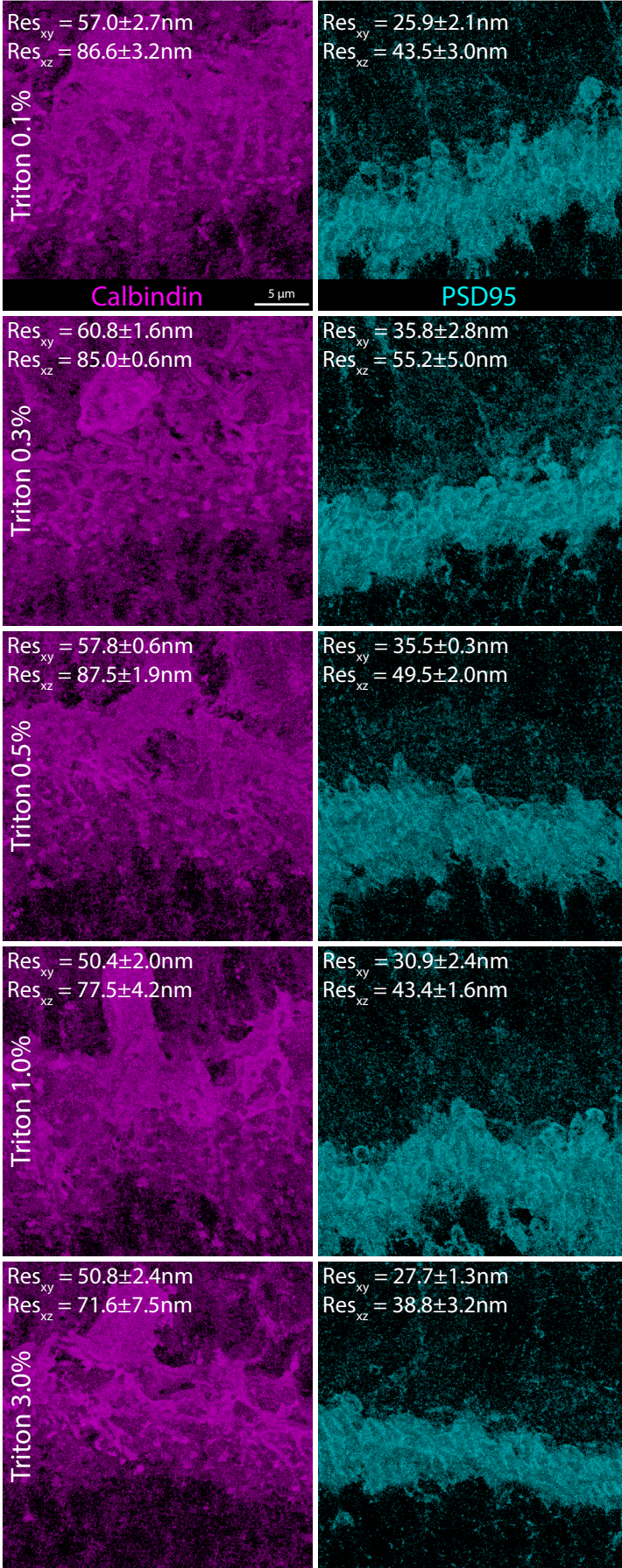
Data S1. Sample and imaging conditions affect image quality and metrics, Related to Figure 4.

Staining - Blocking, NDS

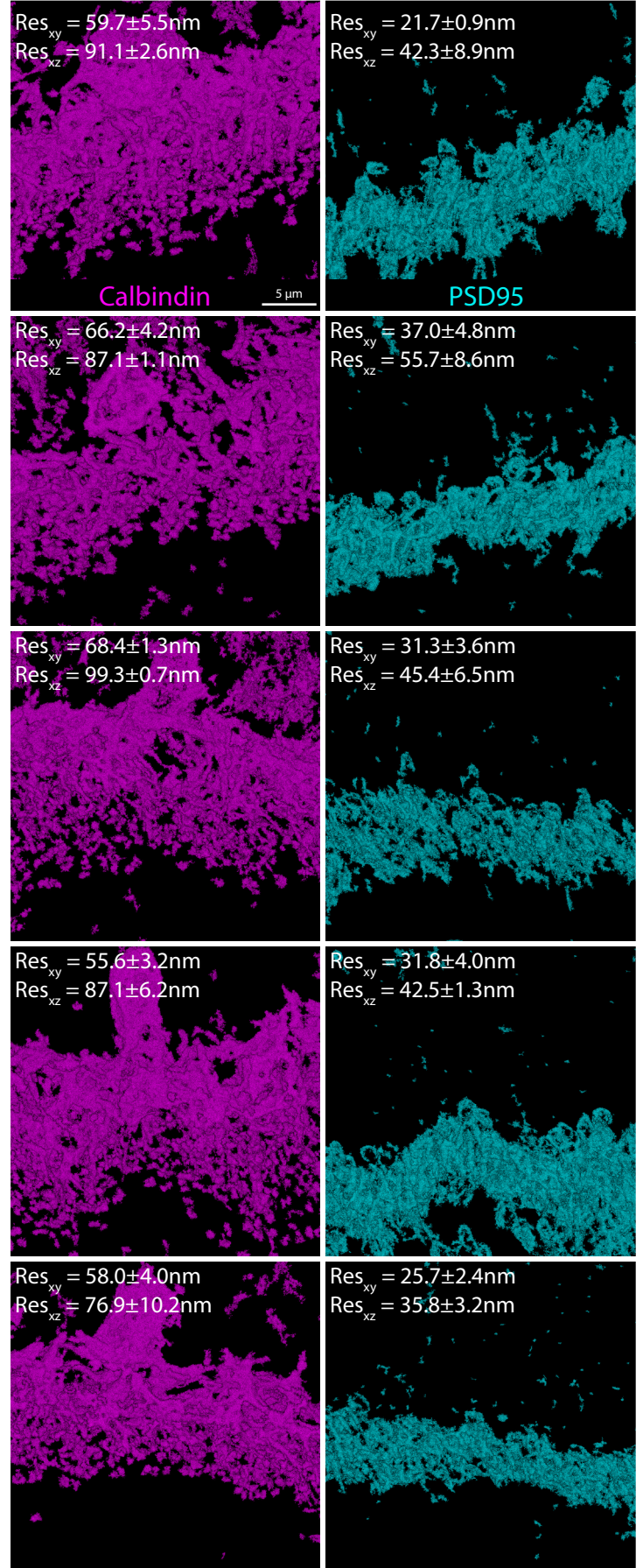


Staining - Blocking, Triton-X100

Unprocessed



Processed



Staining - Blocking, Saponin

Unprocessed

Processed

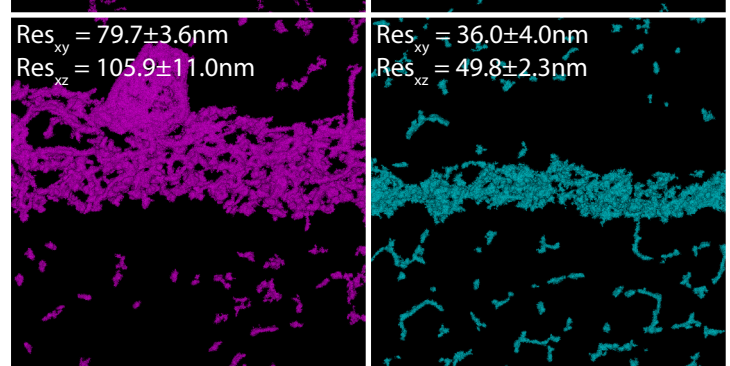
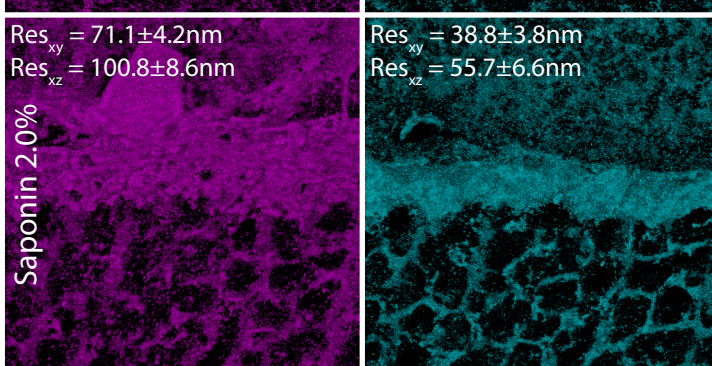
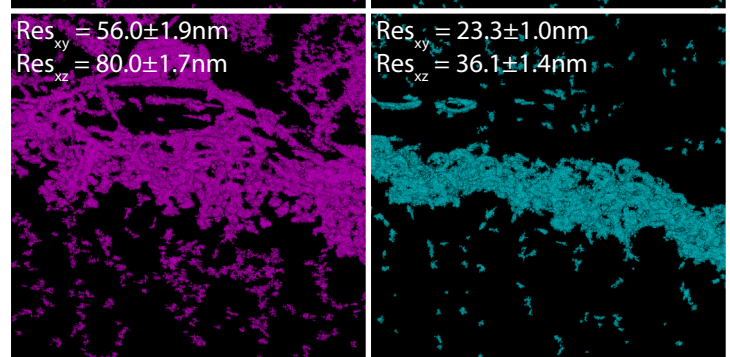
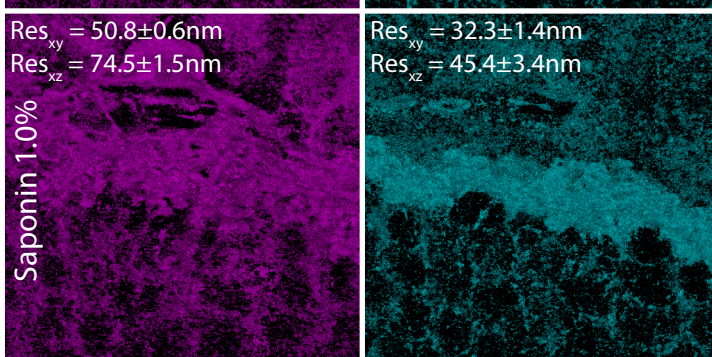
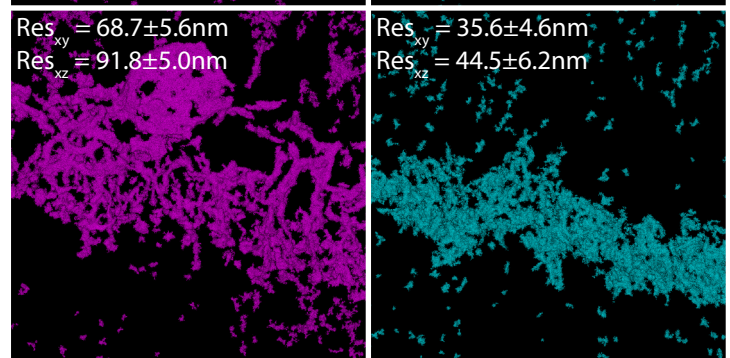
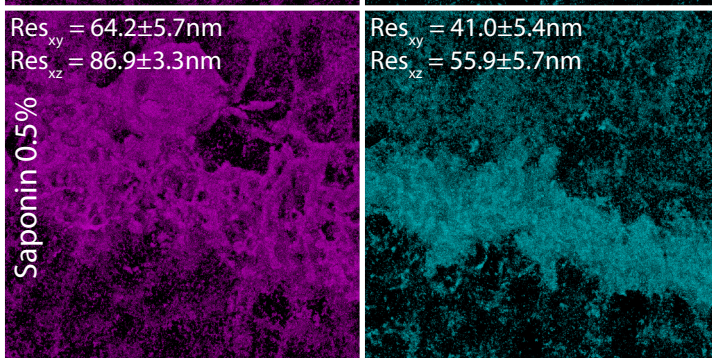
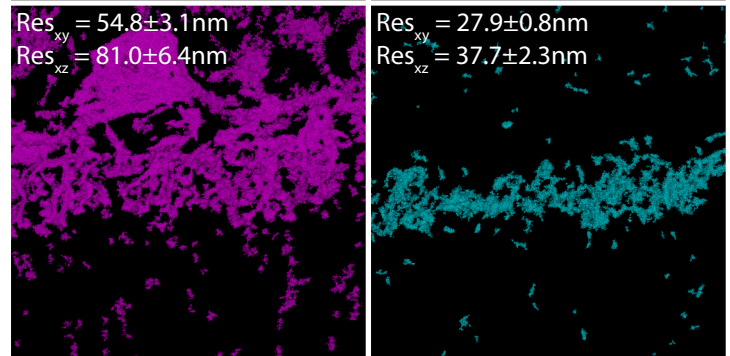
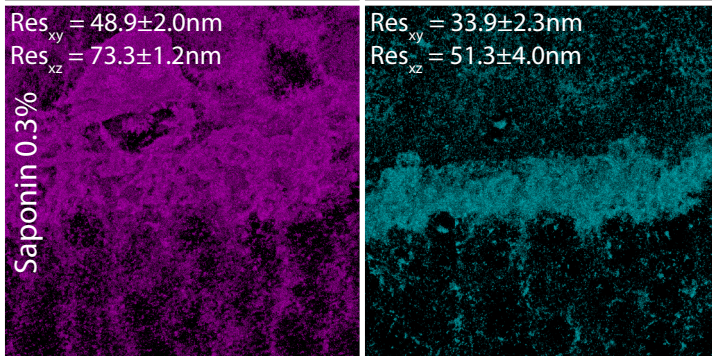
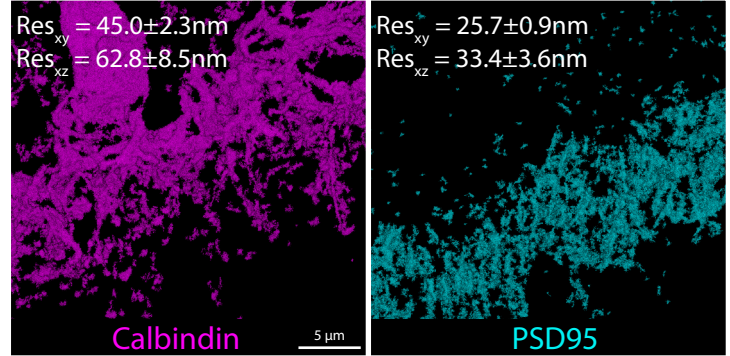
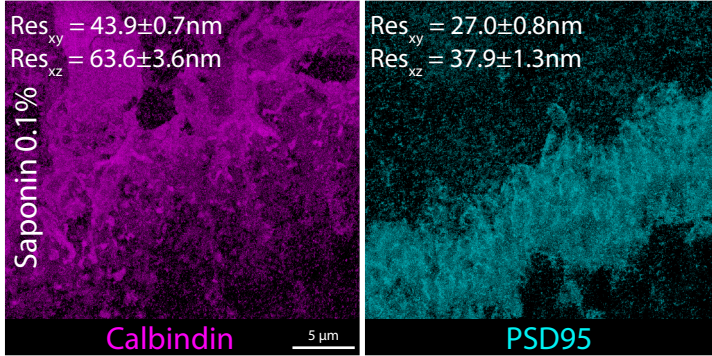
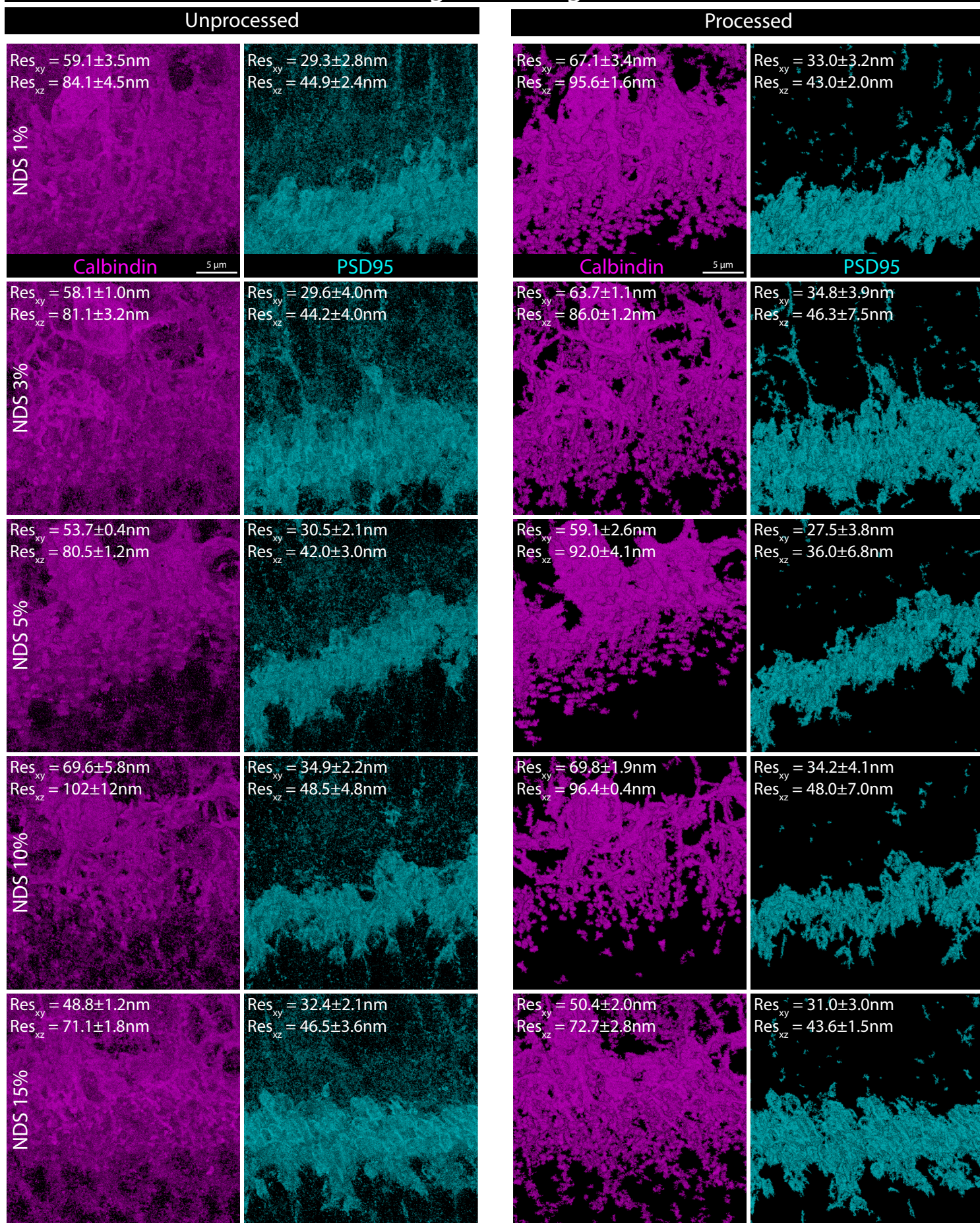


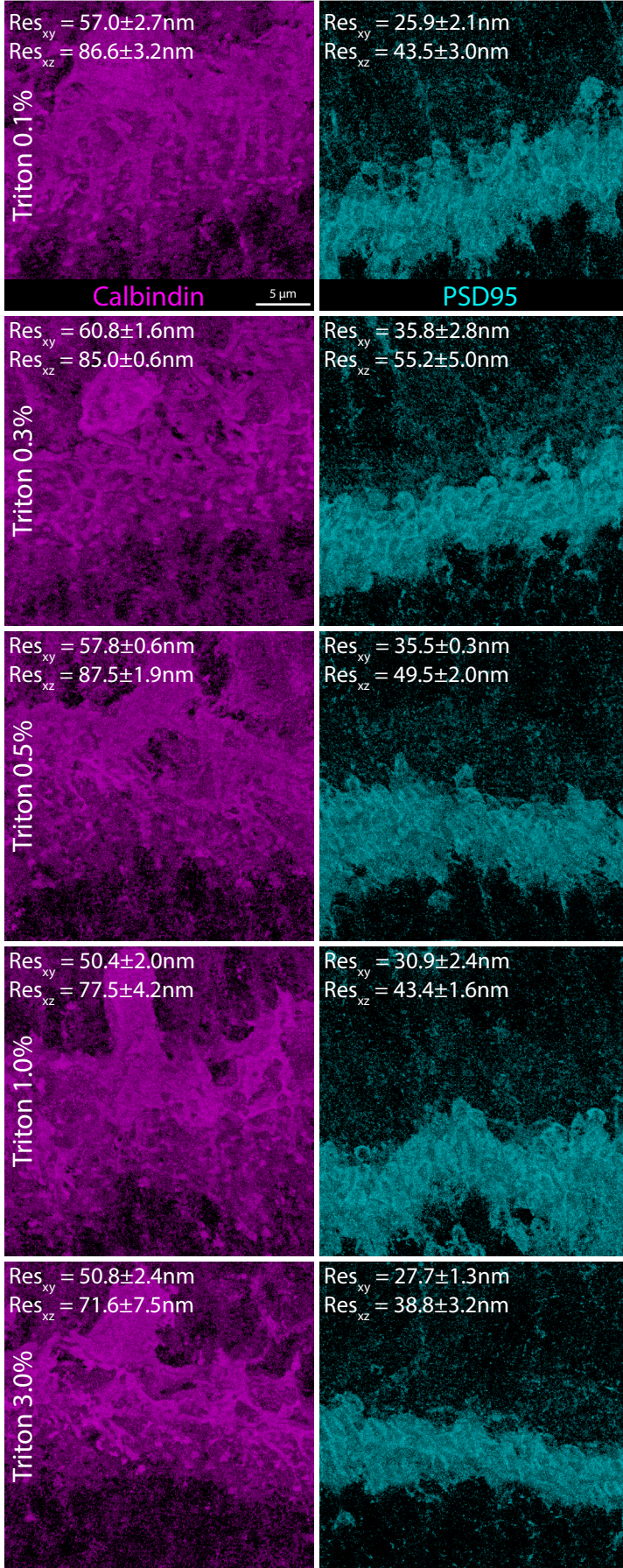
Figure S4. Sample and imaging conditions affect image quality and metrics, Related to Figure 4.

Staining - Blocking, NDS

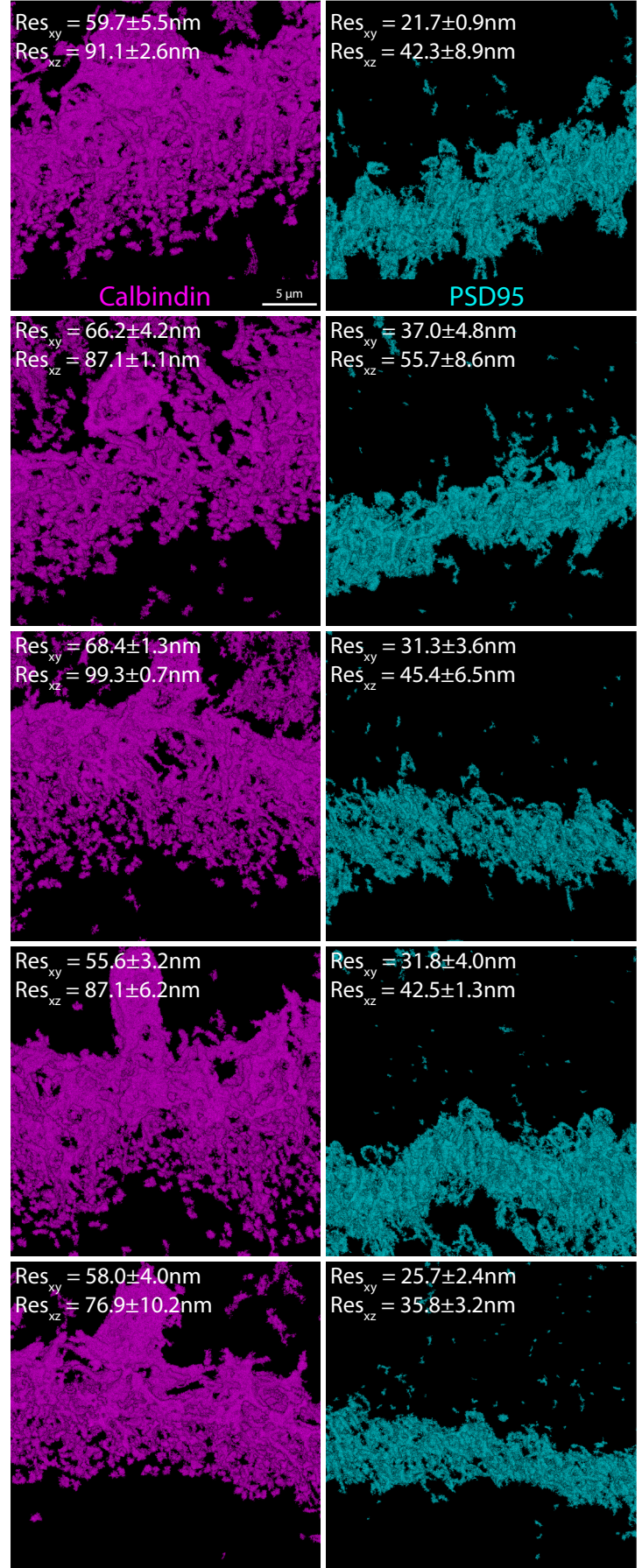


Staining - Blocking, Triton-X100

Unprocessed

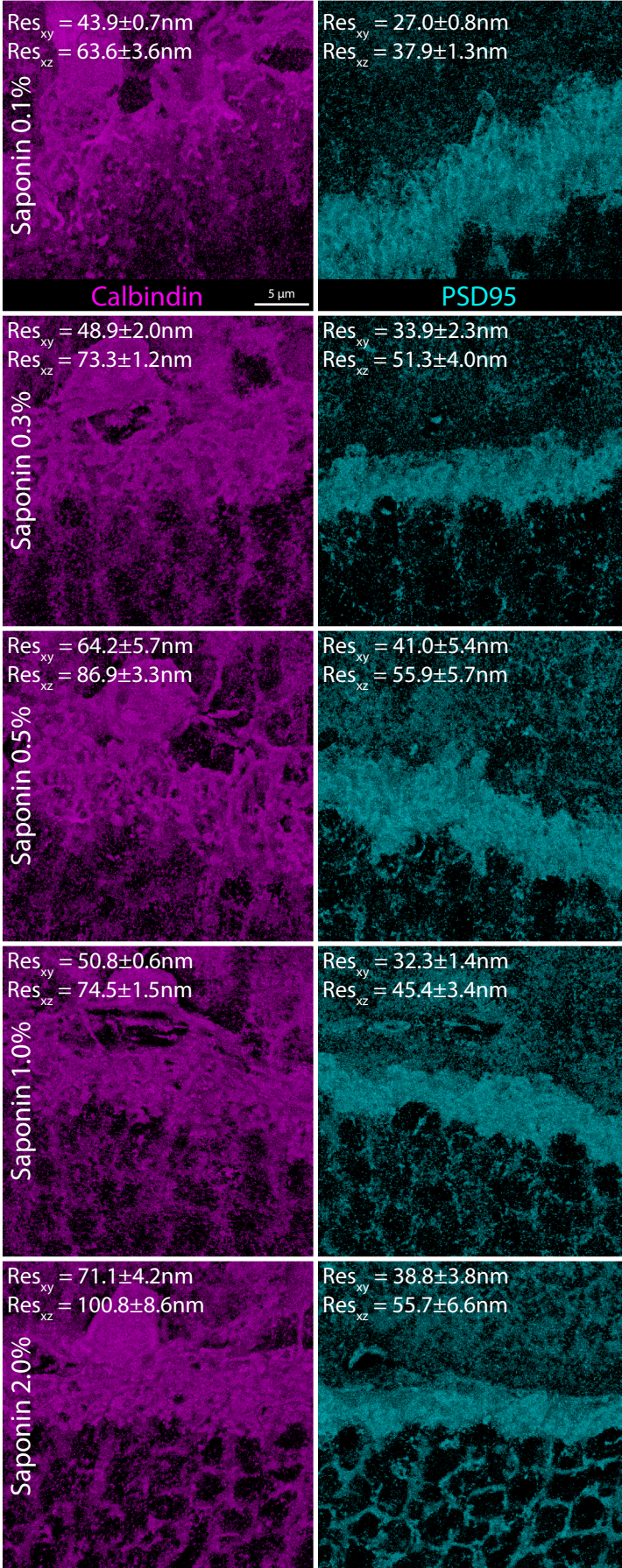


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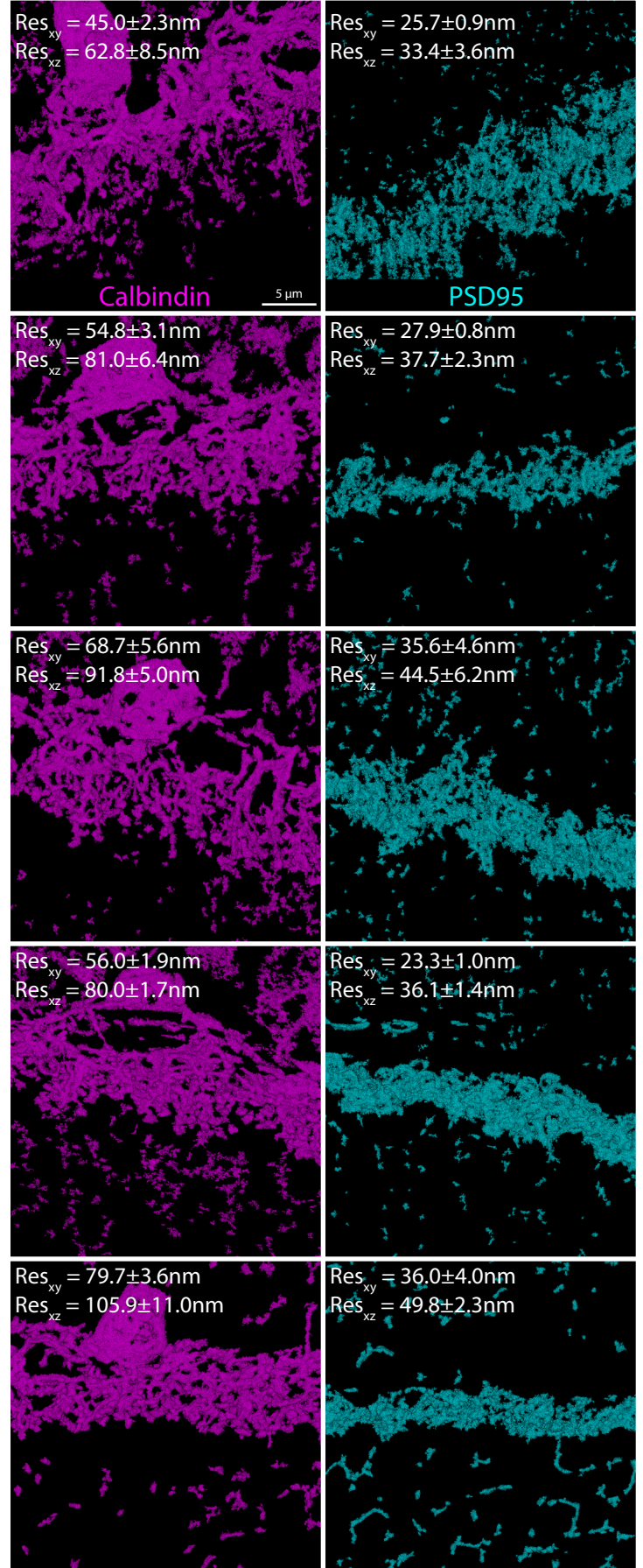


Staining - Blocking, Saponin

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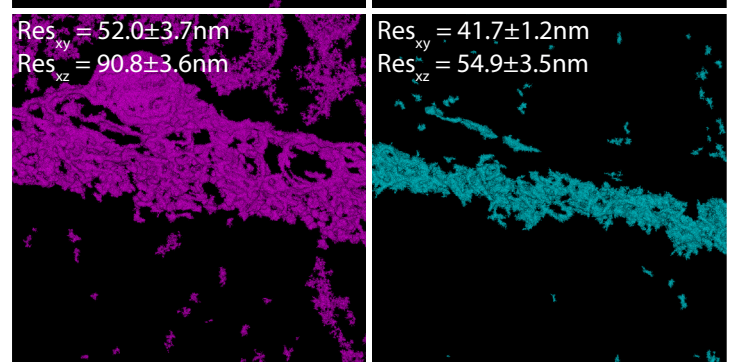
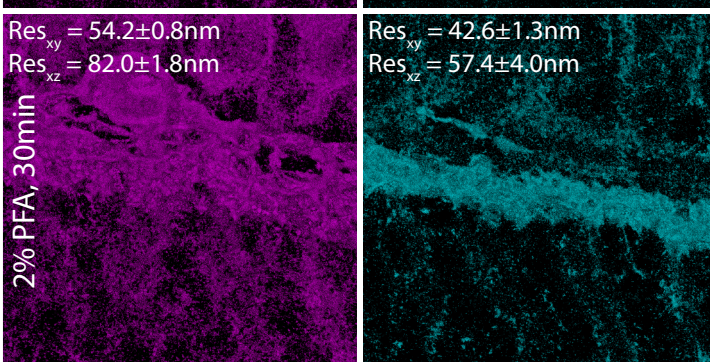
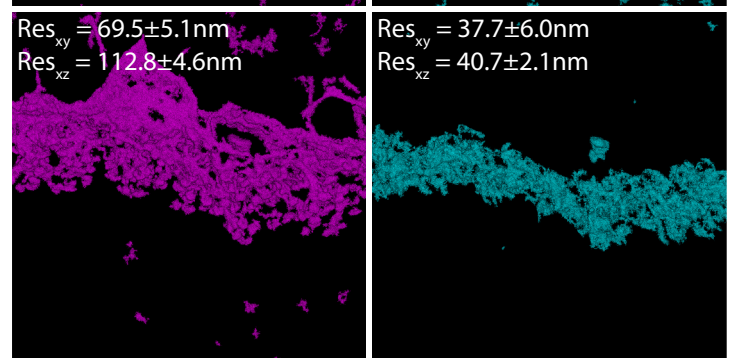
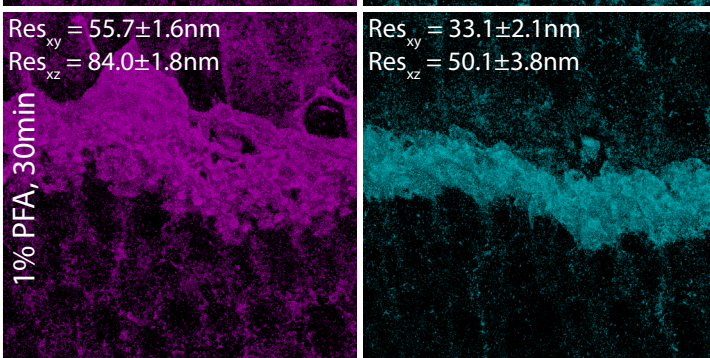
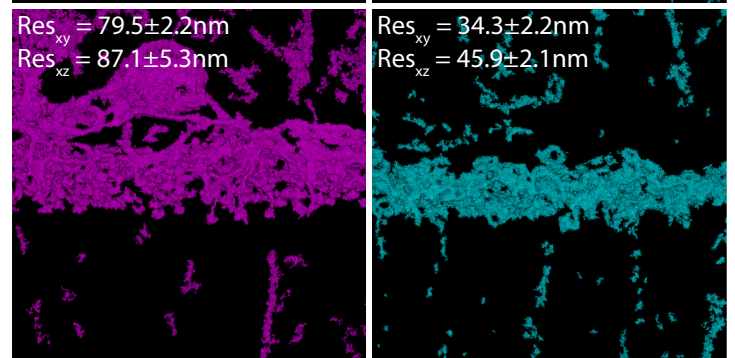
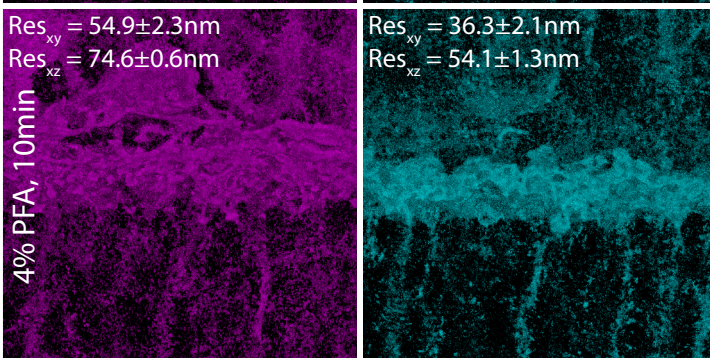
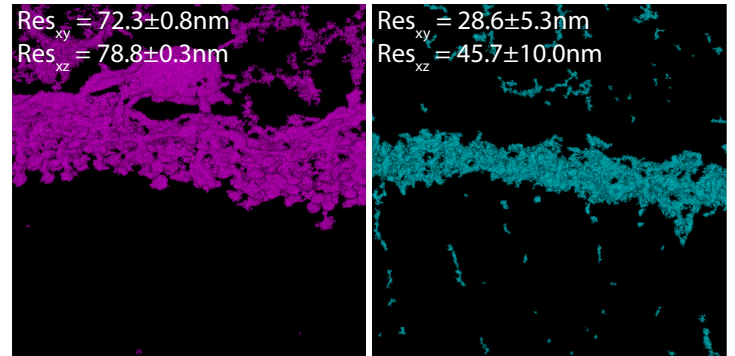
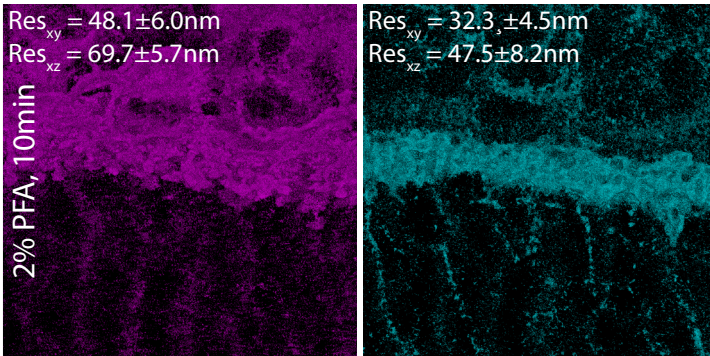
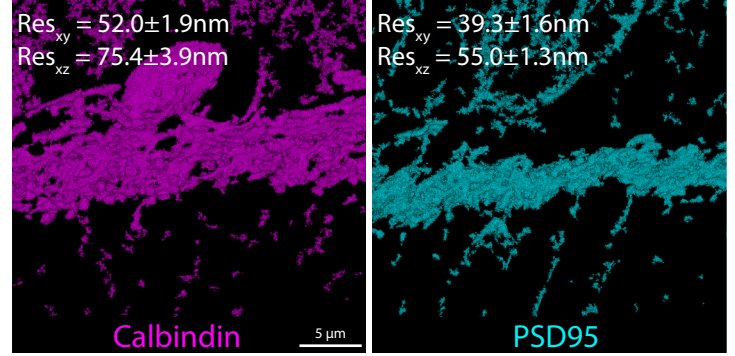
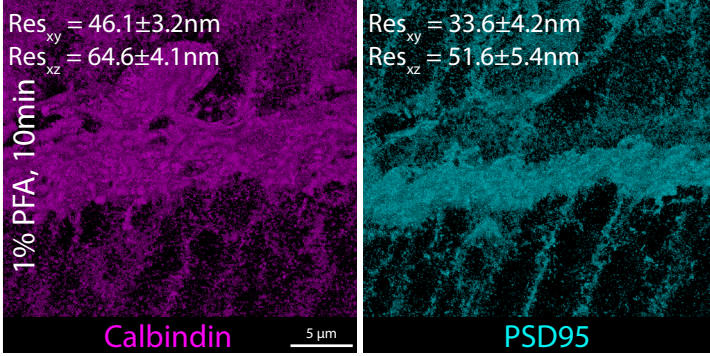
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Staining - Post-Fixation, Time & Concentration

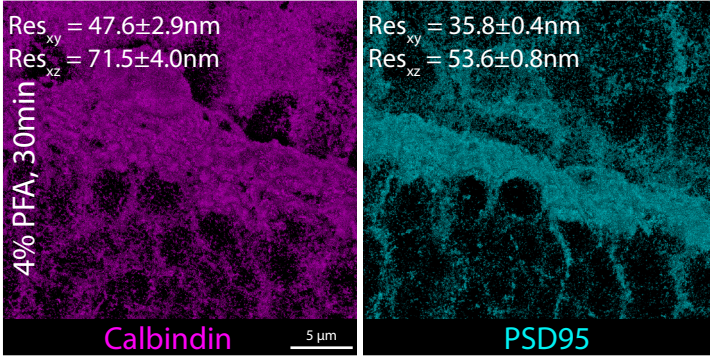
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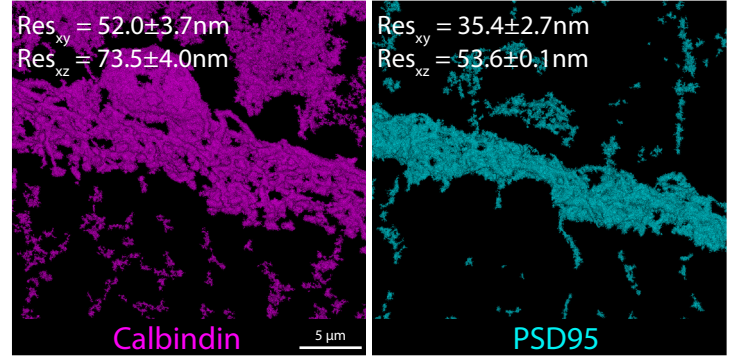


Staining - Post-Fixation, Time & Concentration

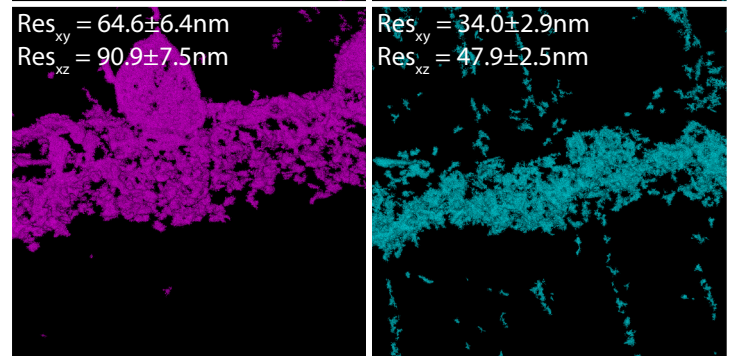
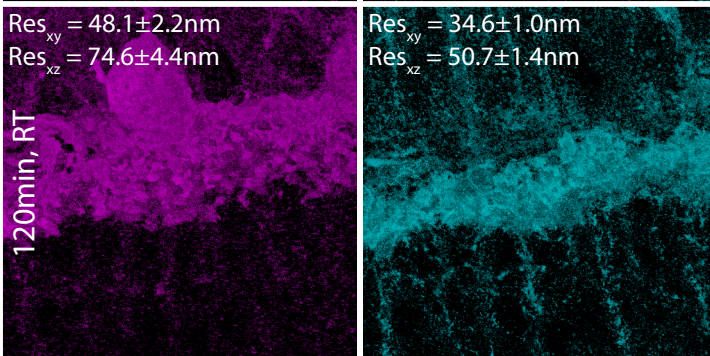
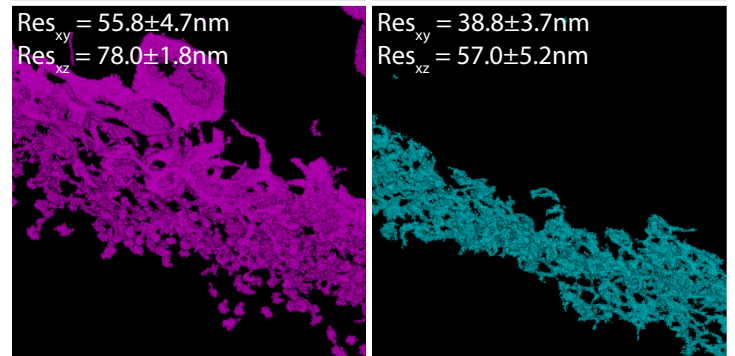
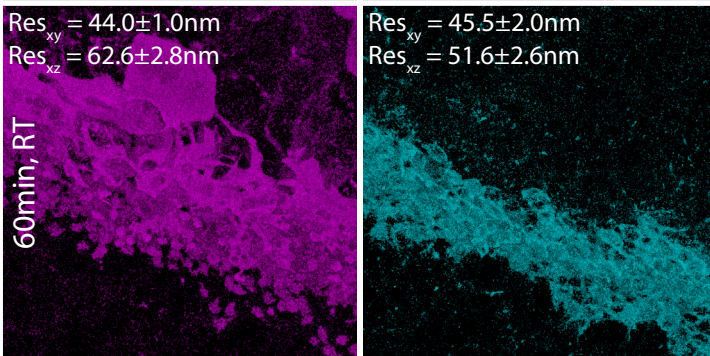
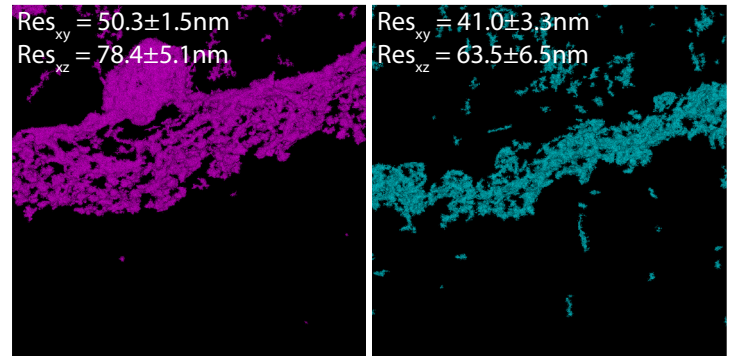
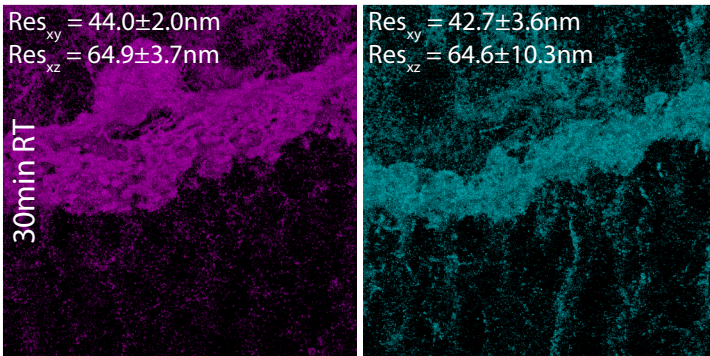
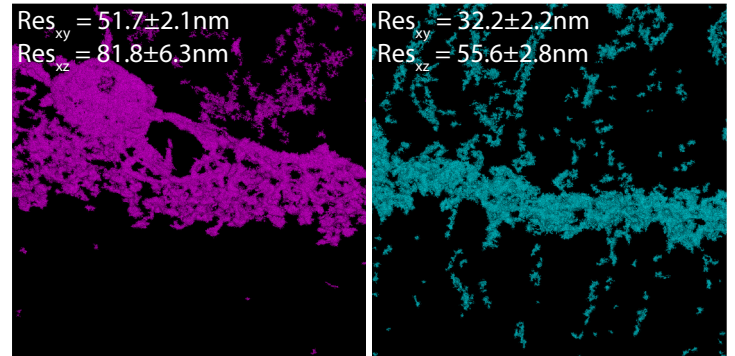
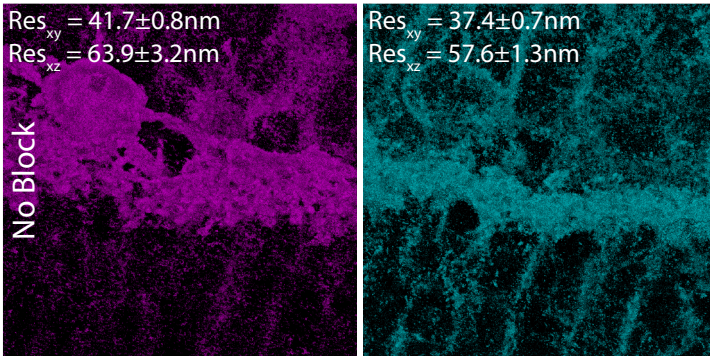
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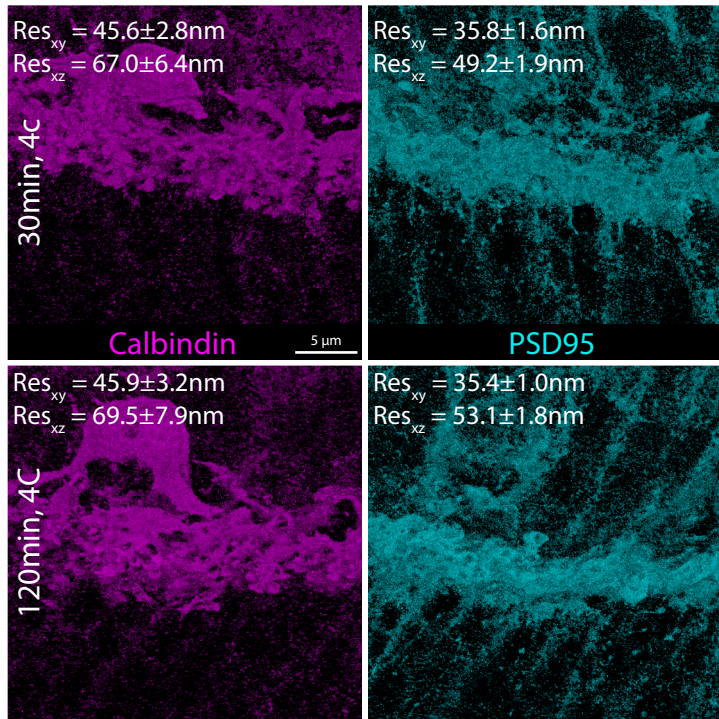


Staining - Blocking, Time & Temperature

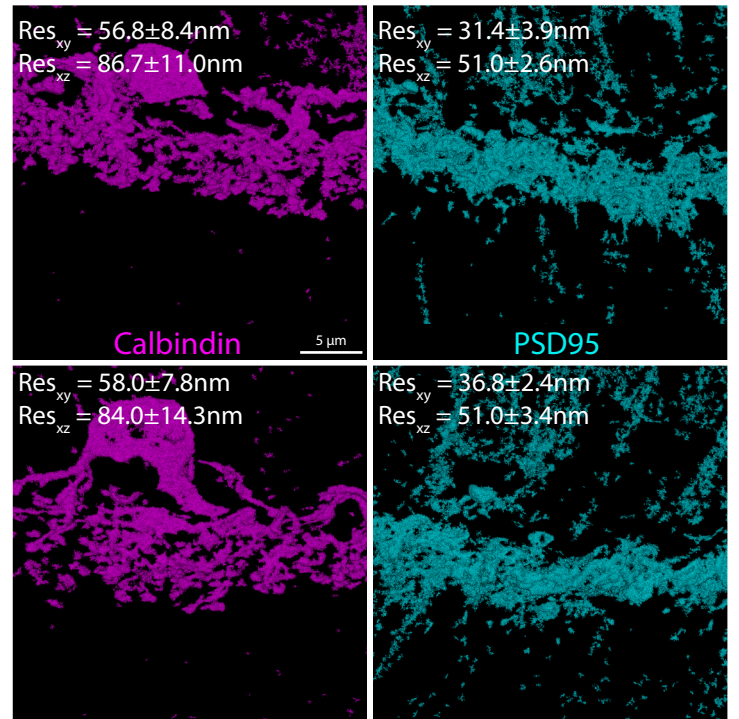


Staining - Blocking, Time & Temperature

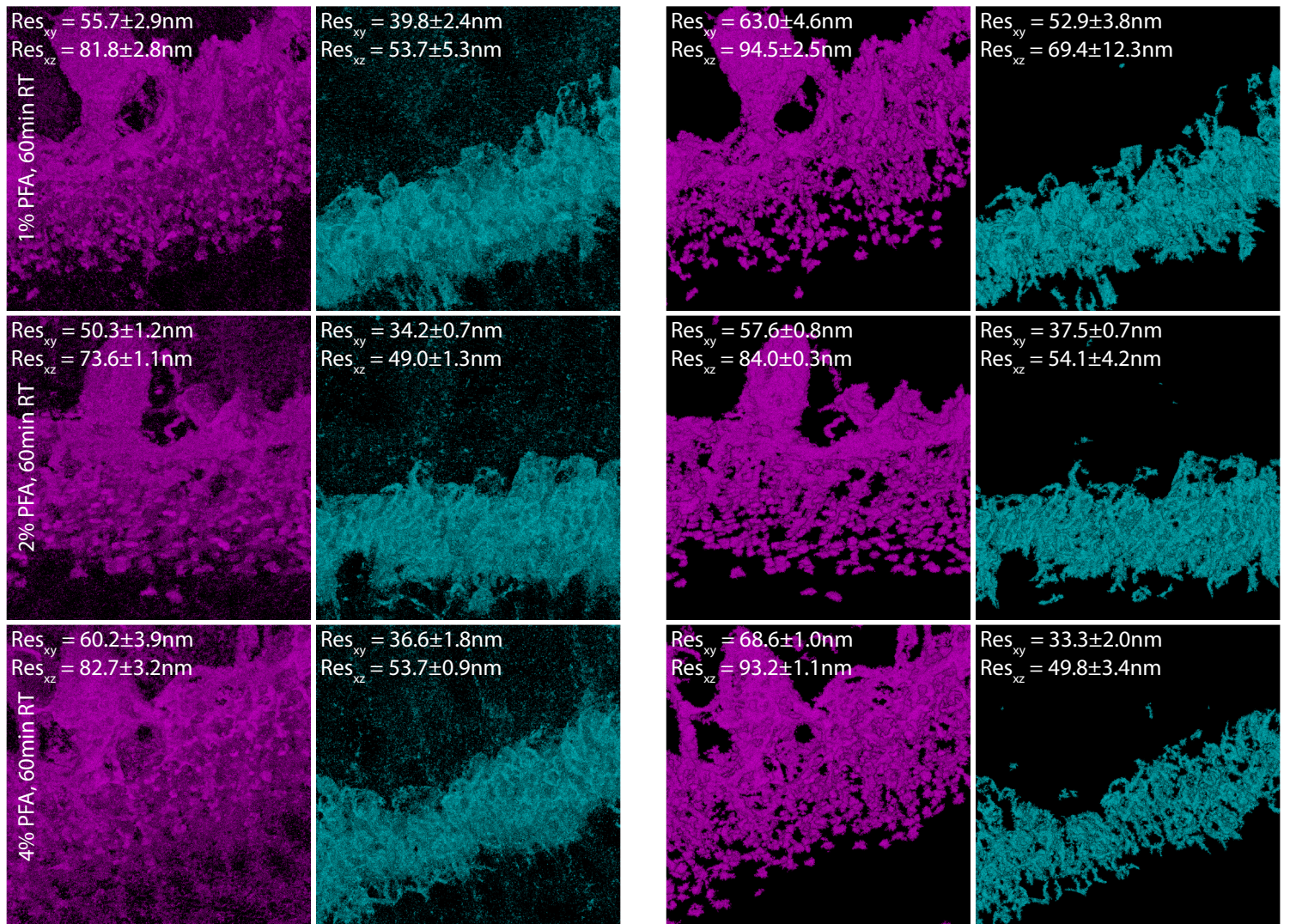
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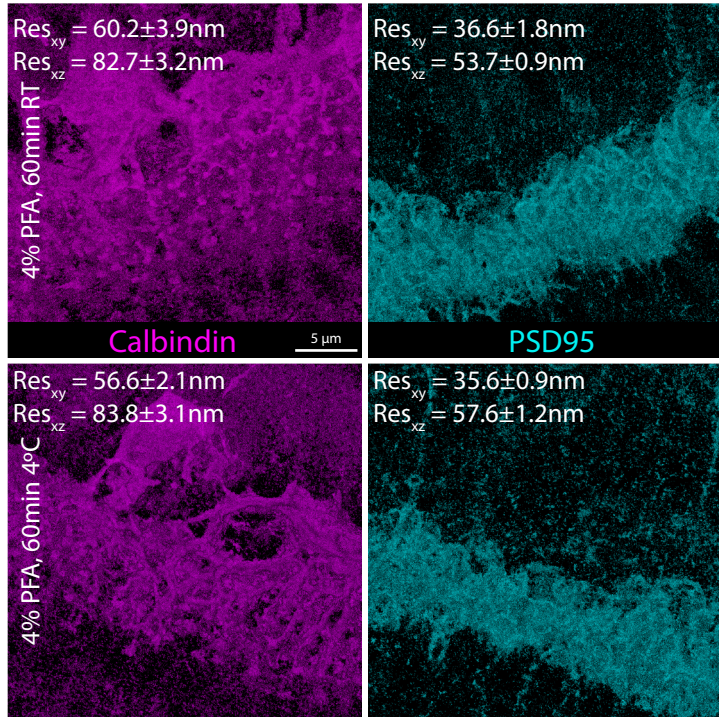


Tissue Preparation - Primary Fixation, PFA Concentration

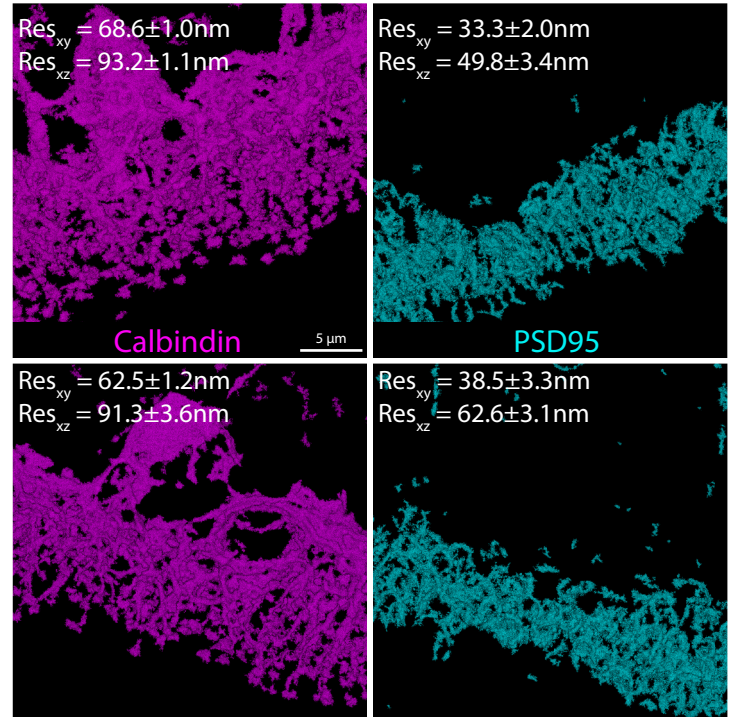


Tissue Preparation - Primary Fixation, Temperature

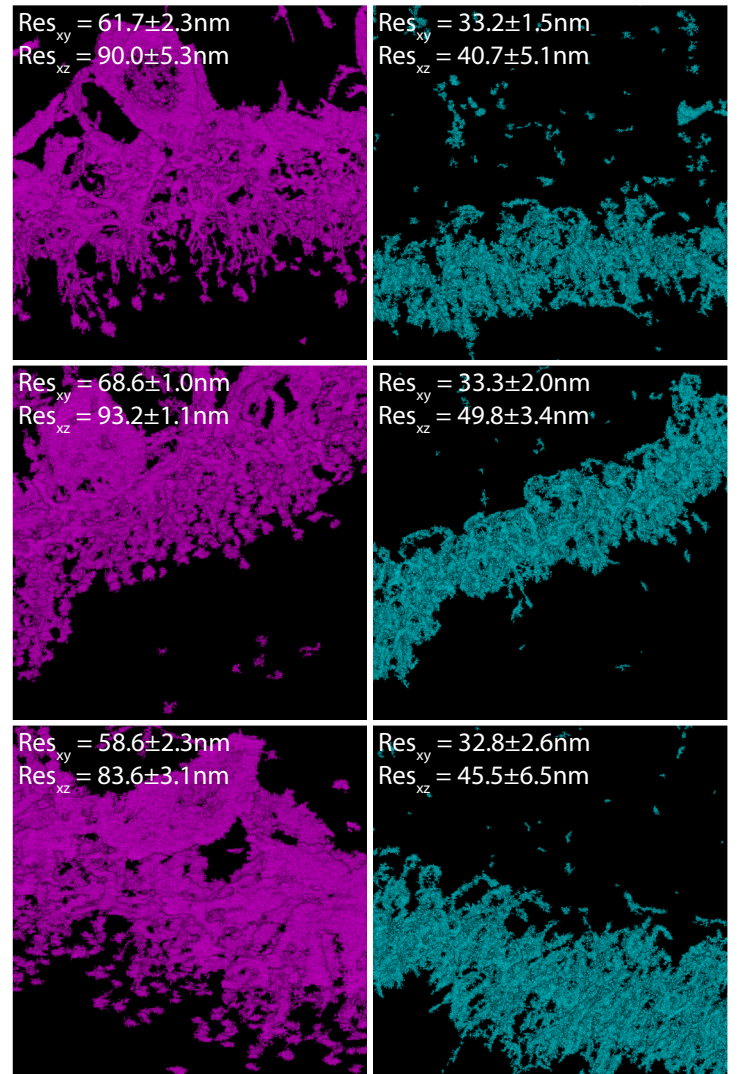
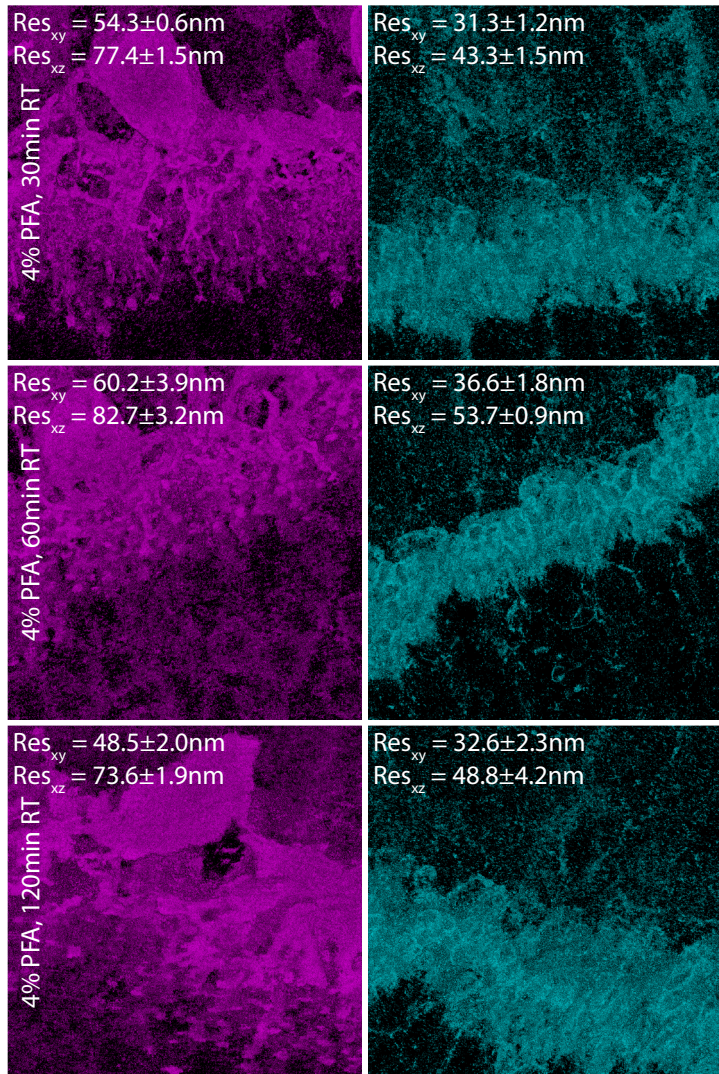
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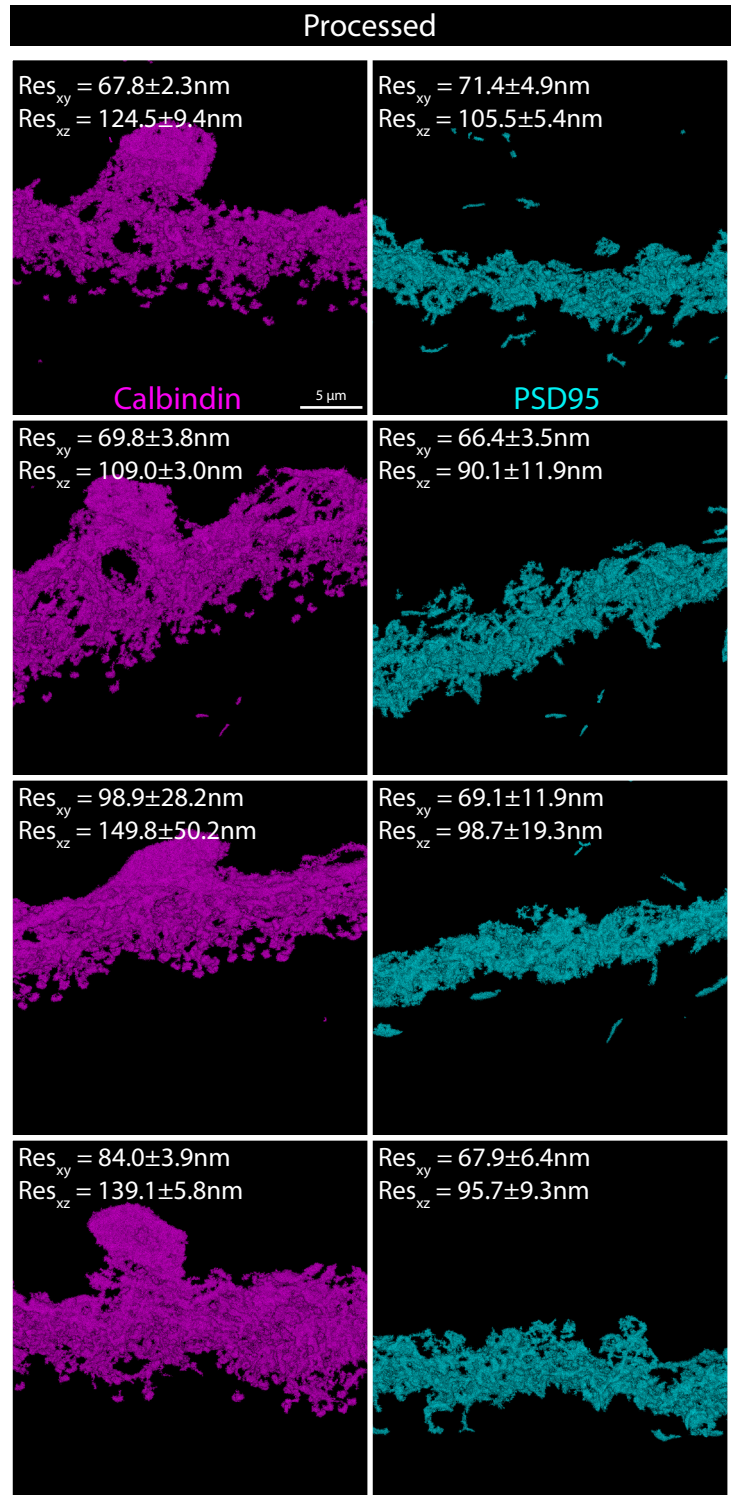
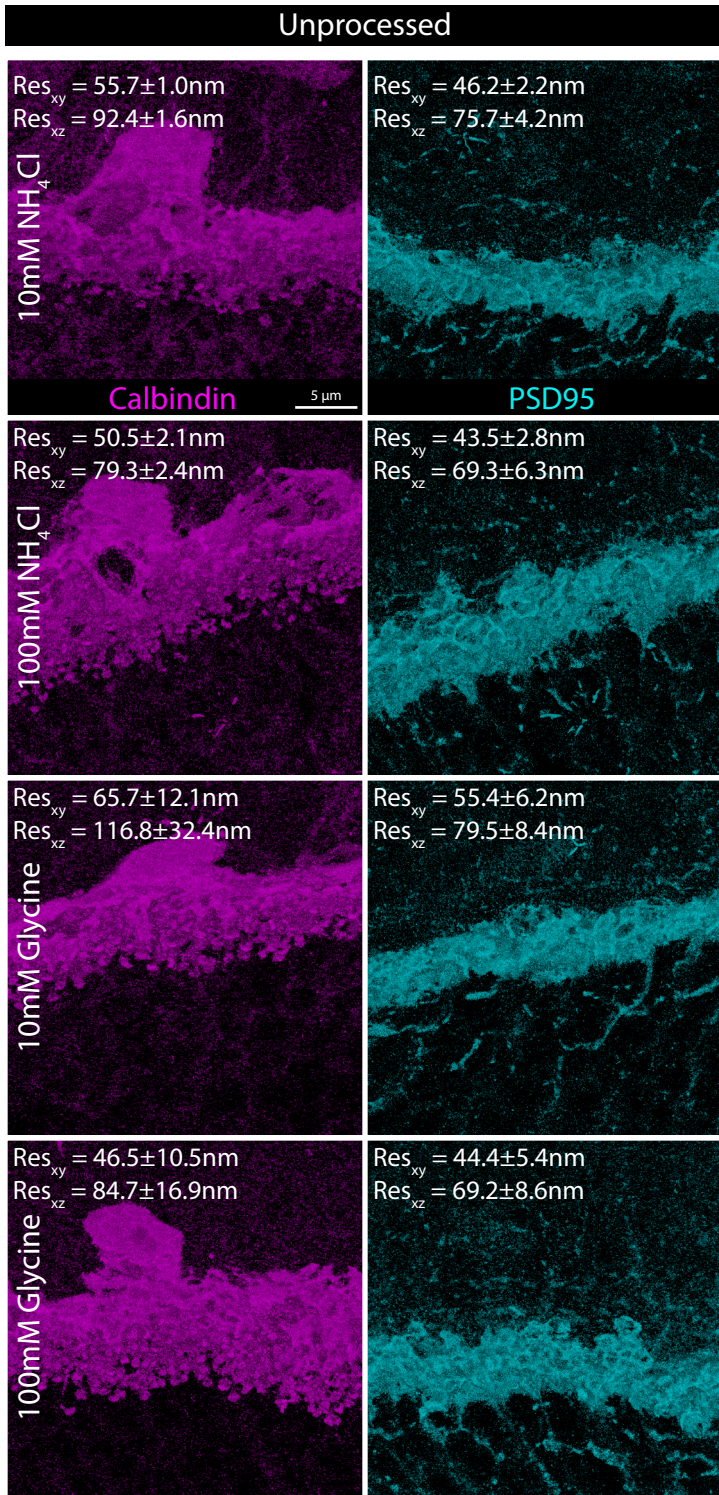
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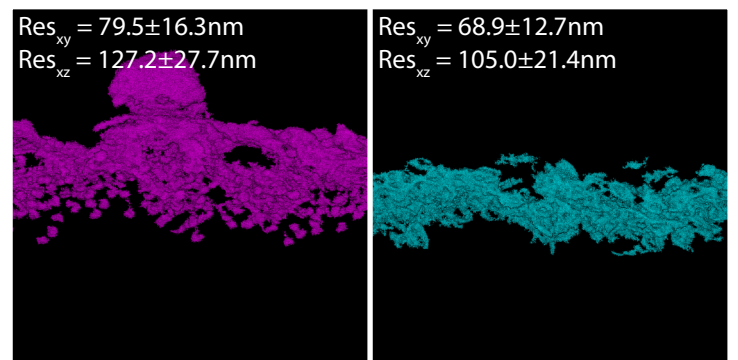
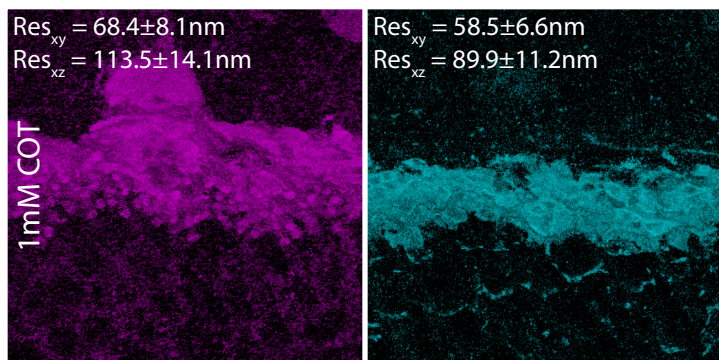
Tissue Preparation - Primary Fixation, Time



Staining - Secondary Quenching, Concentration & Reagent

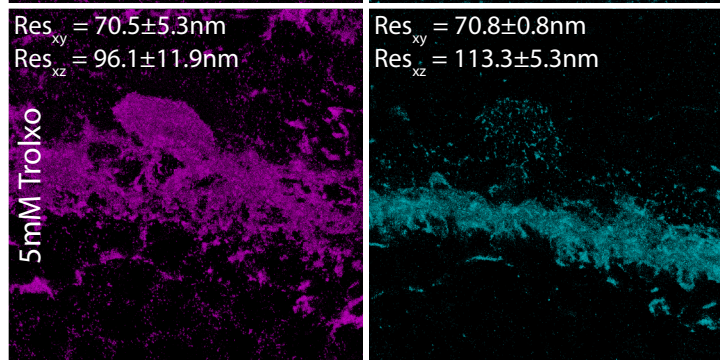
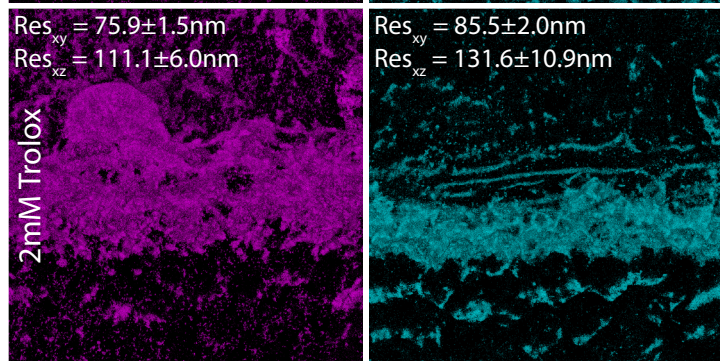
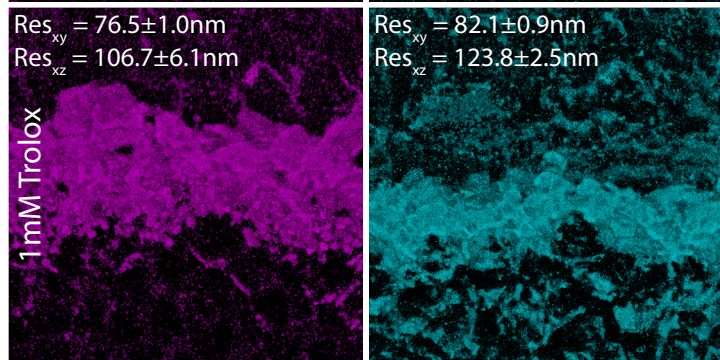
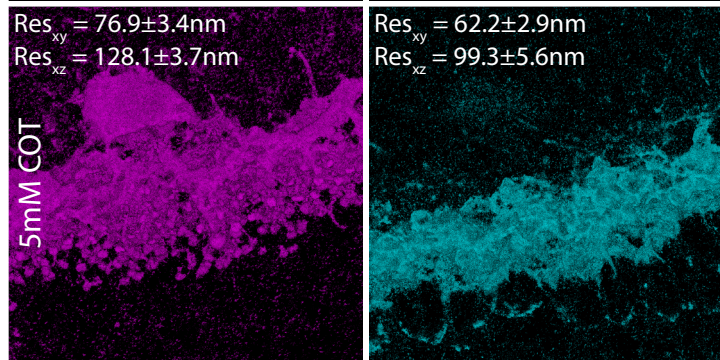
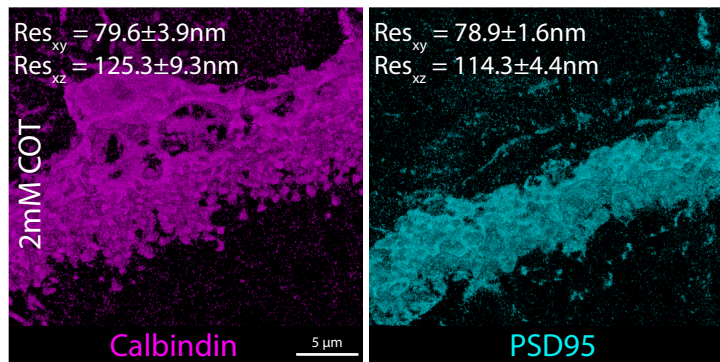


Imaging - Electron Sinks, Concentration & Reagent

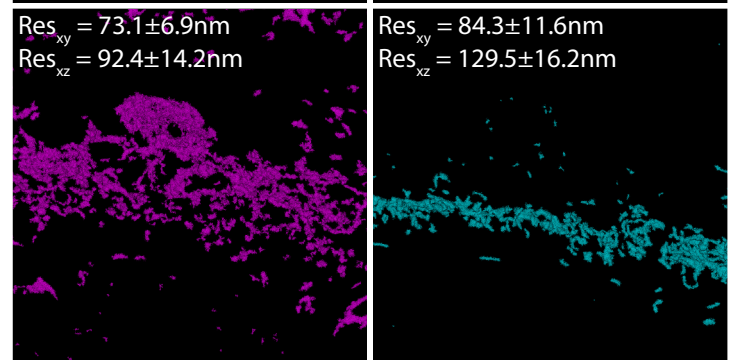
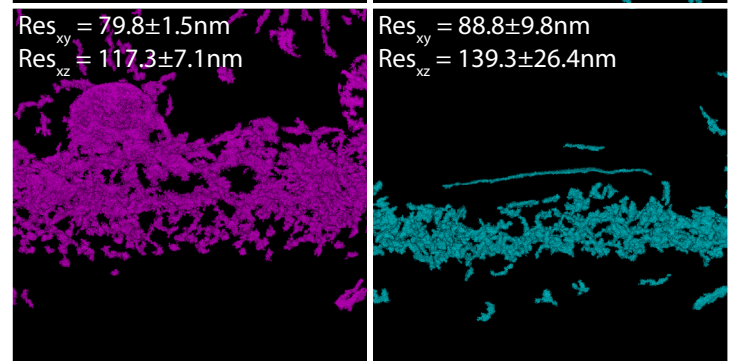
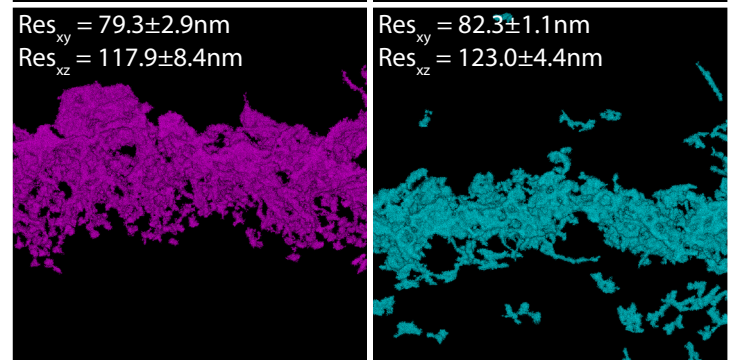
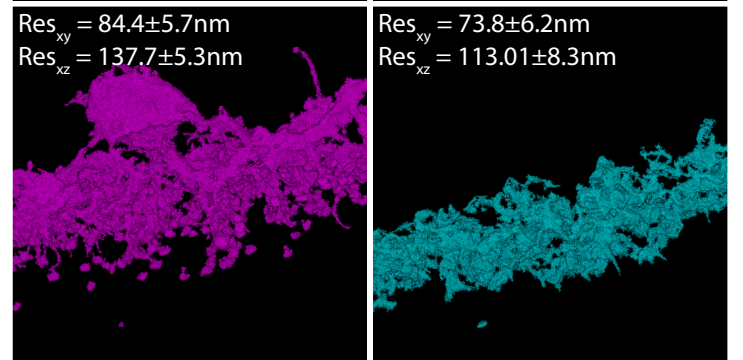
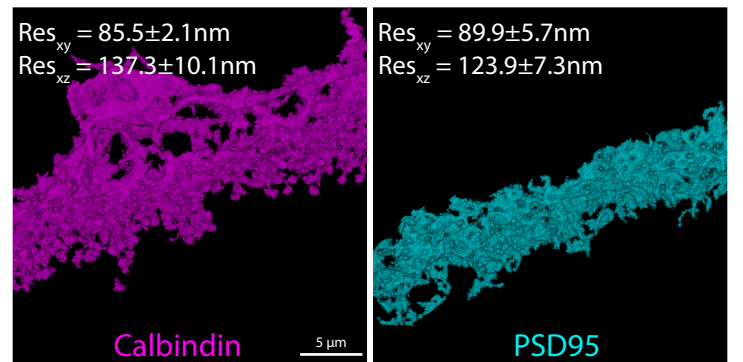


Imaging - Electron Sinks, Concentration & Reagent

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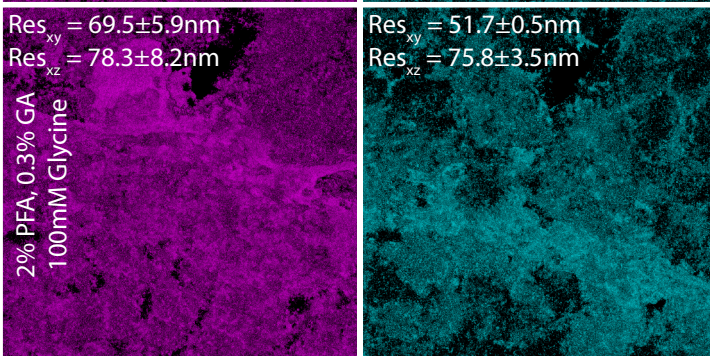
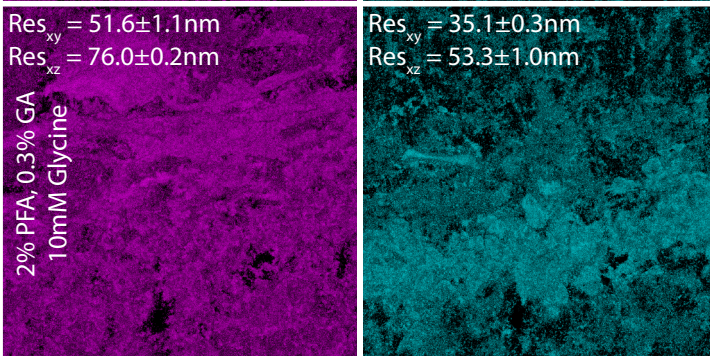
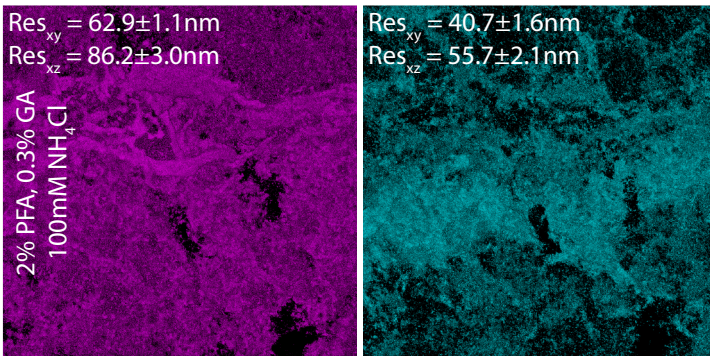
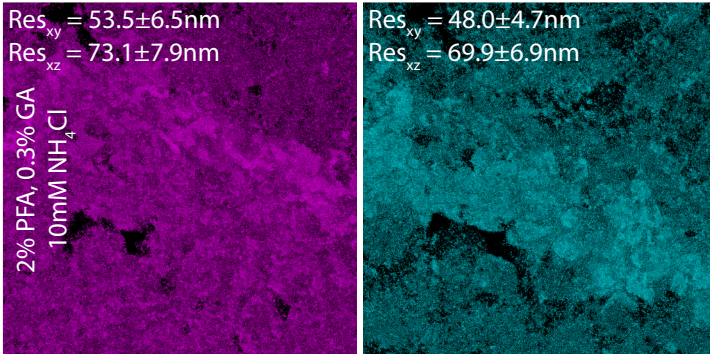
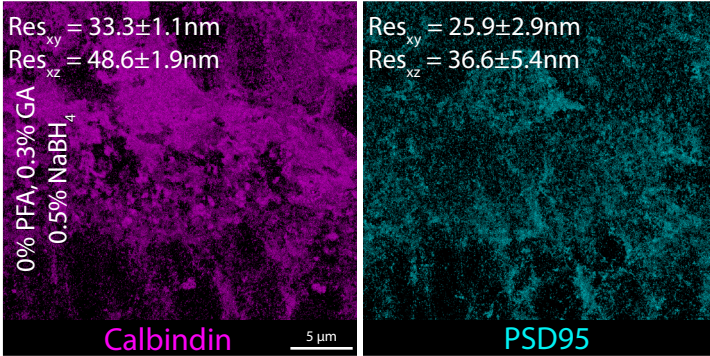


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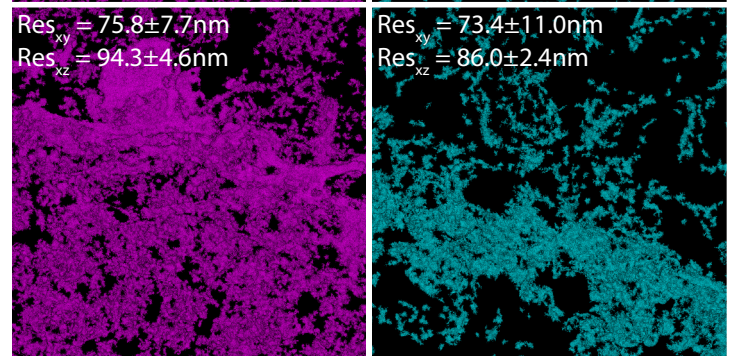
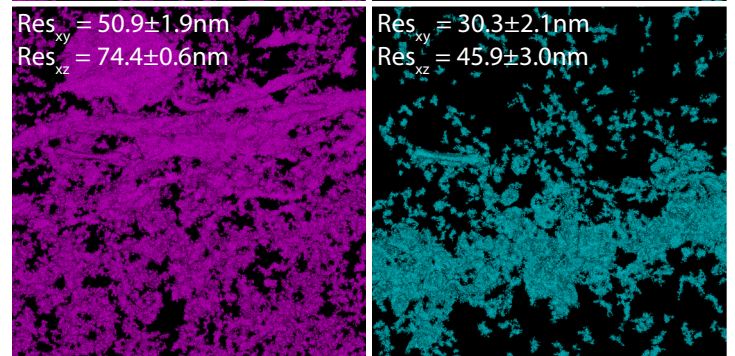
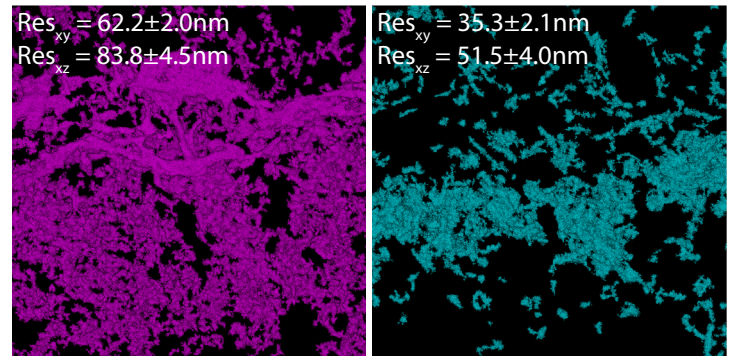
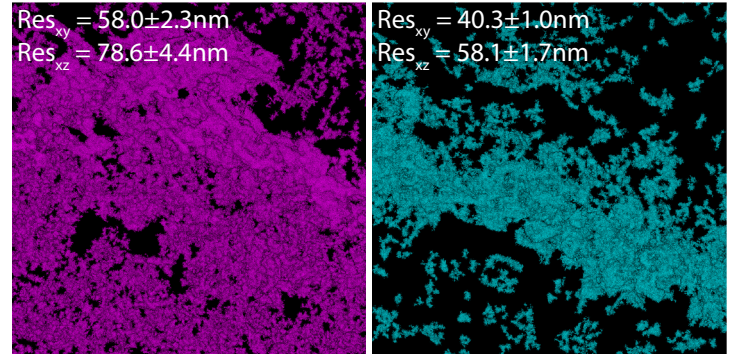
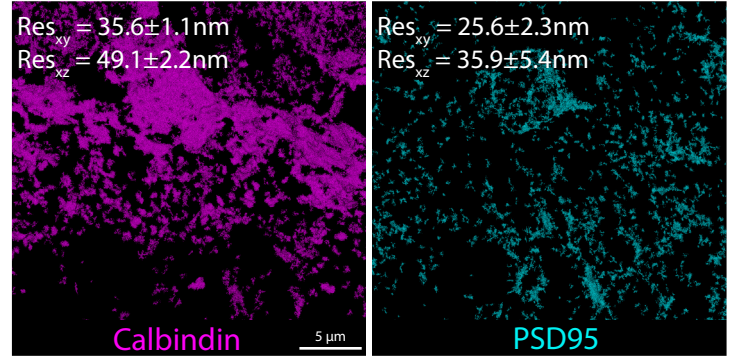


Tissue Preparation-Primary Fixation & Quenching, Reagent & Conc.

Unprocessed

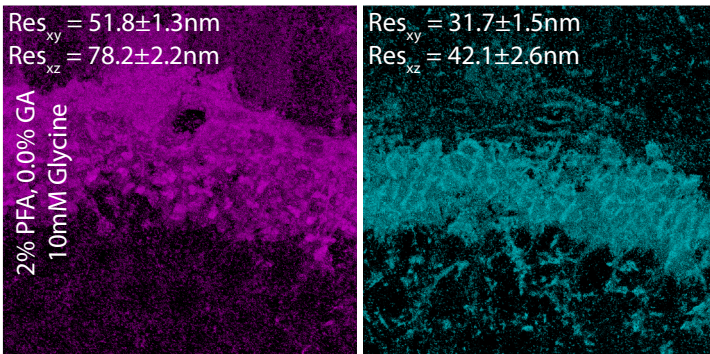
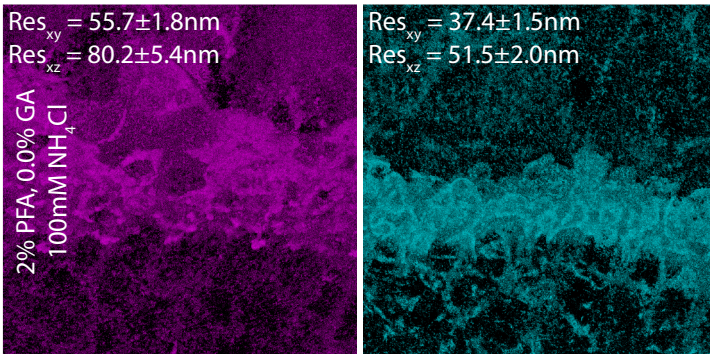
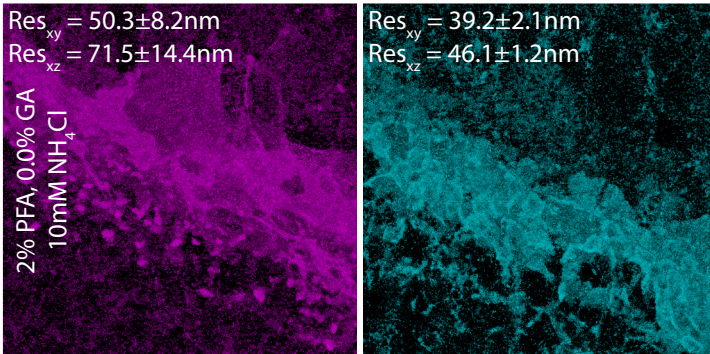
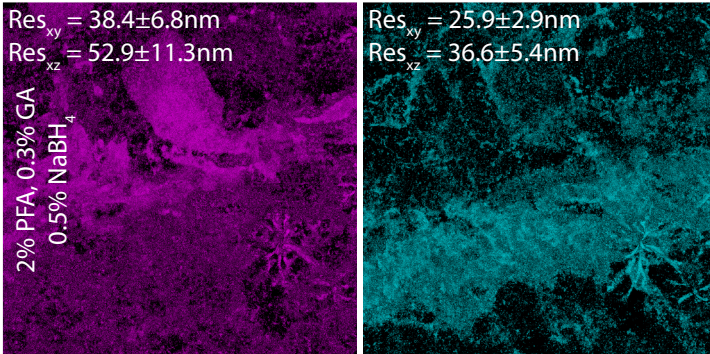
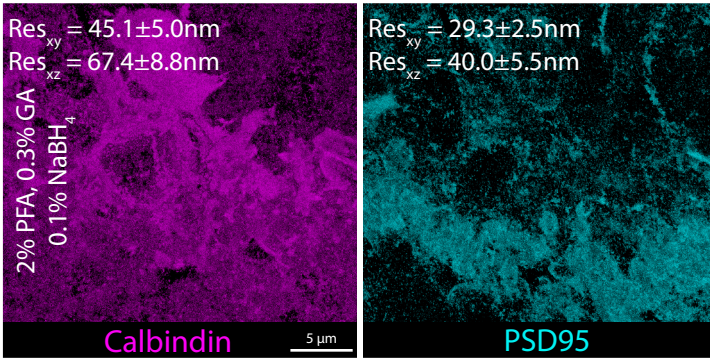


Processed

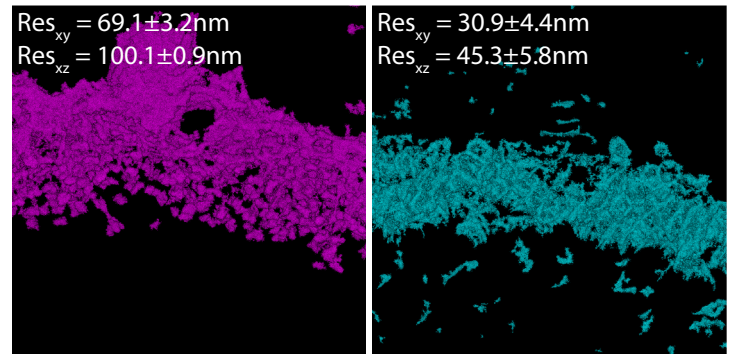
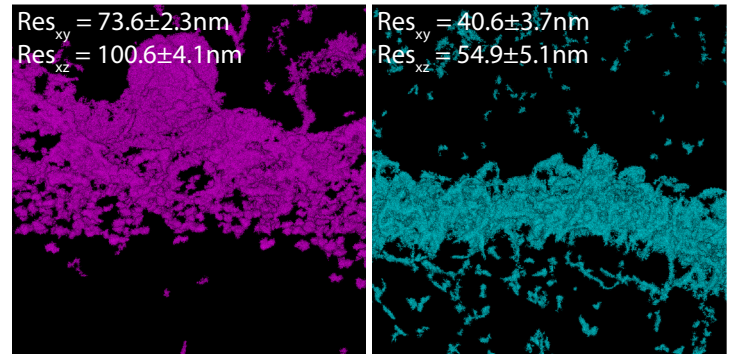
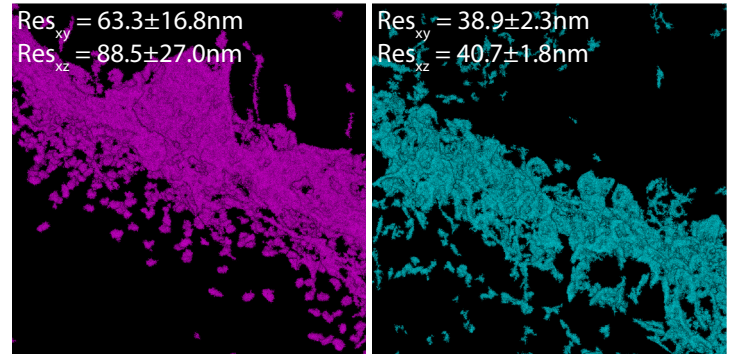
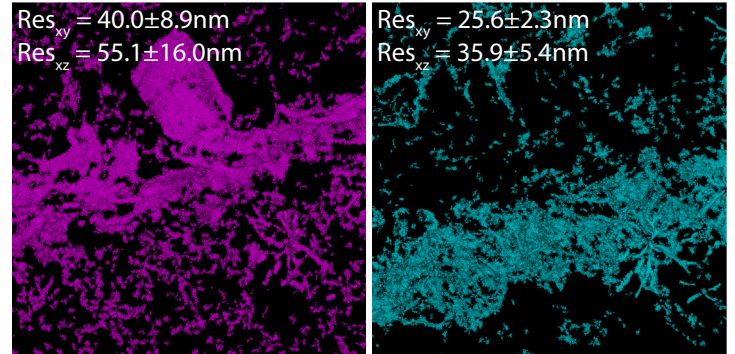
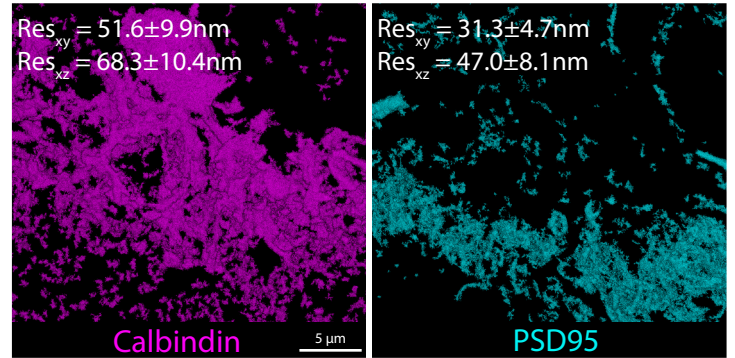


Tissue Preparation-Primary Fixation & Quenching, Reagent & Conc.

Unprocessed

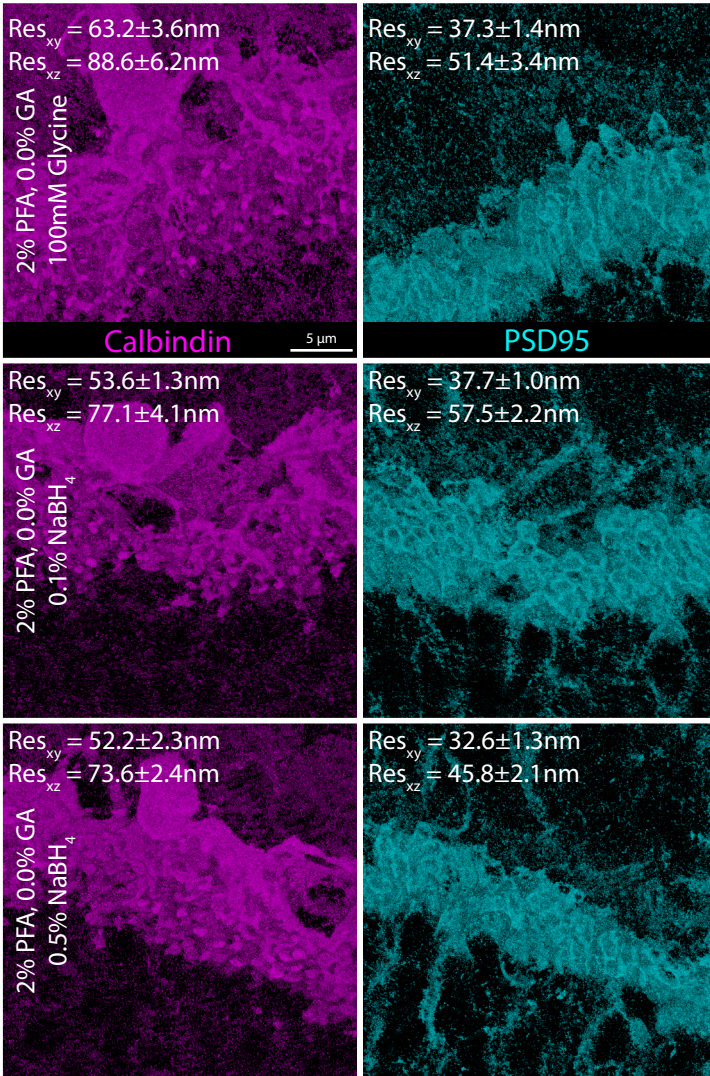


Processed

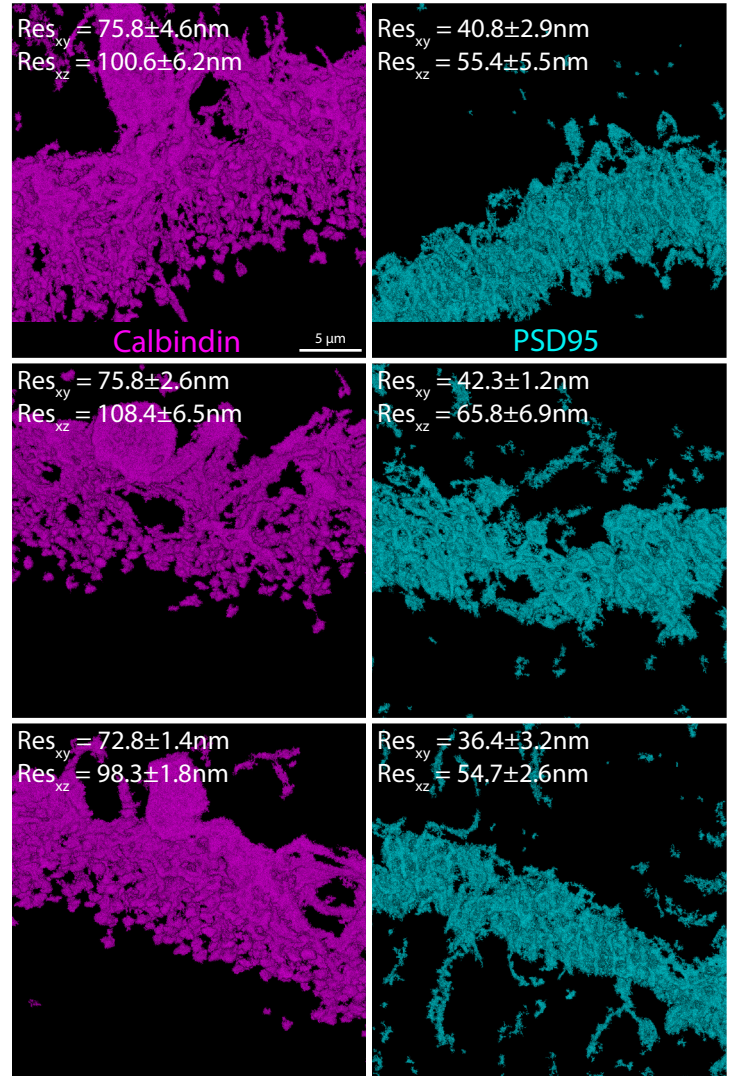


Tissue Preparation-Primary Fixation & Quenching, Reagent & Conc.

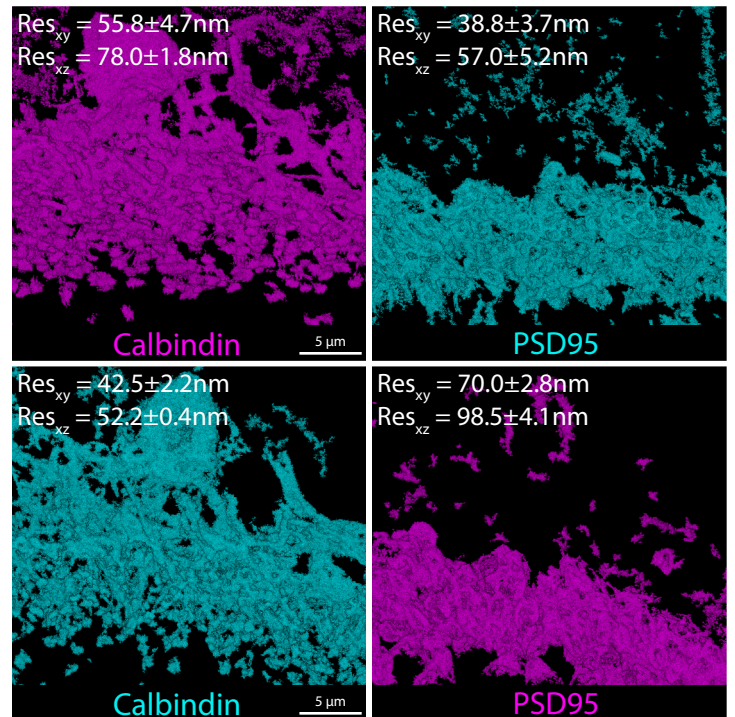
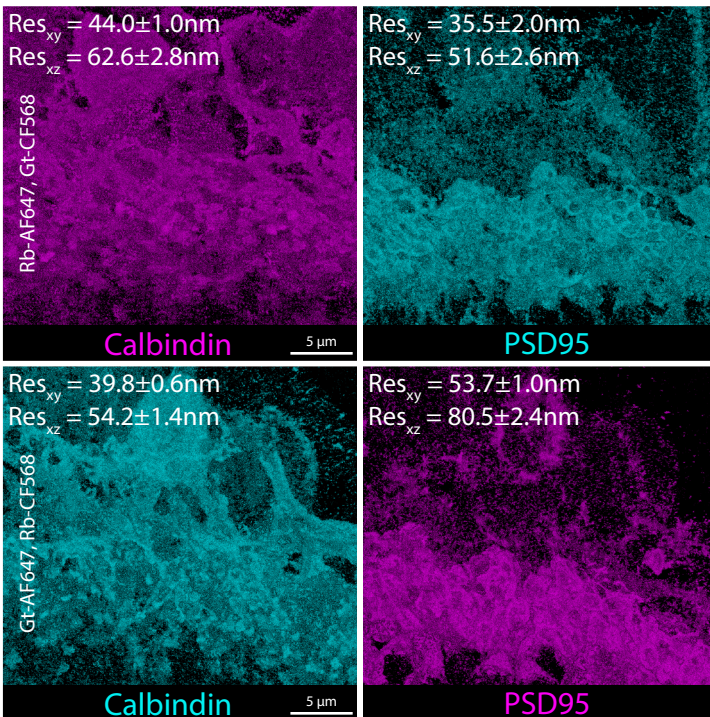
Unprocessed



Processed

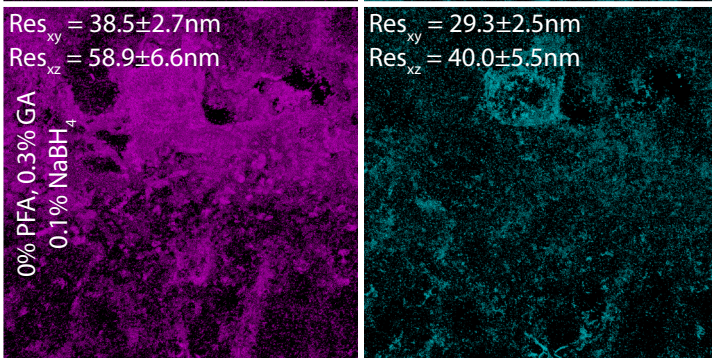
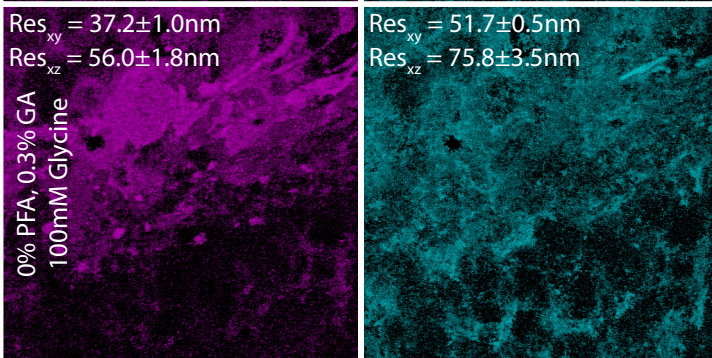
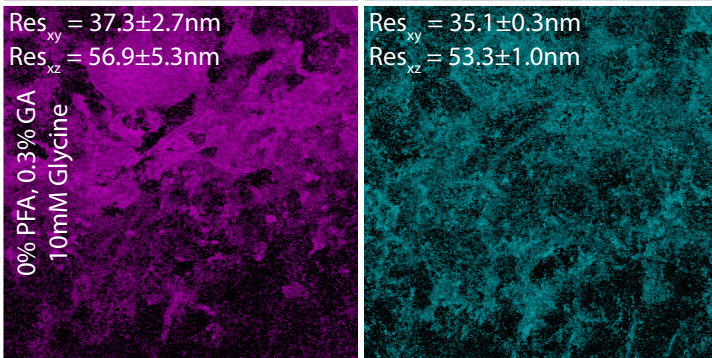
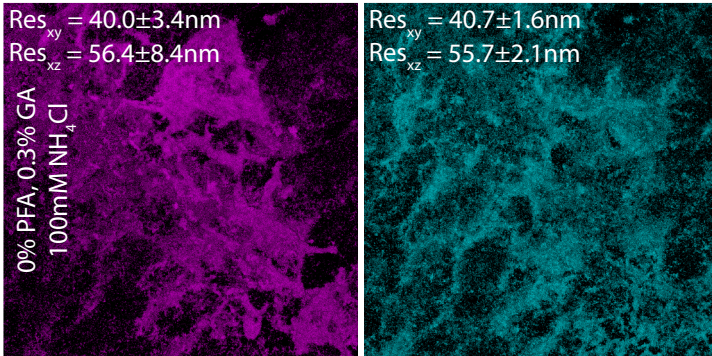
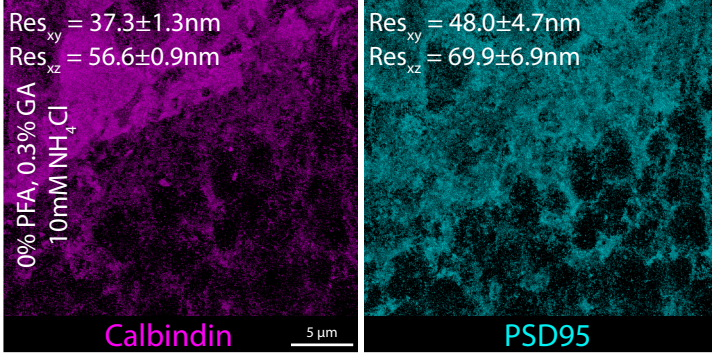


Staining - Secondary, Channel & Primary Target

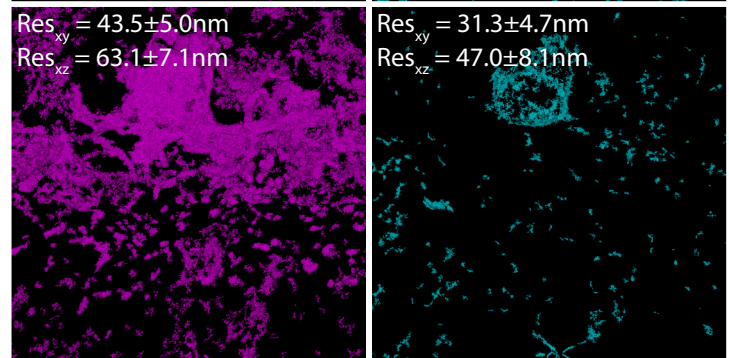
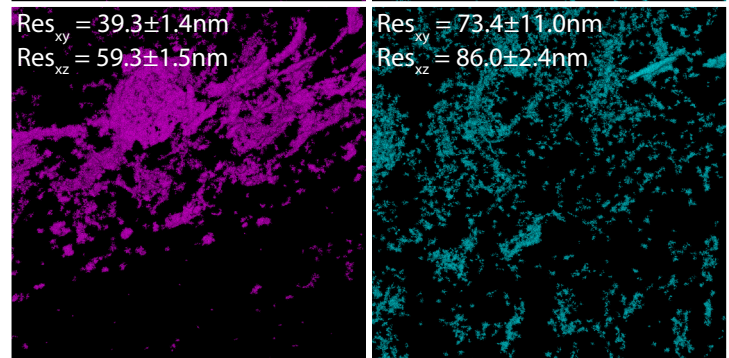
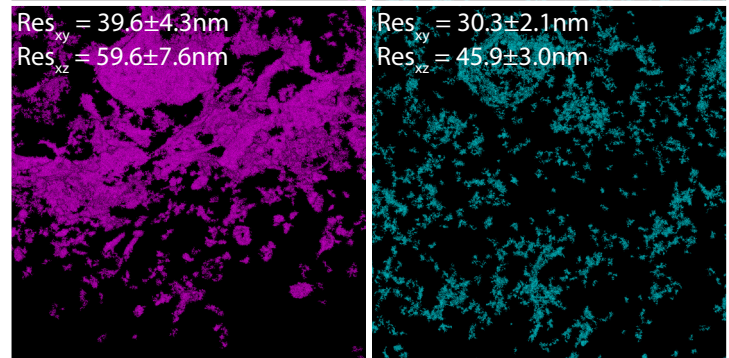
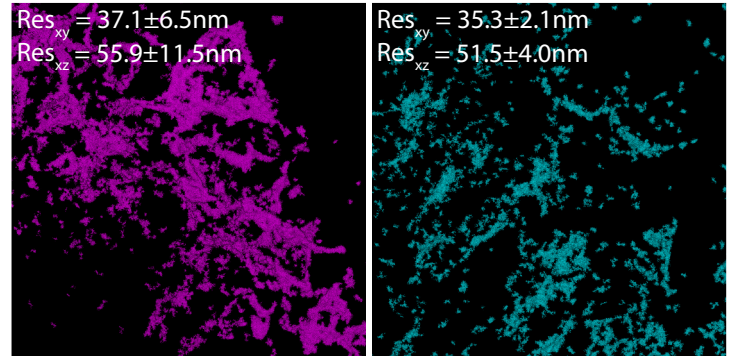
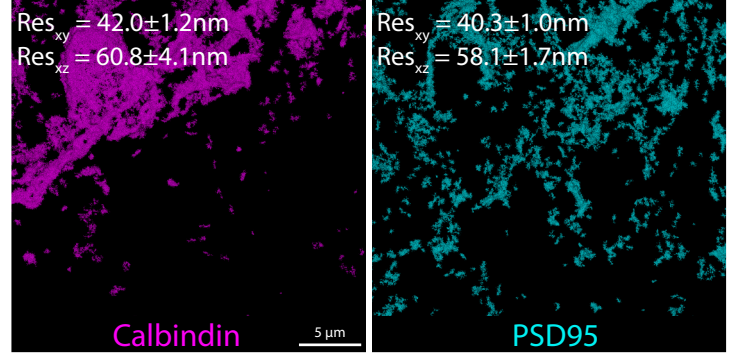


Tissue Preparation-Primary Fixation & Quenching, Reagent & Conc.

Unprocessed

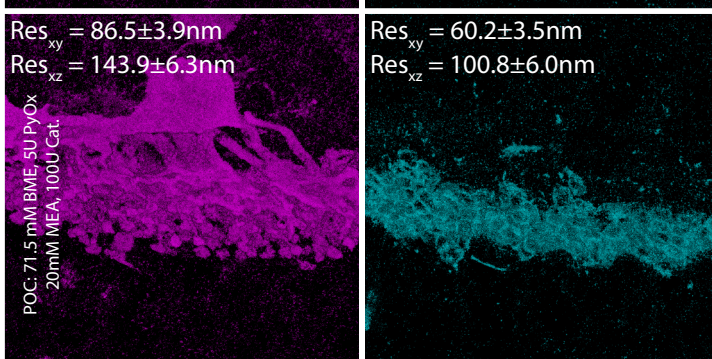
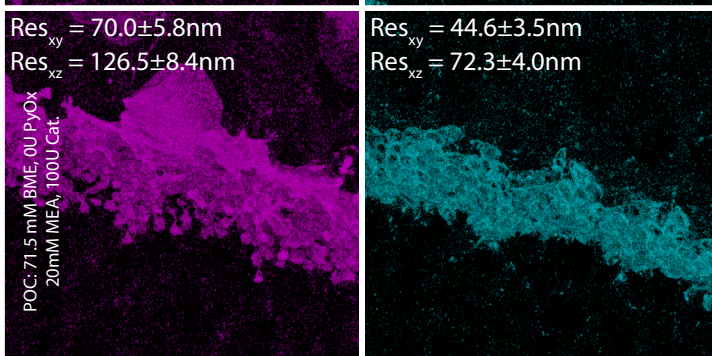
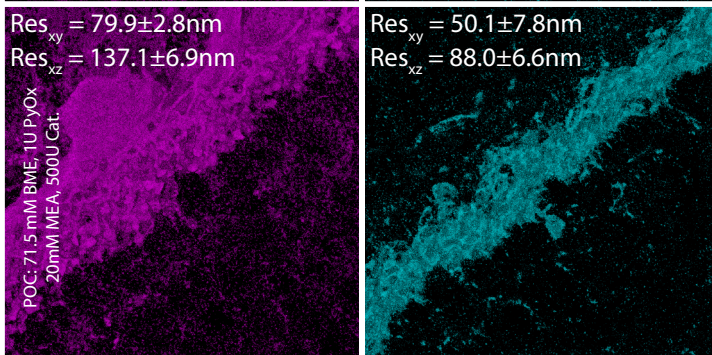
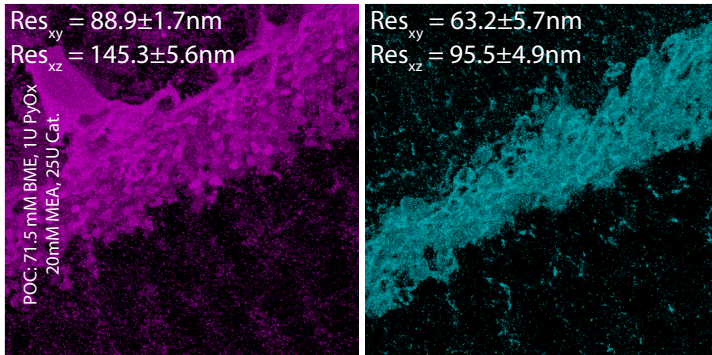
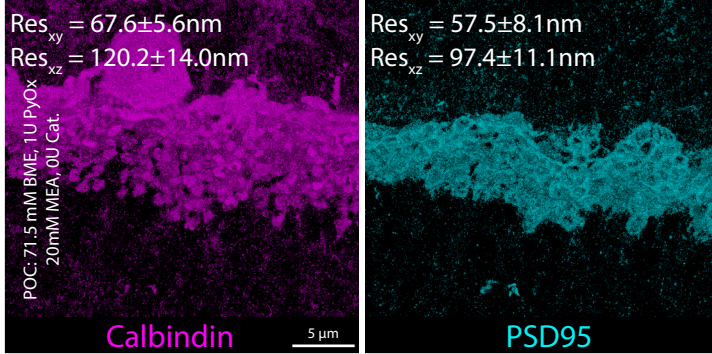


Processed

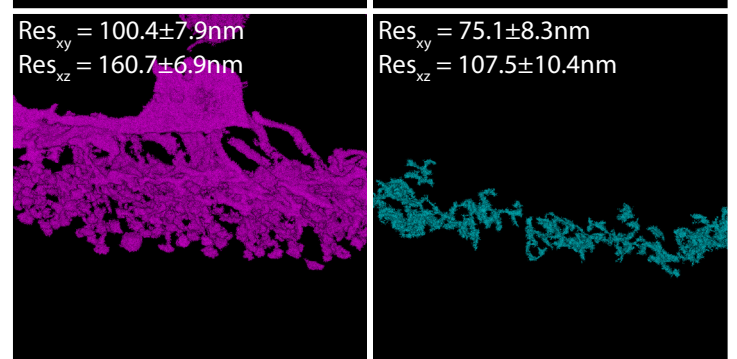
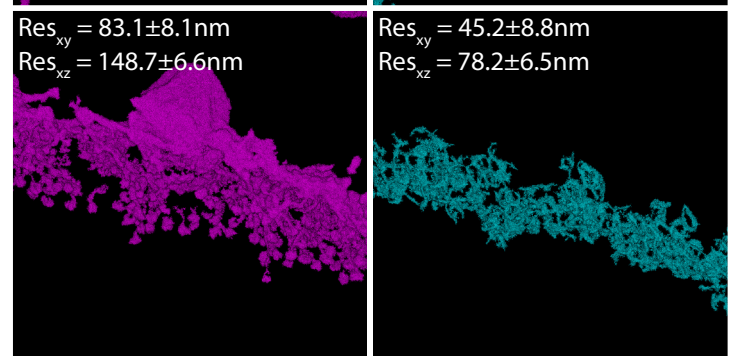
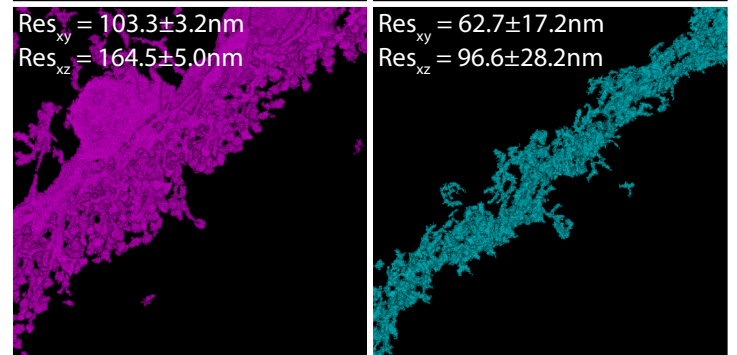
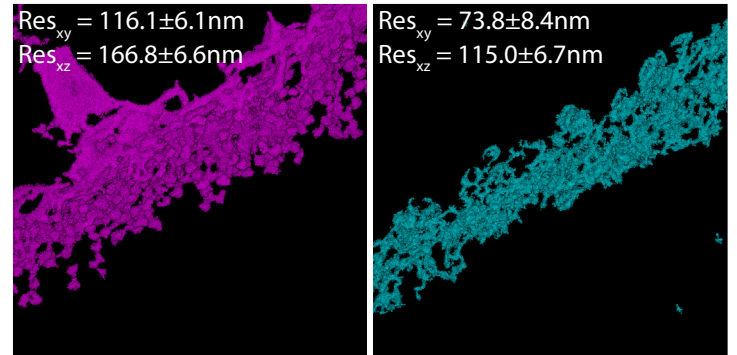
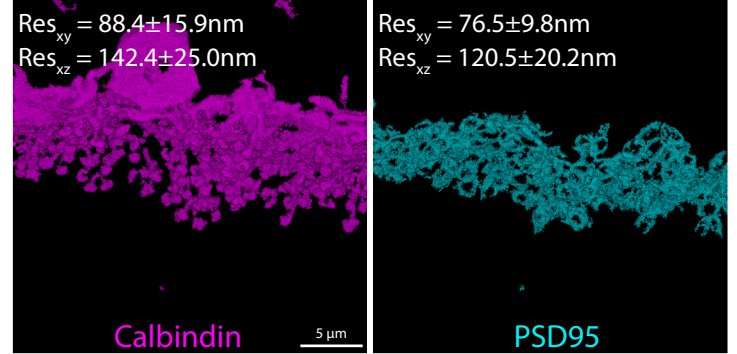


Imaging & Analysis - Imaging Buffer, Pyranose Oxidase Buffer

Unprocessed

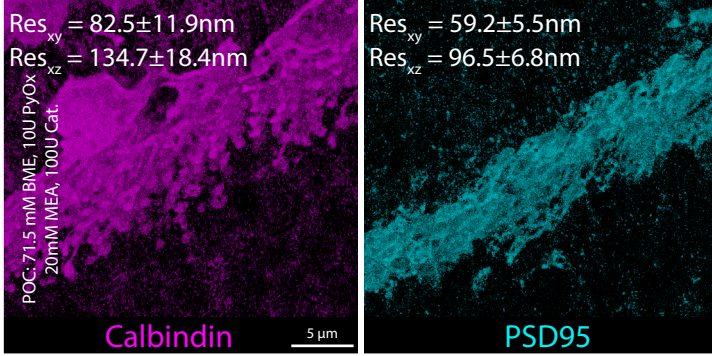


Processed

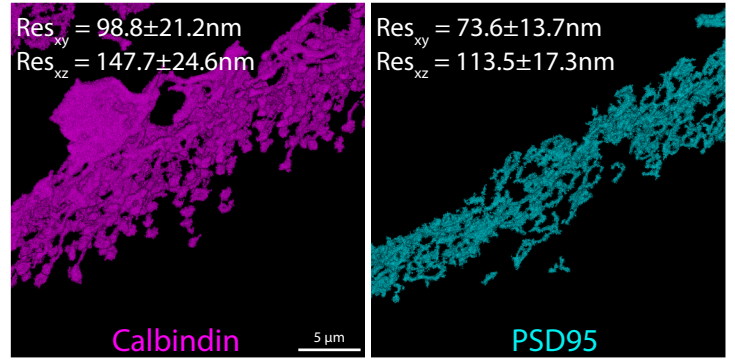


Imaging & Analysis - Imaging Buffer, Pyranose Oxidase Buffer

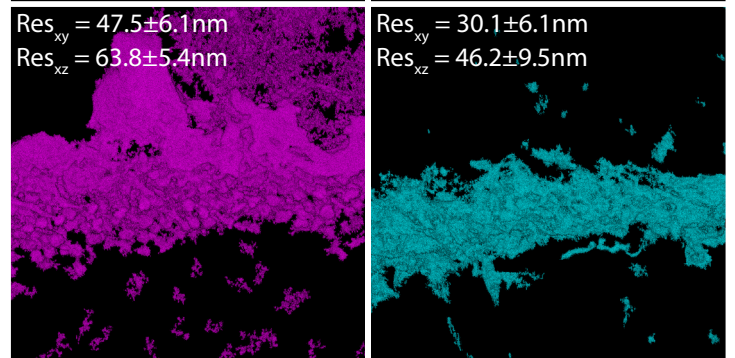
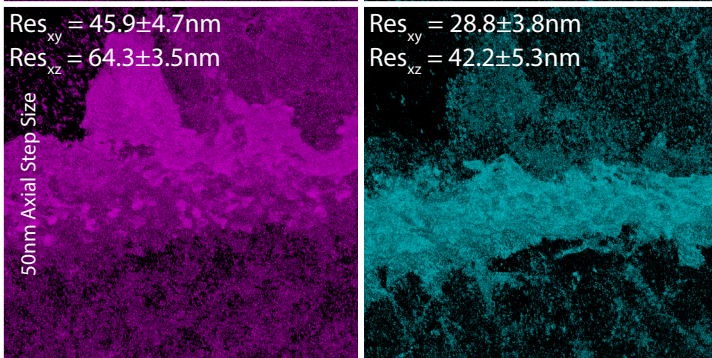
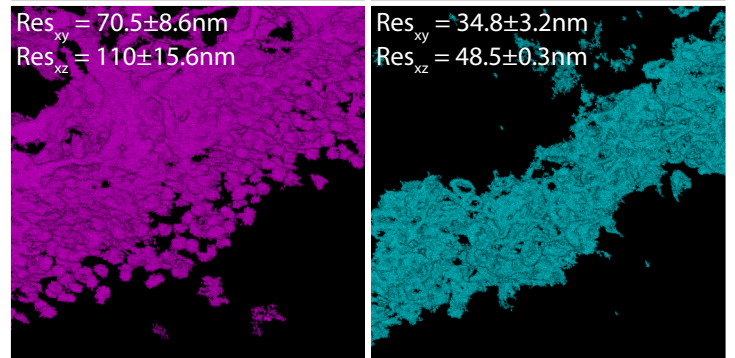
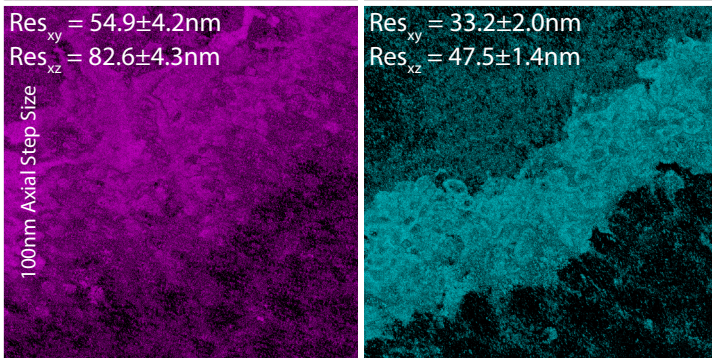
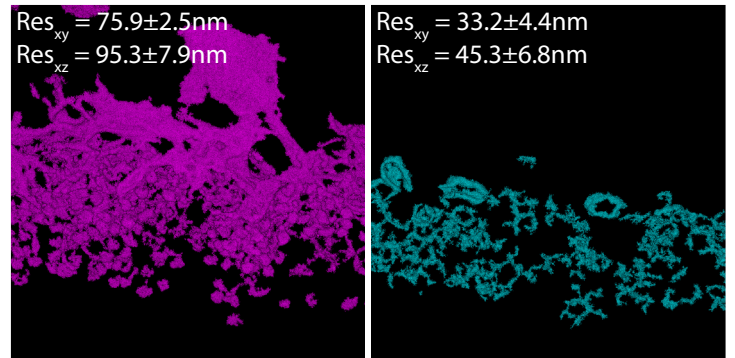
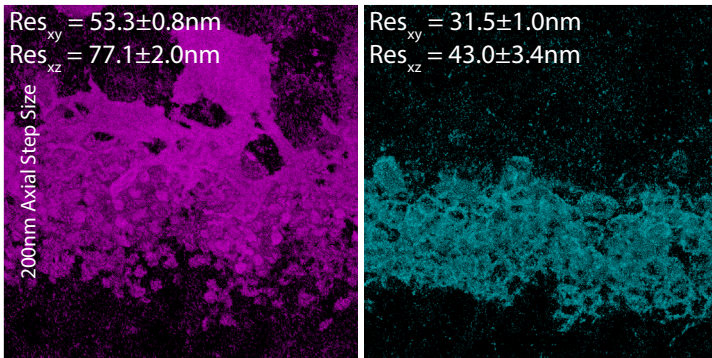
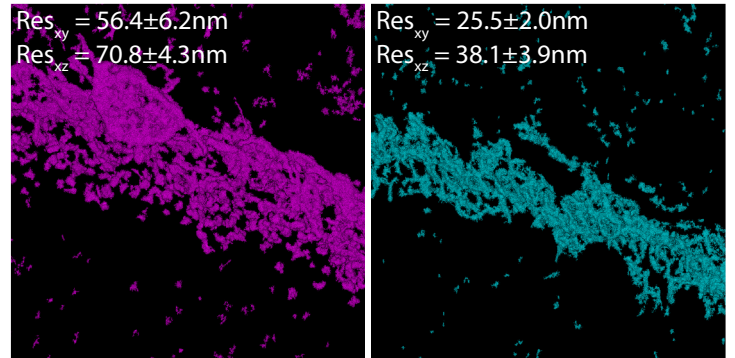
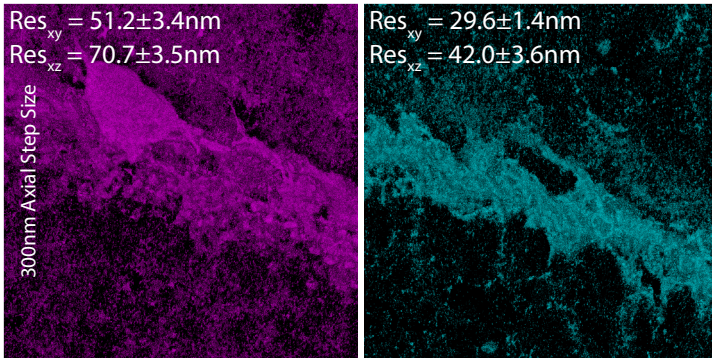
Unprocessed



Processed

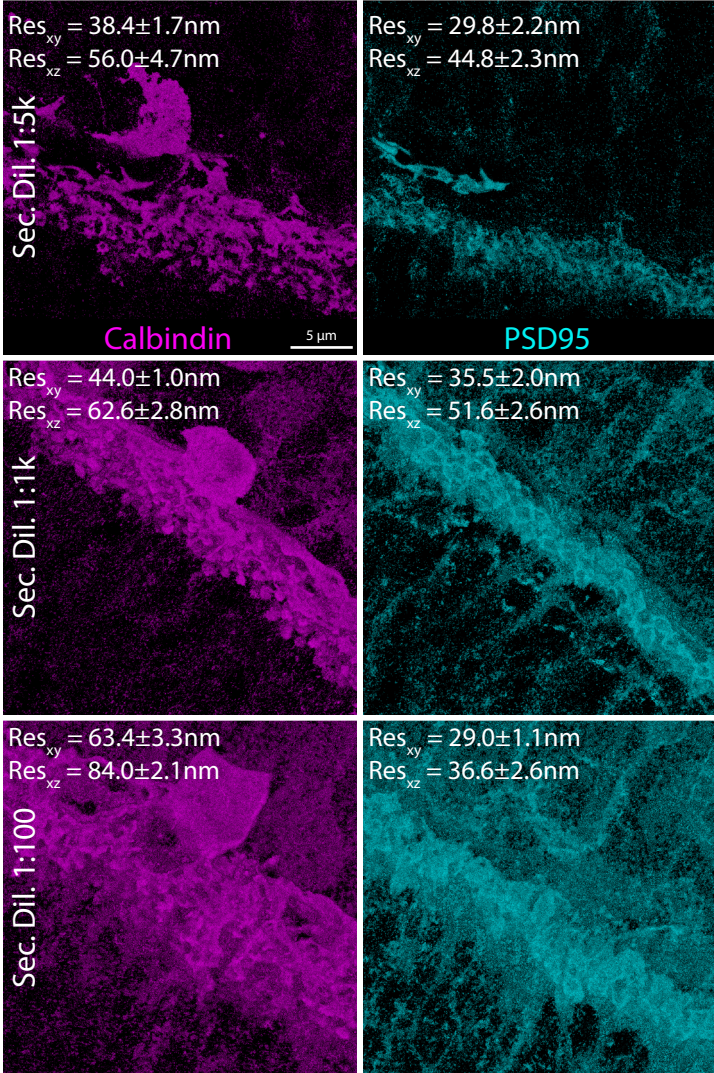


Imaging & Analysis - Image Acquisition, Step Size

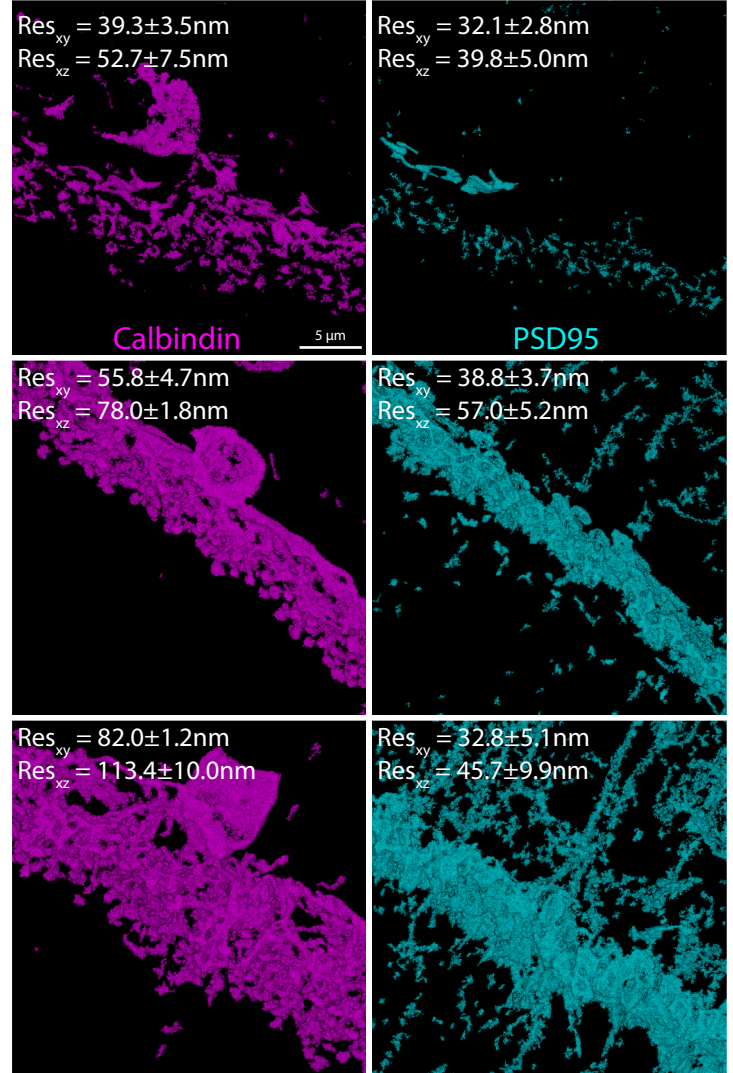


Staining- Secondary, Labeling Density

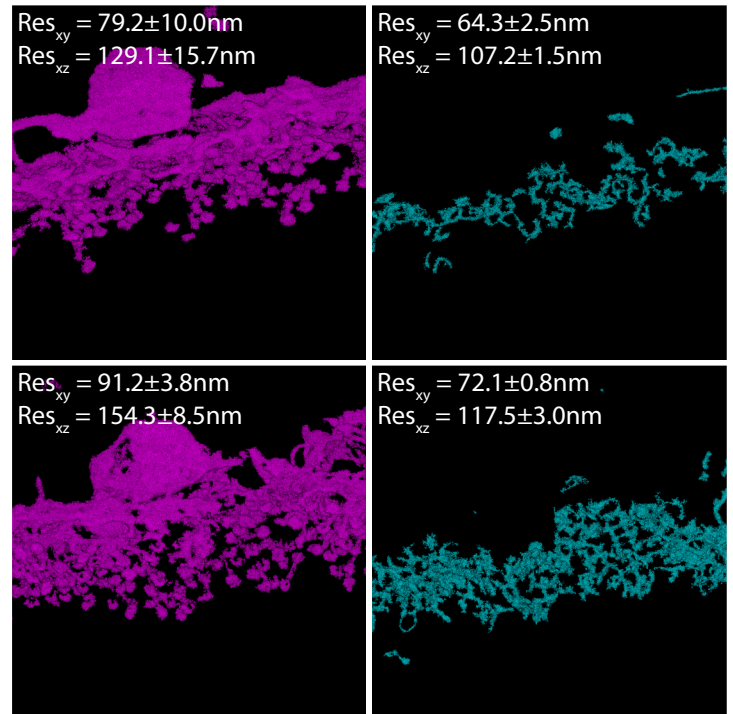
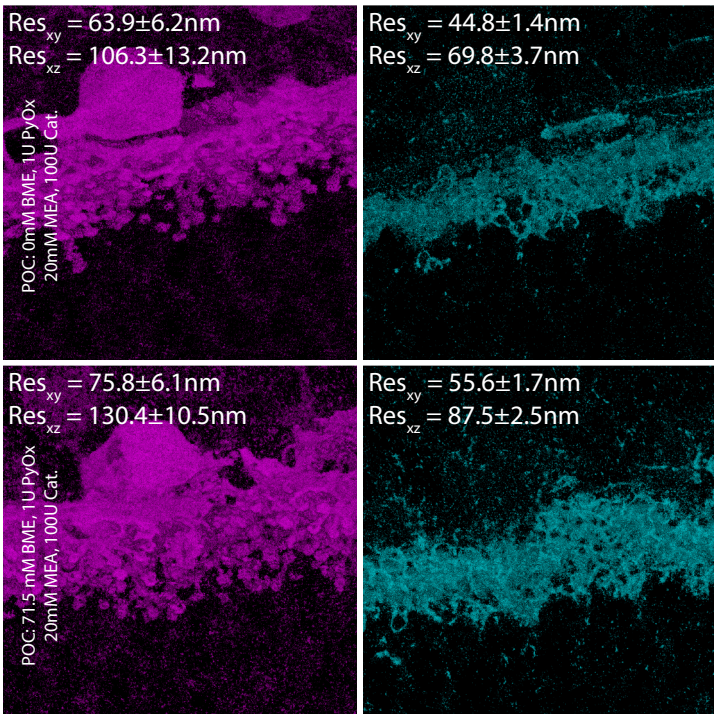
Unprocessed



Processed

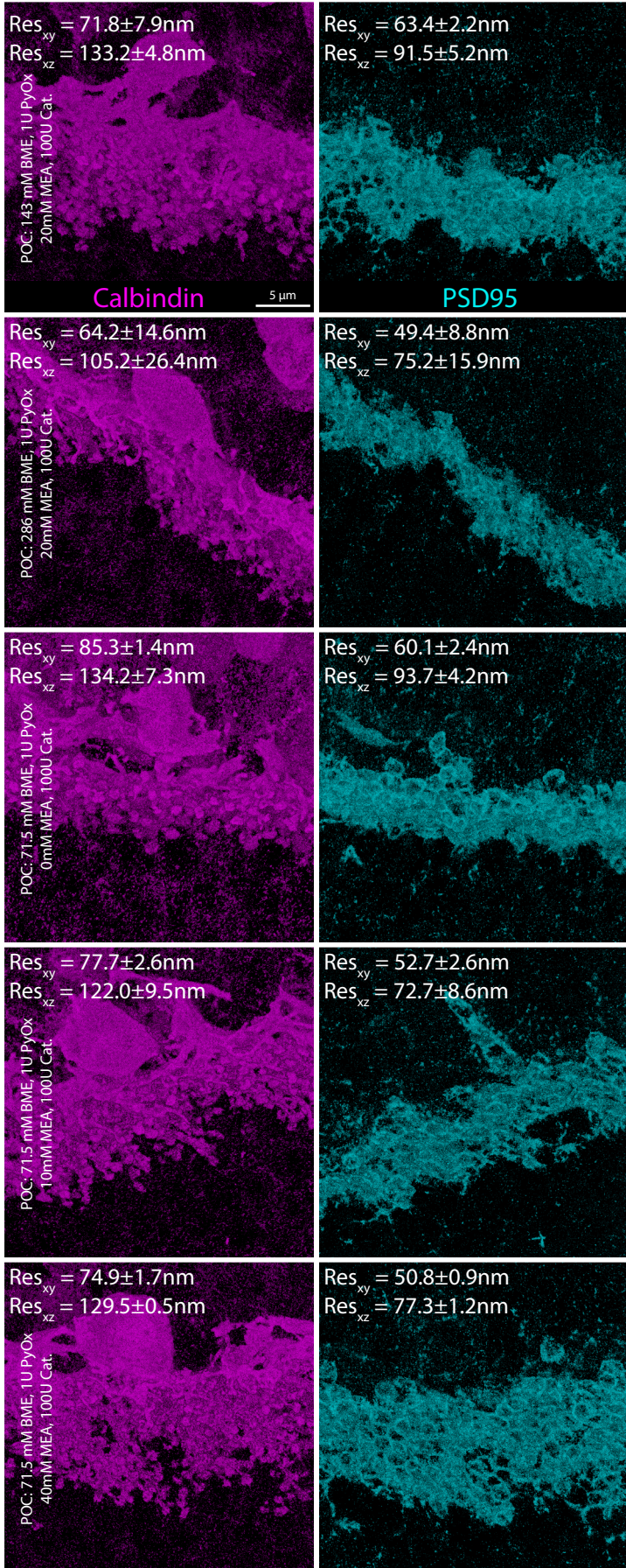


Imaging & Analysis - Imaging Buffer, Pyranose Oxidase Buffer

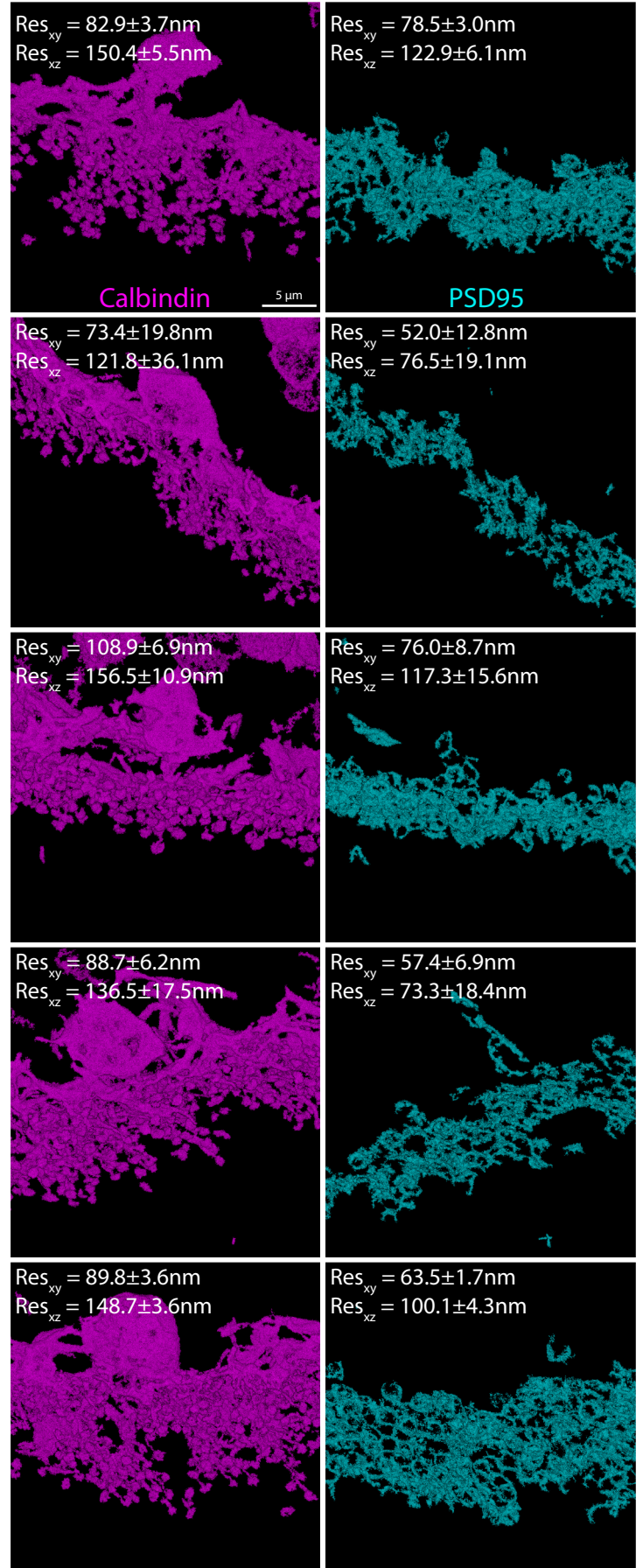


Imaging & Analysis - Imaging Buffer, Pyranose Oxidase Buffer

Unprocessed



Processed



Data S1. Sample and imaging conditions affect image quality and metrics, related to Figure 4.

Representative images of conditions are shown for each channel of a given condition. Unprocessed images are shown alongside their processed counterparts, as well as the resolutions that were measured for each condition. Each row shows a different condition tested. Calbindin-labeled horizontal cells are shown in magenta, and PSD95-labeled rod terminals in cyan, unless otherwise noted. Scale bars = 5 μm .

Supplemental Table 1: Primary antibodies used.

Antigen	Labeling specificity	Source	Dilution (confocal)	Dilution (RAIN-STORM)
Calbindin D-28K	Horizontal cells, subsets of amacrine cells, and retinal ganglion cells	Swant, Cat# CB38a, RRID: AB_10000340	1:5000	1:1000
CD31	Blood vessels, endothelial cells	Fisher, Cat# BDB5500274, RRID: AB_393571	1:200	1:50
Cone arrestin	Cone photoreceptors	Millipore, Cat# AB15282, RRID: AB_11210270	1:2000	1:1000
Collagen IV	Blood vessels	Millipore, Cat# AB769, RRID: AB_92262	1:1000	1:500
Connexin 43	Pericyte gap junctions	Sigma, Cat#C6219, RRID: AB_476857	1:1000	1:500
Desmin	Pericytes	Thermo Fisher, Cat# MA513259, RRID: AB_11000611	1:500	1:500
Dystrophin	Photoreceptor synapses	Abcam, Cat# ab15277, RRID: AB_301813	1:200	1:100
Glutamine synthetase (GS)	Muller glia	BD Biosciences, Cat# 610517, RRID: AB_397879	1:1000	1:500
GFAP	Astrocytes	Sigma, Cat# G3893, RRID: AB_477010	1:500	1:500
Iba1	Microglia	Abcam, Cat# ab5076, RRID: AB_2224402	1:500	1:500
Islet1	ON bipolar cells, starburst amacrine cells, subset of retinal ganglion cells	R&D system, Cat# AF1837, RRID: AB_2126324	1:2000	1:1000
NG2	Pericytes	Abcam, Cat# ab129051, RRID: AB_2877152	1:1000	1:500
PKC α	Rod bipolar cells	Abcam, Cat# ab31, RRID: AB_303507	1:500	1:500
PSD95	Photoreceptor terminals	Abcam, Cat# ab12093, RRID: AB_298846	1:500	1:500
RIBEYE	Ribbon synapses	Synaptic system, Cat#192103, RRID: AB_2086775	1:500	1:500
Secretagogin (SCGN)	Cone bipolar cells	BioVendor, Cat# RD181120100, RRID: AB_2034060	1:1000	1:500
Tau	Microtubule-associated protein	Proteintech, Cat#66499-1-Ig, RRID: AB_2881863	1:1000	1:500
α -Tubulin	α -Tubulin protein	Sigma, Cat#T5168-.2ML, RRID: AB_477579	1:1000	1:500
Tomm20	Mitochondria	Abcam, Cat# ab78547, RRID: AB_2043078	1:1000	1:500
Tyrosine hydroxylase (TH)	Dopaminergic amacrine cell subset	EMD Millipore, Cat# AB1542, RRID: AB_90755	1:2000	1:500
Vesicular glutamate transporter 1 (VGlut1)	Photoreceptor ribbon synapses	Abcam, Cat# ab77822, RRID: AB_2187677	1:500	1:250
Vesicular glutamate transporter 3 (VGlut3)	Subset of amacrine cells	Millipore, Cat#AB5421, RRID: AB_2187832	1:1000	1:500

Supplemental Table 2: Summary of condition variations tested for RAIN-STORM, Related to Figure 1.

Stage	Parameter	Variations	Specifics	Total Localizations Acquired	Background Localizations	AF647 XY Res	CF568 XY Res		
Tissue Preparation	Primary Fixation	Conc./Temp	1% PFA, RT, 60min	8213357	2254413	55.7234	39.7570		
			2% PFA, RT, 60 min	8970285	2398174	50.3231	34.2027		
			4% PFA, RT, 60 min	9146043	3144002	60.1768	36.6071		
			4% PFA, 4C, 60 min	6779812	2732175	56.5597	35.5531		
			0% PFA, 0.3% GA, RT, 60 min	4914685	3059107	38.5014	27.1380		
		Type	2% PFA, 0.3% GA, RT, 60 min	5030786	3181862	45.1080	29.3187		
			4% PFA, RT, 30 min	8642310	3722096	54.3354	31.3137		
			4% PFA, RT, 120 min	10108757	3498390	48.5256	32.5775		
			10mM Glycine	9761923	2841048	51.7930	31.7254		
			100mM Glycine	12209377	3525533	63.2148	37.2768		
	Primary Quenching	Conc./Type	10mM NH3Cl	9751623	3043674	50.2599	39.2483		
			100mM NH3Cl	11649151	3425099	55.7147	37.4105		
			0.1% NaBH4	10066425	2222857	53.5908	37.7183		
			0.5% NaBH4	11155141	2673369	52.2041	32.6448		
			Embedding Method	Thickness	10µm	9146043	3144002	60.1768	36.6071
			20µm	3775049	2494498	63.0147	66.6873		
	Staining	Blocking Buffer	Serum	1% NDS	12554580	3953868	59.1086	29.2762	
				3% NDS	14772243	4423472	58.0771	29.6001	
				5% NDS	10276383	3368744	53.7408	30.4973	
				10% NDS	9029693	2928266	69.5874	34.9191	
15% NDS				13108963	3920780	48.8157	32.4246		
0.1% Triton				9958189	3309736	57.0414	25.8641		
0.2% Triton				13145415	4107653	60.7596	35.5746		
0.5% Triton				9849749	3040177	57.1852	35.4691		
1.0% Triton				10455550	3467400	50.3823	30.9056		
2.0% Triton				10179749	3443009	50.8491	27.7015		
Permeabilizer Type			0.1% Saponin	0.1% Saponin	7443945	3638982	43.9059	26.9680	
				0.3% Saponin	6084083	2769395	48.9074	33.9253	
				0.5% Saponin	6343524	2916079	64.1917	41.0107	
				1.0% Saponin	7614606	3039943	50.7969	32.2521	
				2.0% Saponin	6171508	2849209	71.0758	39.7984	
			Temp/Time	4°C, 30 min	5067694	2089764	45.6442	35.7788	
				4°C, 120 min	5032729	2042575	45.9341	35.4185	
				RT, 30 min	5802683	2586804	43.9987	42.6722	
				RT, 120 min	5932099	2392430	48.0725	34.6311	
				Primary Antibody	Protein Targets	Calbindin			
CAR									
PKCa									
Rbeye									
PSD95									
Desmin									
Iba1									
SCGN									
Dystrophin									
GS									
GFAP									
Islat1									
Connexin43									
a-Tubulin									
Tom20									
CD31									
Collagen IV									
VGLUT1									
VGLUT3									
Tau									
NC2									
AF647 1:100		6073156	1867141			65.7862			
AF647 1:1000		3739319	1475533			43.9605	N/A		
AF647 1:5000		1188206	478033			38.4452			
CF568 1:100		6320942	2239627				30.8011		
CF568 1:1000		3430498	1453105			N/A	35.4525		
CF568 1:5000		855764	445984				29.7523		
Secondary Antibody		Fluorophore/Conc.	1% PFA, 10 min			6260957	2373438	46.1414	33.8572
			2% PFA, 10 min			6750477	2604387	48.1056	32.2951
			4% PFA, 10 min			8659132	3367457	54.9271	36.2804
			1% PFA, 30 min	5374777	2091335	55.9734	33.1415		
			2% PFA, 30 min	7270814	2700660	54.2458	42.6215		
		Conc./Time	4% PFA, 30 min	8267553	2996594	47.6018	35.7969		
			10mM Glycine	5330971	1691966	65.6889	55.4278		
			100mM Glycine	5636215	1918352	46.5097	44.4271		
			10mM NH3Cl	4791254	2077605	55.9535	46.1726		
			100mM NH3Cl	8210985	1932665	52.5240	43.5233		
GLOX		Catalase	0mM BME	13030311	3553994	78.36503	61.88567		
			71.5mM BME	10612112	2538832	70.96677	46.30657		
			143mM BME	11190641	2723165	88.47950	68.97667		
			286mM BME	9332914	2006797	73.10220	72.54603		
			0U Catalase	7104374	1453545	77.87603	71.28623		
			20U Catalase	7137281	2047426	72.29090	76.41330		
			100U Catalase	9595769	2075109	84.14287	65.44140		
			500U Catalase	10195843	2537068	71.02247	64.64857		
			0U Glu. Ox.	7104374	1453545	77.87603	71.28623		
			10U Glu. Ox.	7137281	2047426	72.29090	76.41330		
		Glucose Ox.	20U Glu. Ox.	9595769	2075109	84.14287	65.44140		
			40U Glu. Ox.	10195843	2537068	71.02247	64.64857		
			0mM MEA	10036933	2362114	82.48007	67.48763		
			10mM MEA	11484612	2540791	74.56673	75.99387		
			20mM MEA	8917762	2307084	71.94410	66.66033		
			40mM MEA	9962371	2736120	73.49553	75.60517		
			0mM BME	6344927	2172067	63.94573	44.79663		
			71.5mM BME	6632177	2110500	75.79637	55.62803		
			143mM BME	6865185	1935796	71.76753	63.42270		
			286mM BME	6990130	2025152	64.24200	49.40647		
		PPT	PCD	0U PCD	1878393	1100339	63.68107	42.81973	
				-0.09U PCD	3218723	1402939	68.00433	55.09720	
				-0.17U PCD	3178465	1258718	71.34050	60.59277	
				-0.35U PCD	2138172	1068920	53.55533	44.69093	
				0mM MEA	4388805	1545313	53.09887	54.86183	
			MEA	10mM MEA	4151177	1465681	73.34697	68.12620	
				20mM MEA	3063824	1336807	70.74163	56.03840	
				40mM MEA	2834421	1417678	58.42780	54.92740	
				0mM PCA	1501576	997725	62.61477	45.58707	
				1mM PCA	4425433	1679832	74.27553	74.12297	
POC		BME	2mM PCA	3473750	1608543	72.63673	55.05123		
			4mM PCA	2099282	1104718	72.42687	63.55333		
			0mM BME	6344927	2172067	63.9457	44.7966		
			71.5mM BME	6632177	2110500	75.7964	55.6280		
			143mM BME	6865185	1935796	71.7675	63.4227		
		Pyranose Ox.	286mM BME	6990130	2025152	64.2420	49.4065		
			0U Py. Ox.	6160037	1609622	69.9593	44.6356		
			1U Py. Ox.	7348113	1753847	85.9037	59.7095		
			5U Py. Ox.	6057926	1699933	86.5024	60.1876		
			10U Py. Ox.	7268535	2161980	82.4528	59.1651		
Electron Sinks		MEA	0mM MEA	8338811	2172407	85.3317	60.0705		
			10mM MEA	6045768	1873840	77.7011	52.7179		
			20mM MEA	6072488	2293750	73.6791	55.3963		
			40mM MEA	6479168	1771027	74.9191	50.7731		
	0U Catalase		6053582	1916488	67.6008	57.5033			
	Catalase	20U Catalase	6486951	2078212	88.8967	68.1553			
		100U Catalase	7462499	2963145	83.4481	59.2703			
		500U Catalase	8097364	2179157	79.8785	50.1370			
		1mM Trolox	10297129	2172781	76.4754	82.0630			
		2mM Trolox	5733708	1578968	75.8681	85.4778			
Conc./Type	5mM Trolox	2270131	862326	70.5364	70.7546				
	1mM COT	7249814	2213086	68.4071	53.4930				
	2mM COT	8668903	1677820	79.6458	78.9307				
	5mM COT	7412183	1543031	76.8966	62.1923				
Optimized RAIN-STORM				12804183.3333	7082472.0000	61.1141	31.2234		
2% PFA 4C 60 min, 100mM Gly 4C 60min, 5% NDS, 0.5% Triton, 60min RT Block, 1.500 Ph DI 12h, 1:100 Sec Di 60min, 4% PFA 30min, 100mM NH4Cl 30min, 1U Py. Ox, 143mM BME, 200U Catalase, 40mM MEA, 2mM COT, 200nm Step size, 250f, 3 cycle, 0.16µm, 25p									
85000						1.50E+07	localizations		
20						90	nm		
Abbreviations									
RT	room temperature								
U	Units								