Cell

Supplemental Information

Simple, Scalable Proteomic Imaging for High-Dimensional Profiling of Intact Systems

Evan Murray, Jae Hun Cho, Daniel Goodwin, Taeyun Ku, Justin Swaney, Sung-Yon Kim, Heejin Choi, Young-Gyun Park, Jeong-Yoon Park, Austin Hubbert, Margaret McCue, Sara Vassallo, Naveed Bakh, Matthew P. Frosch, Van J. Wedeen, H. Sebastian Seung, and Kwanghun Chung

Supplemental Experimental Procedures

Mice

Young adult male and female C57BL/6 and Thy1-eGFP-M mice were housed in a reverse 12-hr light/dark cycle with unrestricted access to food and water. All experimental protocols were approved by the MIT Institutional Animal Care and Use Committee and Division of Comparative Medicine and were in accordance with guidelines from the National Institute of Health.

Perfusion

Mice were transcardially perfused with ice-cold PBS and a solution consisting of 4% PFA and 1–4% GA in PBS. Brain tissues were harvested and incubated in the same fixative solution at 4°C for 2–3 days and 2–7 hr at 37°C with gentle shaking to allow for uniform fixation throughout the sample. This incubation time is critical for mitigating the effects of variable perfusion quality and promotes uniform structural and molecular preservation of the sample throughout the SWITCH process, as samples that are not ideally preserved will experience greater loss of biomolecules and a greater degree of sample deformation.

SWITCH-mediated Tissue Preservation

PFA-fixed human samples were washed in a solution consisting of 50% PBS titrated to pH 3 using HCl, 25% 0.1 M HCl, and 25% 0.1 M potassium hydrogen phthalate (KHP). This wash solution was then replaced with fresh solution with the addition of 4–10% GA. The samples were then incubated in this pH 3 solution at 4°C for 2 days with gentle shaking. The acidic pH of this solution greatly slows down the reaction speed of aldehyde fixatives. The solution was then replaced with PBS with the addition of 1–4% GA and the sample was again allowed to incubate for 2–3 days at 4°C and 2–7 hr at 37°C with gentle shaking. The sample was then washed in PBS at room temperature (RT) for 1 day with gentle shaking. After washing, reactive GA within the sample was inactivated by incubation in a solution consisting of 4% glycine and 4% acetamide for 1 day at 37°C with gentle shaking. Finally, the sample was washed for 1 day in PBS at RT with gentle shaking.

GA Gelation Time Experiment

All reagents and containers were first cooled to 4°C and handled on ice. For pH 7 gels, 10 mL of a PBS solution containing 10% BSA was made prior to GA injection. For pH 3, 10 mL of a 0.1 M KHP buffer was titrated to pH 3 with HCl. Once the pH 3 and 7 BSA solutions were prepared, GA

was added to a final concentration of 4% and a timer was started. Gelation was judged by inverting the tube and inspecting for fluid flow. The time required to form rigid gels was recorded in 3 replicates of the pH 3 and 7 condition.

Gel Denaturation Experiment

For acrylamide (AA) gels, 10 mL of a 4% PFA, 5–15% bovine serum albumin (BSA), 4% AA, and 0.25% VA-044 solution was prepared in PBS. The polymerization was carried out under vacuum for 2 hr at 37°C in a 15 mL tube. For GA gels, 10 mL of a 5–15% BSA and 1% GA solution was prepared in PBS and allowed to gel at room temperature for 2 hr in a 15 mL tube. For the epoxide gels, 10 mL of a 5–15% BSA and 15% epoxide (i.e., EX-313, GE31, GE22) solution was prepared in 0.1 M carbonate buffer at pH 9 and allowed to gel at 37°C for 8 hr in a 15 mL tube. Each gel was extracted from the tubes and cut into approximately 5-mm-thick disks. The disks were then washed overnight in PBST to remove any unreacted reagents. After washing, each disk was massed and photographed. The disks were then transferred to an 80°C water bath and incubated overnight. The disks were removed, massed, and photographed again.

Sodium Borohydride Treatment

Sodium borohydride (SB) buffer was made immediately before use by making a 1 mg/ml SB solution with PBS. For 100-µm sections, the tissue was treated 3 times, 10 minutes each. For whole brain, the tissue was treated 3 times, 3 hr each. For both, incubation was done at room temperature without shaking.

Passive Clearing with Thermal Energy

Aqueous clearing solution containing 200 mM SDS, 10 mM lithium hydroxide, 40 mM boric acid, and a variable amount of anti-browning agent (i.e., 0–50 mM sodium sulfite or 0–0.5% [w/v] 1-thioglycerol) was titrated to pH 9 using sodium hydroxide before use. For high temperature clearing, samples were incubated in 40 mL of clearing solution for 8 hr at RT with gentle shaking to allow for the anti-browning agent to diffuse throughout the tissue. Samples were then transferred to a water bath set at 60–80°C. The clearing buffer was replaced if any noticeable color was observed in solution at any point during clearing. To remove the remaining anti-browning agent and SDS after clearing, the samples were washed at 37°C for 24 hr in 40 mL PBST containing 0.02% sodium azide as a preservative.

mRNA FISH

Mice were perfused with ice-cold PBS and then with fixative (4% PFA and 1% GA in PBS). Brains were incubated in the fixative for one day at 4˚C and then 6 hr at RT for post-fixation. Coronal sections were prepared with a vibratome and sections were inactivated at RT for 6 hr, followed by tissue clearing under 37˚C or 70˚C. All solutions were prepared by using diethylpyrocarbonate (DEPC)-treated water. Digoxigenin- and 2,4-dinitrophenol (DNP)-labeled *fos* cRNA probes were detected using horseradish peroxidase-conjugated antibodies. FISH signals were visualized using a tyramide amplification kit (Perkin Elmer).

Refractive Index Matching

A customized refractive index (RI)-matching solution was made by dissolving 50 g diatrizoic acid, 40 g n-methyl-d-glucamine, and 55 g iodixanol per 100 mL water. Cleared samples were incubated in 10 mL of this solution at RT with gentle shaking for 2 days prior to imaging, replacing the solution after the first day. The listed components and their proportions were chosen to adjust the pH and RI for ideal optical clearing (basic pH with RI near 1.47) as well as optimize the osmolarity of the solution to reverse the sample expansion observed after clearing. The contrast agents, diatrizoic acid and iodixanol, significantly affect the RI of the solution, while n-methyl-d-glucamine is used to adjust the pH to more basic values. All components were considered when optimizing for osmolarity. RI was measured using an Abbemat WR/MW automatic multiwavelength refractometer.

Mounting and Imaging

To facilitate the use of long working distance immersion objectives, samples were mounted between a slide glass and a glass-bottom Willco dish. Blu-Tack adhesive was rolled into a cylindrical shape of a thickness slightly greater than that of the sample and was placed in a circular orientation on the slide glass with a small opening to allow addition of immersion medium after chamber construction. The sample was placed within the Blu-Tack circle and the Willco dish was secured onto the adhesive, pressing just firmly enough to make slight contact with the sample. This contact prevents the sample from moving during the imaging process, but minimizes sample deformation. For multiplexed staining experiments, contact was not made with the sample. Taking care to avoid introduction of bubbles, RI-matching solution was injected to fill the void space, and the opening was then closed using fast-curing epoxy glue. Three microscope systems were used for the experiments in this study:

i. Olympus two-photon microscope system (FV1200MPE) equipped with a 25× CLARITY-optimized objective (prototype; NA, 1.0; WD, 8.0 mm), a 10× CLARITY-optimized objective

(XLPLN10XSVMP; NA, 0.6; WD, 8.0 mm), a 10× water-immersion objective (NA, 0.30; WD, 3.6 mm), and a 40× oil-immersion objective (UPLSAPO40XS; NA, 1.25; WD, 0.3 mm). 405, 488, and 635 nm 1p lasers were used;

ii. Leica TCS SP8 microscope system equipped with a white-light laser, spectral detection system, a 20× water-immersion objective (NA, 0.50; WD, 3.5 mm) and a 25× water-immersion objective (NA, 0.95; WD, 2.4 mm).

iii. Custom-made light-sheet microscope equipped with 10× CLARITY-optimized objective (modified from Tomer et al., 2012).

Sample datasets were visualized with IMARIS (Bitplane).

Sample Delabeling

Imaged samples were delabeled in clearing solution at 60–80°C (elution condition) for 1–2 days for large samples and overnight (O/N) for thin samples. Sulfites were added for large samples to prevent browning during the extended delabeling process.

Protein Loss Assay

Mouse brain samples were prepared using various preservation methods, hemisected, and then cut into 1-mm sections. The collection of sections from each hemisphere was massed and then placed into 5 mL of 200 mM SDS clearing solution. The samples were incubated at 37°C with gentle shaking for 2 weeks. A small aliquot was taken from each tube and analyzed using the Bio-Rad DC protein assay kit to quantify the degree of protein loss from the samples.

Microstructure Preservation Assay

A Thy1-eGFP-M mouse was perfused with SWITCH fixative solution and the sample was cut into 1-mm sections. The eGFP expression on the surface of a section was imaged using confocal microscopy and then sample was then subjected to clearing under harsh conditions (200 mM SDS, 80°C) for 1 day. The sample was then labeled using anti-eGFP antibodies and the same region was imaged again using confocal microscopy.

Macrostructure Preservation Assay

Samples processed using SWITCH and CLARITY were cut in 1-mm sections and subjected to clearing under harsh conditions for 1 day. The samples were then mounted in a chamber larger than the size of the sample to prevent compression and imaged using confocal microscopy. RI-

matching solution was used to facilitate imaging of the sample. Cross-sections from the samples were visualized using IMARIS.

Human Tissue Samples

Samples of fixed autopsy tissue were obtained from the Neuropathology Core of the Massachusetts Alzheimer Disease Research Center. Tissue was collected and banked in accordance with approval from the local Institutional Review Board. All samples studied came from subjects without evidence of neurologic disease on clinical grounds at the time of death and without evidence of significant disease processes upon full neuropathologic examination.

Multiplexed Labeling of Thin Samples

A human clinical sample containing visual cortex was obtained and processed using SWITCH. After fixation, 100-μm sections were obtained from the sample and cleared under harsh conditions. In each round, the sample was labeled with DAPI, DyLight 488-conjugated lectin, and a variable antibody using standard immunolabeling procedures. The sample was mounted in a chamber larger than the size of the sample, imaged using confocal microscopy, and then delabeled under harsh conditions O/N. RI-matching solution was used to facilitate imaging of the sample.

Co-Registration of Multiplexed SWITCH Experiments

To register the set of SWITCH experiments from a single tissue, one of the experimental rounds for a tissue is arbitrarily chosen as the fixed reference to which the rest of the experiments will be registered. Each experiment has one fluorescence channel dedicated to lectin, which allows the software to identify distinctive points in the vasculature in order to achieve the fine morphological adjustments across the tissue volume. We used a 3D Harris Corner detector (Harris and Stephens, 1988) to find those keypoints and a 3D modification of the SIFT descriptor (Lowe, 2004; Scovanner et al., 2007) to calculate correspondences. For robustness, we instituted a variation of RANSAC (Fischler and Bolles, 1981) to test affine transformations on local subvolumes, confirming that keypoint correspondences between experiments were legitimate. Finally, with the validated keypoints, a thin plate spline interpolation (Bookstein, 1989) was implemented to warp the tissue in a physically plausible manner.

Each experiment was processed individually by eye before using software to calculate the registration: The size of the interrogated tissues, approximately 1 mm^3 in volume with 1.09 μm × 1.09 μm × 1.99 μm resolution, required imaging subvolumes that were stitched together using the

Leica or Olympus microscope software. The resulting tissue volume, a 4-dimensional object for the three spatial coordinates and the fluorescence channel, was examined by human eye using FIJI (Schindelin et al., 2012) to ensure image quality and a common orientation across experiments. Additionally, a rectangular crop was made around the tissue to remove unnecessary, blank voxels. Each individual experiment was then processed using a MATLAB computational pipeline developed for SWITCH and shared online via Github (https://github.com/dgoodwin208/Registration).

The registration pipeline has five primary steps based on the Lectin channel of each experiment. First, the image volume is partitioned into 25 subvolumes for parallelization of work and robustness checking in later steps. Each subvolume then identifies distinguishable keypoints using a 3D Harris Corner Detector and uses a 3D modification of SIFT written by Scovanner (http://www.cs.ucf.edu/~pscovann/) to create a descriptor vector associated with the keypoint. Note that we calculated keypoints and descriptors at multiple scale levels, achieved by convolution with Gaussian kernels of progressive size, to ensure a sufficient degree of scale invariance to the detected descriptors to successfully find matches despite differences in microscope setups across experiments. The calculations for keypoints and descriptors was often calculated in parallel across subvolumes to save time.

The third step is that each subvolume searches for corresponding keypoints in the appropriate subvolume in the reference experiment using the SIFT metric for measuring similarity between descriptors. To accomplish this we used the open-source VLFeat (http://www.vlfeat.org) implementation of SIFT matching algorithm. The fourth step is that the keypoint correspondences are validated via calculating affine transformations of random subsets of 4 corresponding keypoints, and the number of inliers of the resulting transformation assessed by a voxel distance threshold of 3 pixels. Each time a pair of corresponding points is counted as an inlier, it receives a vote, and after the order of 10^6 affine transformations, correspondence pairs with at least 80% of the votes of the highest voted pair are kept as legitimate correspondences. Finally, the validated correspondences are used to calculate a thin plate spline for the entire volume using an open-source TPS implementation written by Yang, Foong and Ong (http://www.mathworks.com/matlabcentral/fileexchange/47409-glmdtps-registration-method/content/GLMD_Demo/src/TPS3D.m), resulting in a highly accurate warp to match the morphology of the reference experiment.

Semi-automatic Identification of Cells and Blood Vessels

Image volumes were displayed and analyzed using custom-built graphical user interface software developed with Delphi XE4 (Embarcadero Technologies). Each image section was preprocessed to correct the inhomogeneous illumination at each image tile. In detail, we subtracted the mean intensity of a 100 × 100 μm^2 window centered to each pixel from its intensity to uniformize the background intensity. A different algorithm was devised specifically for each marker to semi-automatically detect the centroid location and soma size of all cellular objects and vascular pixels. In general, a spherical soma volume was isolated according to the best contrast between intrasomal pixels and background pixels by increasing the size of concentric spheres, and the soma size was determined as the spherical diameter. After this automatic detection process, we corrected misidentified cell bodies manually, and the portion of the correction was less than 10%. We applied normalization of foreground signal and a Gaussian filter to the NeuN channel prior to the analysis. SMI-32^+ cells were fully recognized manually according to their characteristic feature of the soma connected to a vertically oriented fiber with a large nuclear shadow, and the determination of their coordinates and soma sizes was aided by an automation module of the software. Each section in the lectin channel was converted to a vascular pixel mask image according to a customized threshold, and unconnected small clusters of pixels were removed.

Quantitative Analysis of the Co-registered Image Channels

We used a series of custom-built software developed with Delphi XE4 for the quantitative analysis. For co-expression analysis of two or more markers, an initial decision was made by checking whether the centroids of cells in each channel fell within a 5-μm distance. This classification was then manually verified with a quick review software tool. Especially, $NeuN^-$ neurons were carefully reviewed, and any weak NeuN signal that changed synchronously with the other marker signal was identified as $NeuN^+$. Cell density, vascular density, and cell- or pixel-to-vessel distance along cortical depth were obtained from their average of 50- or 100-μm window with a 50- or 100-μm interval, and data points of less than 10 objects were excluded from plotting. Cell and vascular densities were corrected to exclude the dead volume outside the tissue in the ROI. The cell-to-vessel distance was calculated as a distance from the centroid location of the cell to the nearest vascular pixel. Cells or pixels positioned at the sections containing any incomplete vascular information ($z \leq 24$ μm or $z \geq 80$ μm) were excluded from the analysis of distance to nearest vessel. The distribution profile of cell-to-vessel distance was obtained with a 3-μm interval.

SWITCH-mediated Myelinated Fiber Labeling

To create a DiD solution for myelinated fiber labeling, 1 mg of DiD powder was dissolved in 200 µL of a solution consisting of 10 mM SDS in PBS (SWITCH-Off). For 1-mm mouse sections, samples were incubated in SWITCH-Off solution O/N with gentle shaking at 37°C. The solution was replaced with a volume of fresh SWITCH-Off buffer that was sufficient to cover the sample, and 1 µL of the DiD solution was added. The sample was allowed to incubate for 1 day at 37°C with gentle shaking, at which point the sample was moved to a large volume of PBST (SWITCH-On) for 1 day at 37°C with gentle shaking. The sample was imaged using confocal microscopy. RI-matching solution was used to facilitate imaging of the sample.

For mouse hemispheres, the sample was incubated in SWITCH-Off solution at 37°C O/N with gentle shaking and then transferred to a volume of fresh SWITCH-Off solution sufficient to cover the sample, at which point 2 µL of the DiD solution was added. The sample was incubated in this solution at 37°C for 4 days with gentle shaking and then moved to a large volume of SWITCH-On solution for 1 day at 37°C with gentle shaking. RI-matching solution was used to facilitate imaging of the sample.

Orientation Analysis of Myelinated Fibers

Analysis was performed on planar images in the xy, yz, and xz planes using OrientationJ (http://bigwww.epfl.ch/demo/orientation/). Specifically, OrientationJ was used to calculate the preferred orientation of each pixel (ranging from −90° to 90°) using the corresponding finite difference gradient. This generates planar images whose pixel values correspond to the angular component in that plane (i.e., xy planar image contains θ_{xy}). After obtaining this orientational information in xy, yz, and xz for all slices, the separate components of the orientation (i.e., θ_{xy} contains x and y components of the orientation) are added together to yield three-dimensional orientation vectors. The orientation vectors represent the orientations of the fibers and the fascicles. These orientation vectors can be binned according to their angles to yield information about how the fibers and the fascicles are distributed in terms of their orientation. This information can then be used to predict what angle of intersection these fibers make. Specifically, each peak in the histogram is identified and the subpopulation is estimated based on the FWHM. These are then assigned to either fibers or fascicles based on observation (i.e., in the xy plane, the image shows that fibers make a vertical/horizontal grid while the fascicles make more of a diagonal/diagonal diversion; this means that the peaks near 0 and 90 (which is equivalent to −90) correspond to the fibers, and the peaks near −45 and 45 correspond to the fascicles). After obtaining the total populations of the entire volume, the intersections are estimated by subtracting the corresponding two peaks and then scaling that result by the FWHM. Then, assuming that all

fibers and fascicles have similar pixel counts, the fraction of fibers making certain intersections can be determined.

Autocorrelation Analysis of Myelinated Fibers

The analysis using the finite difference gradient is a nonlinear process that may introduce error to the analysis. The error is compounded by the fact that the z-resolution of the volume is almost three times lower than the x- and y-resolutions. A more accurate approach would be to use autocorrelation. (The finite difference gradient acts as a high pass filter for the autocorrelation.) Autocorrelation would show all the peak distributions in a more non-biased manner. As such, we calculated the autocorrelation in the volume image using MATLAB. Specifically, we used the Fourier convolution theorem with 3DFFT and a periodic boundary condition to calculate the autocorrelation of the volume image filtered with a Gaussian window; then, we transformed the resulting autocorrelation data in Cartesian coordinates to spherical coordinates and integrated out the radial component to visualize the data.

SWITCH-mediated Antibody Labeling

Samples were first equilibrated in a large volume of SWITCH-Off solution (0.5 mM SDS in PBS). Samples were then moved to a volume a SWITCH-Off solution just large enough to cover the sample and containing 20 µL of antibody solution (for histone H3 staining of 1-mm-thick tissue blocks.) Care should be taken to ensure that the final concentration of SDS in the SWITCH-Off solution is appropriate after the addition of antibody solution. The amount of antibody solution necessary will depend on the target identity. The samples were incubated in this antibody solution for 12 hr at 37°C with gentle shaking. Samples were then transferred to 10 mL of SWITCH-On solution (PBST) and were washed for 12 hr at 37°C with gentle shaking.

Table S2. A Statistics of All Combinations of Six Cell Markers, Related to Figure 3.

Antigen						Cortical layer					
GFAP	NeuN	SMI-32	CB	CR	PV	I	II	III	IV	V	VI
−	−	−	−	−	+	0	3	11	5	1	7
−	−	−	−	+	−	4	15	42	7	5	1
−	−	−	−	+	+	None					
−	−	−	+	−	−	2	0	14	1	3	8
−	−	−	+	−	+	0	2	4	5	1	15
−	−	−	+	+	−	1	11	12	3	1	3
−	−	−	+	+	+	None					
−	−	+	−	−	−	None					
−	−	+	−	−	+	None					
−	−	+	−	+	−	None					
−	−	+	−	+	+	None					
−	−	+	+	−	−	None					
−	−	+	+	−	+	None					
−	−	+	+	+	−	None					
−	−	+	+	+	+	None					
−	+	−	−	−	−	59	828	1,007	1,197	1,158	2,030
−	+	−	−	−	+	0	8	38	57	32	49
−	+	−	−	+	−	15	56	93	15	4	6
−	+	−	−	+	+	None					
−	+	−	+	−	−	1	77	448	22	43	83
−	+	−	+	−	+	0	10	64	66	49	13
−	+	−	+	+	−	2	23	34	4	1	4
−	+	−	+	+	+	None					
−	+	+	−	−	−	0	3	122	24	78	10
−	+	+	−	−	+	0	0	1	0	0	0
−	+	+	−	+	−	None					
−	+	+	−	+	+	None					
−	+	+	+	−	−	0	0	4	1	0	0
−	+	+	+	−	+	0	0	2	3	0	0
−	+	+	+	+	−	None					
−	+	+	+	+	+	None					
+	−	−	−	−	−	42	123	367	152	238	256
+	−	−	−	−	+	None					
+	−	−	−	+	−	None					
+	−	−	−	+	+	None					
+	−	−	+	−	−	None					
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+	+	+	−	−	−	None
+	+	+	−	−	+	None
+	+	+	−	+	−	None
+	+	+	−	+	+	None
+	+	+	+	−	−	None
+	+	+	+	−	+	None
+	+	+	+	+	−	None
+	+	+	+	+	+	None

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