Nanobody immunostaining for correlated light and electron microscopy with preservation of ultrastructure

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Morphological and molecular characteristics determine the function of biological tissues. Attempts to combine immunofluorescence and electron microscopy invariably compromise the quality of the ultrastructure of tissue sections. We developed NATIVE, a correlated light and electron microscopy approach that preserves ultrastructure while showing the locations of multiple molecular moieties, even deep within tissues. This technique allowed the large-scale 3D reconstruction of a volume of mouse hippocampal CA3 tissue at nanometer resolution.

The correlation between the ultrastructure of a biological tissue and its molecular attributes poses major challenges, a problem acute in the study of the brain. Permeabilization of its membranes for immunolabeling will degrade the quality of any electron microscopy (EM) image of a biological specimen. Techniques that do not require permeabilization include transgenic labeling with fluorescent proteins, tags that generate a product visible in the EM, and post-embedding immunogold-EM1–3. None of these techniques, however, are useful if the goal is to display many different molecular markers in the same sample.

The number of different cell types in the brain is vast, and their organization remains poorly understood. In principle, correlation of immunofluorescence images with EM images of the same sample (CLEM) could provide the molecular identities of cells within an EM image. To avoid problems with permeabilization, antibodies can be applied after sectioning of a resin-infiltrated block, as in Array Tomography4. However, individual staining of hundreds or thousands of sections is labor-intensive. The resin that infiltrates the tissue must be less hydrophobic than the hardest resins, making it difficult to obtain thin sections (<50 nm)5. Instead of using OsO4, alternative heavy metal stains may avoid epitope destruction, but with attendant loss of ultrastructural quality7. Clearing and expansion technologies compatible with immunostaining or fluorescent protein expression have improved the resolution of volumetric fluorescence imaging5,8. However, rather than staining all cells and organelles, as in EM, only the fluorescently labeled signals are visible. Moreover, these clearing and expansion methods eliminate the possibility of subsequently doing high-resolution EM on the same samples.

Our goal was to overcome the aforementioned problems by combining pre-embedding immunofluorescence and EM with preservation of ultrastructure. We developed nanobody-assisted tissue immunostaining for volumetric EM (NATIVE). NATIVE is based on the use of nanobodies, single-domain antibody fragments derived from camelid heavy chain–only antibodies6, as immuno-

Fig. 1 | Optimization of NATIVE. a, Brain tissue from YFP-16 mice, stained by a GFP-specific nanobody (Enh). Image is representative of seven independent experiments. b–e, Penetration comparison: tissues were stained with nanobodies (b, c) and commercial full-size antibodies (d, e) against GFP. Images are representative of two independent experiments. Images were taken at cross-sections to show penetration with (b, d) or without (c, e) ECS. (Nb, nanobody; Ab, full-size antibody; ECS, extracellular space). f, Proposed dual mechanism for enhanced penetration of nanobodies. Route 1 (R1): direct penetration of chemically fixed cell membrane; route 2 (R2): penetration through preserved extracellular space.
fluorescence staining agents, which allowed us to fully decouple immunofluorescence techniques from the subsequent staining for EM (no harsh permeabilization, special resins, osmium substitutes or clearing agents were required). Therefore, light and EM can each operate without compromise and at full capability, an advantage over existing CLEM strategies.

The cytoplasm of aldehyde-fixed tissues is poorly accessible to full-size antibodies, but permeability might be sufficient for nanobodies (Fig. 1). We selected a GFP-specific nanobody (Enh), labeled it with an organic fluorophore AF647 using sortase

9,10, then tested its suitability in NATIVE. We stained paraformaldehyde-fixed brain slices from a YFP-16 transgenic mouse, which expresses a yellow GFP variant in its neurons under control of the Thy-1 promoter

11, with AF647-conjugated GFP-specific nanobody (Enh). The YFP signal correlated well with Enh staining (Fig. 1a), indicating that intracellular epitopes are accessible to nanobodies after chemical fixation. To see whether maintenance of extracellular space (ECS)

12 improves penetration of the nanobody, we tested Enh nanobody on 500-μm-thick brain sections fixed in the presence of sucrose, in order to prevent cell swelling. Enh with preservation of ECS penetrated deeply, with good staining intensity up to 100μm on the side of application (48-h staining at 4 °C; Fig. 1b,c), beyond the imaging depth of confocal microscopes when operated without tissue clearing. An anti-GFP monoclonal antibody (~150 kDa) only stained the very surface without permeabilization. (Fig. 1d,e).

Preservation of ECS for NATIVE (Fig. 1f) allows deeper penetration of the staining agent, better represents the natural condition of brain tissue for more accurate ultrastructure and facilitates segmentation of EM data

12. We expanded the selection of nanobodies to VHH-DC13 (ref. 13), VHH-21 (ref. 14), and VHH-E9 (ref. 15), which recognize cell surface markers integrin-αM (CD11b; microglia) and lymphocyte antigen Ly-6C/6G (endothelial cells) and intracellular marker glial fibrillary acidic protein (GFAP), respectively. We sortagged VHH-DC13, VHH-21 and VHH-E9, each to a different oligoglycine-modified fluorophore (AF488, AF647 and TexasRed, respectively). We subsequently performed confocal fluorescence imaging on nanobody-stained, intact, thick tissue sections retrieved from perfusion-fixed (4% paraformaldehyde) and ECS-preserved (3% sucrose) animals. We used laser scanning confocal microscopy with a 40 × 1.3 NA oil immersion lens in z-stack mode to reconstruct a 400 μm × 400 μm × 42 μm volume of mouse hippocampal CA3 (xyz pixel size: x–y, 0.208 μm; z, 1 μm; Fig. 2a, Supplementary Figs. 1 and 2 and Supplementary Videos 1 and 2). Confocal imaging was immediately followed by secondary chemical fixation (2.5% glutaraldehyde + 2% paraformaldehyde) in preparation for EM.

We used an automated tape-collecting ultramicrotome (ATUM) to collect 800 serial sections of 50 nm thickness on carbon-coated (conductive) Kapton tape, covering 40 μm of depth. Strips of tape containing the sections were affixed to silicon wafers and imaged using a scanning electron microscope

16,17. We acquired a low-resolution EM volume of 400 μm × 400 μm × 13 μm and a high-resolution EM volume of 40 μm × 40 μm × 13 μm that coincided with part of the confocal volume (Fig. 2b, Supplementary Fig. 3 and Supplementary Video 3).

We used the low-resolution EM data to map the position of the sections on the wafers for automated EM imaging and to locate the region imaged by fluorescence

12. However, the confocal and EM stacks were rotated, tilted and stretched differently. To obtain registration of the two data sets, we performed a 3D affine transformation based on 16 corresponding-point pairs placed on chromatin, blood vessel branches and lysosomes in both the fluorescence microscopy and the EM datasets. In any small region of the aligned confocal and EM image stacks we could identify fluorescently labeled molecular markers and their ultrastructural correlates. In Fig. 2c, the fluorescence images of the CA3 region of the hippocampus identify microglial cells (yellow) and astrocytes (red) near the endothelial cells of a blood vessel (green), presumably representing the blood–brain barrier and nuclei (blue).

The preservation of ultrastructure in NATIVE-stained samples appeared identical to staining of EM samples without immunostaining or fluorescence imaging. Somatic vesicles, synapse contacts, mitochondria, intracellular microtubule bundles, axons and
dendritic processes were well preserved with excellent membrane contrast throughout the entire CLEM dataset (40 μm; Supplementary Figs. 4 and 5). In contrast, the use of 0.1% Triton as a mild permeabilization reagent resulted in deterioration of membrane structure with loss of fine processes (Fig. 2d and Supplementary Fig. 6).

We succeeded in segmenting glia, a difficult target in brain tissue reconstruction. Glia show variability in the shape of their processes and have small membranous extrusions that are below the diffraction limit of confocal fluorescence microscopes. Using a computer-assisted manual segmentation approach (VAST)16, we segmented both a nanobody-stained astrocyte and a nanobody-stained microglial cell (Fig. 3a and full 3D view Supplementary Fig. 7, Supplementary Video 4). The spatial orientation and cell-cell contacts were well preserved by NATIVE in 3D reconstruction, showing the advantage of EM for ascertaining fine structure (Fig. 3b,c). We found a process of a microglial cell that envelopes parts of other cells and some debris (Fig. 3b, arrowhead), which is consistent with the role of microglia in synapse elimination18. A magnified section from this region of the cell (Fig. 3d and Supplementary Fig. 8) shows finger-like protrusions near debris and other cellular material that was not immunolabeled. We identified an expanded perivascular terminal structure (endfoot) of the astrocyte on the surface of the blood vessel, along with smaller contacts from the same cell (Fig. 3c,e). We also identified a tripartite synapse (Fig. 3e, arrowheads), where the astrocyte forms a sheath surrounding a pair of presynaptic and postsynaptic elements19 (Fig. 3f). Correlation of the GFAP signal in fluorescence with GFAP fiber bundles in the electron micrographs (Supplementary Fig. 9) shows a continuous distribution of this fibrillary protein around the nucleus, forming a belt-like structure around the astrocyte’s dumbbell-shaped nucleus (Fig. 3g,h, Supplementary Figs. 10 and 11 and Supplementary Videos 5 and 6).

NATIVE-enabled 3D-CLEM also provides a reference for robust proofreading during serial-section EM reconstruction. EM reconstruction guided by fluorescence provides a useful check for manual tracing and maybe automated segmentation (Fig. 3b,c).

In conclusion, the NATIVE technique, by use of fluorescently tagged nanobodies, allows, in the same sample, imaging without compromising the quality of images in either the fluorescence or the EM regimen. We expect that light sheet or super-resolution microscopy can also be coupled to NATIVE. For the EM module, automated serial section EM enables routine and rapid volumetric tissue volumes at nanometer-scale resolution. NATIVE should allow exploration of biological samples other than brain. Data analysis will be limited primarily by computational capacity20, but a larger selection of nanobodies will be critical as well1.
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Author contributions

X.L. and T.F. conceived, designed and implemented NATIVE with contributions from all authors. R.S. assisted in collecting serial sections, EM imaging and aligning EM images. D.B. provided the tracing tool and performed EM-LM alignment and 3D rendering. C.G. led segmentation efforts with contributions from X.L., T.F., and J.C. J.L. and H.P. supervised the work. All authors contributed to data analysis. All authors contributed to writing of the manuscript.

Competing interests

A provisional patent application has been filed.

Additional information

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Methods

Step-by-step instructions for the NATIVE workflow are available as a Supplementary Protocol and online at Protocol Exchange^{22}.

Sortase reaction. Detailed protocols have been described previously^{22}. Briefly, in a reaction buffer containing Tris-HCl (50 mM, pH 7.5), CaCl2 (10 mM), NaCl (150 mM), N-terminal triglycine-modified peptides/organic fluorophores (500 μM) and LPETG-containing VHVs (100 μM) were added, followed by the addition of sortase A pentamutant (2.5 μM, Addgene: 51140). After incubation at 12 °C with gentle agitation for 2–4 h, Ni-NTA beads were added to the reaction mixture with gentle shaking for another 30 min at 4 °C. At the end of incubation, the total mixture with beads was directly loaded onto a PD-10 column (GE Healthcare).

Fractions corresponding to the desired product failed to stick to Ni-NTA beads and were retrieved in the void volume to yield pure VH/H-fluorophore conjugate. The labeled VH was adjusted to a final concentration of 1 mg/mL and stored at −80 °C with 10% (v/v) glycerol.

Perfusion and fixation. Adult C57BL/6 mice, except where noted, were used in developing NATIVE (all animal procedures were performed according to US National Institutes of Health guidelines and approved by the Committee on Animal Care at Harvard University). Mice were anesthetized with isoflurane inhalation and subsequently perfused transcardially with a Masterflex Peristaltic pump at a flow rate of 10 mL/min. 15 mL carbonated aCSF buffer was perfused to clear the blood, followed by 30 mL ice-cold fixative mixture of 3% sucrose, 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were carefully removed from the skull to avoid damage to the tissue. On a Leica VT1000 S vibratory blade microtome, 120 μm slices were cut at 4 °C in the fixative mixture. Slices were transferred into fresh fixative solution and post-fixed for 24 h with gentle agitation at 4 °C.

Nanobody staining. Fixed brain slices were trimmed into smaller pieces containing the target area and transferred to a clean 24-well plate. After rinsing 3×5 min in 0.1 M PBS, a solution containing 0.1 M glycine and 0.05% sodium azide (glycine blocking solution) was added. Nanobody mixture staining solution was made by diluting fluorophore-conjugated nanobody stock solutions (~1 mg/mL) 1:250 in the glycine blocking solution. After 20 min of blocking, 1 mL of the nanobody staining mixture was added to each well. Nanobody staining was performed for 4 h at 4 °C with gentle agitation. Samples were then rinsed 3×5 min in 0.1 M PBS. Nuclei were stained with Hoechst solution (1:5,000 dilution in 0.1 M PBS) for 5 min and transferred to 1% uranyl acetate solution. The sample stayed in uranyl acetate solution at 4 °C overnight, protected from light. On the next day, the sample was warmed up to 50 °C in the same uranyl acetate solution for 1 h. The sample was washed 3×5 min and ready for embedding.

Resin embedding. The sample was dehydrated through a graded acetone series (25, 50, 75, 100, 5 min each) and then placed in anhydrous acetone for 10 min. After infiltration in 25% EPON-acetone for 1 h, 50% EPON-acetone for 2 h and 75% EPON-acetone for 2 h, the sample was placed in 100% EPON overnight on a rotator. The infiltrated sample was then transferred to the embedding capsule and incubated in fresh EPON in a 60 °C oven for two days.

Serial ultrathin sections collection with ATUM. The block face of the sample cylinder was trimmed into a 1.5×1.5 mm square containing the ROI imaged by light microscopy. The low-resolution image was used as reference for trimming. 50-nm-thin sections were cut using a Leica ultramicrotome and collected on a home-built automated tape collecting system on Kapton tape that was carbon coated and plasma treated. Upon collection of the sections, the tape was cut into appropriate lengths and mounted on 100-mm silicon wafers.

EM imaging. The sections were imaged with a Zeiss Sigma scanning electron microscope using a backscattered electron detector. A few sections were mapped at low resolution and compared with the light microscopy (LM) images. Blood vessels and nuclei were employed as fiducial markers in correlating the LM and EM images to locate the ROI. High-resolution images of the ROI (12k×12k pixel image, 5 nm per pixel) were acquired with the working distance of ~7.3 mm, 8 keV incident electron energy and dwell time of 2 μs. The EM image stack was aligned by Fiji’s plugin “Linear Stack Alignment with SIFT” to form a coherent 3D EM volume.

LM-EM coregistration and data processing. LM images and aligned EM images were loaded into VAST separately (https://software.rc.fas.harvard.edu/lichtman/vast/). Characteristic features visible in both image stacks were identified, including chromatin, branching of blood vessels and lysosomes, and corresponding point pairs were placed in each feature in the two stacks. The coordinates of these points in both LM and EM were recorded, and a 3D affine transformation was calculated for translating the LM coordinates to the EM coordinates using the pseudoinverse method. We then translated the LM image stack to the EM coordinate system and loaded both stacks in VAST together. The microglia, the astrocyte and the blood vessel endothelial cell were traced in VAST. Traced objects and isosurfaces of the fluorescence were extracted as 3D surfaces and rendered in 3ds Max (Autodesk Inc).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Code availability. The Matlab codes to compute a 3D transformation are available as Supplementary Software.

Data availability

The authors declare that the main data supporting the findings of this study within the article and its Supplementary Information files are available. The high-resolution EM, low-resolution EM and aligned LM image stacks can be downloaded from https://software.rc.fas.harvard.edu/lichtman/temp/NATIVE.

References

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| ☒   | ☒ Clearly defined error bars |
| ☒   | ☒ State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

The light microscopy images were taken on Zeiss LSM 880 with the software Zen; the electron microscopy images were taken on Zeiss Sigma SEM with the software Atlas5.

Data analysis

The EM image stack was aligned by Fiji; the 3D affine transformation was computed by Matlab; cell tracing was conducted on VAST (https://software.rc.fas.harvard.edu/lichtman/vast/); 3D renderings were performed on 3ds Max.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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The author declare that the main data supporting the findings of this study are available within the article and its Supplementary Information files. The complete LM and EM image stack are available from the corresponding author upon request.

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Life sciences study design

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Sample size
We specified the sample size for each experiment in the Figure legends. These numbers include only the repetitions after the method was fully optimized (i.e. the staining, imaging and data analysis results were reliably reproduced in those experiments). Since the subject of our manuscript is to establish a new immunostaining and imaging method rather than a particular biological finding, we believe this sample size is sufficient to verify the performance of our method.

Data exclusions
No data were excluded.

Replication
Experimental results were reliably reproduced.

Randomization
There was no need for randomization in this technique development.

Blinding
The blinding test we performed was to give random samples stained with either nanobodies or antibodies to J.W.L.. J.W.L. determined which group the sample belonged to after checking the epifluorescence. All the eight samples were successfully determined by J.W.L.

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
| --- | ---------------------- |
| ☑ | Unique biological materials |
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☑ | Palaeontology |
| ☑ | Animals and other organisms |
| ☑ | Human research participants |

Methods

| n/a | Involved in the study |
| --- | ---------------------- |
| ☑ | ChiP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials
The fluorophore conjugated nanobodies used in this work are not currently commercially available. The sequences of those nanobodies can be found in the corresponding references and the detailed protocol of the fluorophore conjugation is provided in the Supporting Information.
**Antibodies**

| Antibodies used | GFP tag polyclonal antibodies conjugated with Alexa 647 was purchased from ThermoFisher, Catalog number A-31852, Lot number 1755557, stock concentration 2mg/mL, immunofluorescence dilution 8 μg/mL. |
|-----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Validation      | Certificates of analysis (Lot number 1755557) can be found on company's website.                                                                                                                       |

**Animals and other organisms**

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| Laboratory animals | Adult (P90+) male and female C57Bl/6 mice were used in this work. All animal procedures were performed according to US National Institutes of Health guidelines and approved by the Committee on Animal Care at Harvard University. |
|--------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Wild animals       | No wild animals were used in this study.                                                                                                                                                           |
| Field-collected samples | No field-collected samples were used in this study.                                                                                                                                              |