Visualization and molecular characterization of whole-brain vascular networks with capillary resolution

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Structural elucidation and molecular scrutiny of cerebral vasculature is crucial for understanding the functions and diseases of the brain. Here, we introduce SeeNet, a method for near-complete three-dimensional visualization of cerebral vascular networks with high signal-to-noise ratios compatible with molecular phenotyping. SeeNet employs perfusion of a multifunctional crosslinker, vascular casting by temperature-controlled polymerization of hybrid hydrogels, and a bile salt-based tissue-clearing technique optimized for observation of vascular connectivity. SeeNet is capable of whole-brain visualization of molecularly characterized cerebral vasculatures at the single-microvessel level. Moreover, SeeNet reveals a hitherto unidentified vascular pathway bridging cerebral and hippocampal vessels, thus serving as a potential tool to evaluate the connectivity of cerebral vasculature.

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Cerebral vasculature is an anatomically and histologically inhomogeneous three-dimensional (3D) traffic network for blood cells, nutrients, oxygen, metabolic wastes, signaling molecules, etc. Although structural and molecular analyses of cerebral vasculature are promising for providing fundamental insights into cerebral circulation and cerebrovascular diseases, which are major causes of death and disability, a few studies have captured detailed structural information of molecularly identified vascular networks due to technical limitations (Supplementary Tables 1 and 2).

Here, we introduce SeeNet, a method for 3D visualization and molecular characterization of the entire vascular network, including virtually all microvessels, in the whole brain. SeeNet includes (i) intravascular perfusion of a blood–brain barrier (BBB)-impermeable fluorescent crosslinker, (ii) thermally controlled polymerization of a hybrid hydrogel, and (iii) bile salt-based tissue clearing.

Results
Thermally controlled vascular casting with hybrid hydrogel.
Classic methods for vascular casting using solidifying polymers, such as resin, fail to allow visualization of all vessels because the polymers often become clogged in microvessels. To suppress clogging, we conceived a strategy in which polymerization was initiated after complete perfusion of cerebral vessels with monomer acrylamide and a BBB-impermeable fluorescent crosslinker. We designed a fluorescent compound termed RITC–Dex–GMA consisting of rhodamine isothiocyanate (RITC), a fluorescent group; glycidyl methacrylate (GMA), a crosslinkable group; and dextran (Dex), a macromolecule that makes the compound have a molecular weight large enough to prevent it from crossing the BBB and small enough to not clog microvessels (Fig. 1a). RITC–Dex–GMA can be synthesized through a few simple chemical reactions and can easily be purified with ethanol precipitation (Supplementary Fig. 1a, b). To regulate the timing of the polymerization of RITC–Dex–GMA and acrylamide, we adopted the thermal initiator VA-044; note that VA-044 triggers polymerization when heated to 37 °C (Supplementary Fig. 1c, d). After intravascular perfusion with phosphate-buffered saline (PBS) and paraformaldehyde (PFA), mice were further polymerized when heated to 37 °C (Supplementary Fig. 1c, the thermal initiator VA-044; note that VA-044 triggers and 6 slices from 9, 10, and 6 mice, respectively, Dex–GMA versus lectin (i.v.): P = 0.201 × 10^{-9}, Q_{2,2} = 16.7; Dex–GMA versus lectin (perfused): P = 0.201 × 10^{-9}, Q_{2,2} = 8.27; P = 0.294 × 10^{-10}, F_{2,22} = 69.9, Tukey’s test after one-way ANOVA). The SN ratio of transcardially perfused lectin was significantly higher than that of intravenously injected lectin (Fig. 1d, n = 6 and 10 slices from 6 and 10 mice, respectively, P = 4.53 × 10^{-4}, Q_{2,2} = 6.42, Tukey’s test after one-way ANOVA). However, as previously reported in the liver, vasculature staining with transcardial perfusion of lecithin was spotty (Supplementary Fig. 3). The overlap ratio of RITC–Dex–GMA-casted vessels and CD31-immunopositive vessels was 98.2 ± 0.8%, which was significantly higher than the overlap ratio 59.4 ± 4.4% (intravenous injection) and 64.4 ± 7.3% (perfused) for Texas Red-conjugated lectin (Fig. 1e, mean ± SD of 9, 10, and 6 slices from 9, 10, and 6 mice, respectively, Dex–GMA versus lectin (i.v.): P = 2.39 × 10^{-10}, Q_{2,2} = 16.4; Dex–GMA versus lectin (perfused): P = 3.51 × 10^{-6}, Q_{2,2} = 12.4; lectin (i.v.) versus lectin (perfused): P = 0.401, Q_{2,2} = 1.86, P = 1.59 × 10^{-10}, F_{2,2} = 74.5, Tukey’s test after one-way ANOVA). Therefore, thermally controlled post hoc polymerization of perfused RITC–Dex–GMA could be used to visualize nearly all of the cerebral vasculature with a high SN ratio.

We also compared the staining performance between our method and a gelatin–fluorescein isothiocyanate (FITC)–albumin gel perfusion method, which is reported to be able to label cerebral vasculature with a high SN ratio (Supplementary Fig. 4a). Unexpectedly, however, we noticed that the gelatin–FITC–albumin gel perfused samples were only partially stained with anti-CD31 (Supplementary Fig. 4b–d). The percentage of CD31-positive areas in slices was 4.37 ± 0.94%, in contrast to the expected 9.25 ± 1.12% when CD31 is successfully stained (Supplementary Fig. 4e, mean ± SD of 5 and 9 slices from 5 and 9 mice, respectively, P = 5.83 × 10^{-6}, t_{12} = 7.66, Student’s t-test). A possible explanation for this incomplete staining could be that the warm PFA used in this method altered the efficiency of fixation or the antigenicity of CD31. To examine whether the antigenicity was affected, we compared the staining patterns of other protein markers for neurons (NeuN), astrocytes (GFAP), and microglia (Iba-1) in samples that were fixed using a standard method (PFA), our method (Dex–GMA), or the gelatin–FITC–albumin method (Supplementary Fig. 5a–c). The immunostaining patterns in Dex–GMA-treated samples were similar to those of PFA-fixed samples. However, in gelatin–FITC–albumin-treated samples, the immunosignals were deteriorated, and the staining patterns were not spatially even. The percentages of immunopositive areas to entire areas were similar between PFA-treated and Dex–GMA-treated samples, whereas the percentages were lower in gelatin albumin-treated samples (Supplementary Fig. 5d; P = 3.47 × 10^{-3}, F_{2,12} = 9.44, one-way ANOVA; PFA versus Dex–GMA: P = 0.46, Q_{2,12} = 1.73; Dex–GMA versus gelatin–albumin: ***P = 3.13 × 10^{-3}, Q_{2,12} = 5.97; PFA versus gelatin–albumin: *P = 2.79 × 10^{-2}, Q_{2,12} = 4.238, Tukey’s test. Supplementary Fig. 5e; P = 9.19 × 10^{-3}, H_{2,12} = 9.38, Kruskal–Wallis test; PFA versus Dex–GMA: P = 0.10, Q_{2,12} = 0.99; Dex–GMA versus gelatin–albumin: *P = 2.45 × 10^{-2}, Q_{2,12} = 2.61; PFA versus gelatin–albumin: *P = 2.45 × 10^{-2}, Q_{2,12} = 2.61, Steel-Dwass test. Supplementary Fig. 5f; P = 7.60 × 10^{-4}, F_{2,12} = 13.9, one-way ANOVA; PFA versus Dex–GMA: P = 8.48 × 10^{-2}, Q_{2,12} = 3.342; Dex–GMA versus gelatin–albumin: ***P = 5.48 × 10^{-4}, Q_{2,12} = 7.43; PFA versus gelatin–albumin: **P = 3.35 × 10^{-2}, Q_{2,12} = 4.09, Tukey’s test.). These data indicate that the antigenicity was preserved in Dex–GMA-treated and PFA-fixed samples but was altered in gelatin–FITC–albumin-treated samples. In the gelatin–FITC–albumin gel perfusion method, we occasionally observed clusters of CD31-positive microvessels that were not stained by FITC, suggesting that gelatin–FITC–albumin gels were clogged in some brain regions (Supplementary Fig. 4f). To quantitatively compare the staining performance between the two methods, we labeled endothelial cells using the intravenously injected lectin-conjugated dye for a reference (Supplementary Fig. 6a, b). Unlike anti-CD31 staining, the performances of lectin labeling were similar between the two methods, possibly because the labeling was carried out before the perfusion of warmed PFA and the change in the antigenicity...
Fig. 1 Near-complete visualization of cerebral vasculature using thermally controlled polymerization of a BBB-impermeable fluorescent crosslinker. a Design of the BBB impermeable fluorescent crosslinker RITC–Dex–GMA. The fluorophore RITC and the crosslinkable group GMA are chemically linked through dextran, a BBB-impermeable molecule. b Strategic concept of vascular casting using a hybrid hydrogel. Red circles and gray dots indicate RITC–Dex–GMA and acrylamide, respectively. c Top: A confocal image of a sagittal section of an adult mouse brain treated with vascular casting using RITC–Dex–GMA, acrylamide, and VA-044. Scale bar = 1 mm. Bottom: Magnified images of RITC-Dex-GMA-casted vasculature of different brain regions (red) and endothelial cells counterstained using anti-CD31 in the same fields (green). Scale bar = 100 μm. d SN ratios of the fluorescence intensities in lectin-based and Dex-GMA-based visualization (n = 10, 6, and 9 slices from 10, 6, and 9 mice, respectively; box plots indicate median and 25–75% interquartile ranges; whiskers cover 10–90% quantiles. P = 2.94 × 10^-10, F2,22 = 69.9, one-way ANOVA; Dex-GMA versus lectin (i.v.); ***P = 1.59 × 10^-10, Q2,22 = 16.7; Dex-GMA versus lectin (perfused); **P = 2.01 × 10^-5, Q2,22 = 8.27; lectin (i.v.) versus lectin (perfused); ***P = 4.53 × 10^-4, Q2,22 = 6.42, Tukey’s test). e Overlap ratios between anti-CD31 immunoreactivity and Texas Red/RITC fluorescence in lectin-based and Dex-GMA-based visualization (59.4 ± 9.45%, 64.4 ± 7.32%, and 98.2 ± 0.8%, mean ± SD of 10, 6, and 9 slices from 10, 6, and 9 mice, respectively; box plots indicate median and 25–75% interquartile ranges; whiskers cover 10–90% quantiles. P = 1.59 × 10^-10, F2,22 = 74.5, one-way ANOVA; Dex-GMA versus lectin (i.v.); ***P = 2.39 × 10^-10, Q2,22 = 16.4; Dex-GMA versus lectin (perfused); **P = 3.51 × 10^-8, Q2,22 = 12.4; lectin (i.v.) versus lectin (perfused); P = 0.401, Q2,22 = 1.86, Tukey’s test). Source data are provided as a Source Data file.
for analyzing the molecular and structural characteristics of cerebral vasculature.

**Vascular connectivity-retaining delipidation by a bile salt.** For the visualization of the 3D structure of vascular networks, conventional casting methods use corrosive chemicals and remove surrounding parenchymal tissues. We sought to replace this classical strategy with optical tissue clearing that retains molecular information in the brain parenchyma. First, we conducted tissue clearing by delipidation with sodium dodecyl sulfate (SDS) for 1 week\textsuperscript{5,13} followed by refractive index (RI)-matched in ScaleCUBIC-2 for 2 d. In the SDS-treated group, the white matter remained opaque, and the sample swelled. In the SDC-treated group, the cleared brain was still pale yellow, but the white matter was transparent. The sample swelled to a lesser extent. The grid interval is 2 mm.

**Fig. 2 Rapid tissue clearing with sodium deoxycholate.** a, b Time-series of photographs of a brain taken at each step of tissue clearing. The fixed brain was treated with EDTA for 12 h followed by SDS a or SDC b for 7 d (daily refreshed), washed with PBS and refractive index (RI)-matched in ScaleCUBIC-2 for 2 d. In the SDS-treated group, the white matter remained opaque, and the sample swelled. In the SDC-treated group, the cleared brain was still pale yellow, but the white matter was transparent. The sample swelled to a lesser extent. The grid interval is 2 mm. c The light transmittance (380–780 nm) of fixed whole brains treated with SDS and ScaleCUBIC-2 (dark blue), with SDC and ScaleCUBIC-2 (light blue) or with PBS (black) (n = 8 brains each, P = 2.22 × 10\textsuperscript{−10}, F\textsubscript{2,28} = 4.56 × 10\textsuperscript{5}, two-way ANOVA (between groups)). d Percentages of opaque area in the whole brains treated with SDS and ScaleCUBIC-2, with SDC and ScaleCUBIC-2, or with PBS (n = 8 brains each; box plots indicate median and 25–75% interquartile ranges, P = 2.22 × 10\textsuperscript{−16}, F\textsubscript{2,21} = 4.08 × 10\textsuperscript{3}, one-way ANOVA; SDC versus SDS: ***P = 7.47 × 10\textsuperscript{−14}, Q\textsubscript{2,21} = 26.73; SDS versus PBS: ***P = 4.36 × 10\textsuperscript{−14}, Q\textsubscript{2,21} = 94.78; SDC versus PBS: ***P = 4.36 × 10\textsuperscript{−14}, Q\textsubscript{2,21} = 121.5, Tukey’s test). e Time course of tissue expansion during clearing (n = 8 brains each, P = 2.22 × 10\textsuperscript{−16}, F\textsubscript{2,21} = 6.66 × 10\textsuperscript{2}, one-way ANOVA; SDC versus SDS: ***P = 4.41 × 10\textsuperscript{−14}, Q\textsubscript{2,21} = 33.15; SDC versus PBS: ***P = 9.92 × 10\textsuperscript{−11}, Q\textsubscript{2,21} = 17.69; SDS versus PBS: ***P = 4.36 × 10\textsuperscript{−14}, Q\textsubscript{2,21} = 50.84, Tukey’s test). Error bars represent standard deviations. Source data are provided as a Source Data file.
inapplicable for use in animal tissues\textsuperscript{13,24}, we found that it made the mouse brain transparent after 2-d RI matching with ScaleCUBIC-2, at least under our fixation condition (Fig. 2b). The average light-transmittance ratio of SDC-cleared tissues was slightly lower than that of SDS-cleared tissues (Fig. 2c, \( n = 8 \) brains each, \( P = 2.22 \times 10^{-16}, F_{2,89} = 4.56 \times 10^{3}, \) two-way ANOVA), but SDC-cleared tissues exhibited a spatially uniform RI across the specimens and had no opaque areas (Fig. 2d, \( n = 8 \) brains each, \( P = 7.47 \times 10^{-14}, Q_{2,21} = 26.73; \) SDC versus SDS, \( P = 2.22 \times 10^{-16}, F_{2,21} = 4.08 \times 10^{3}, \) Tukey’s test after one-way ANOVA). Moreover, swelling was significantly reduced in SDC-cleared tissues (Fig. 2c, \( n = 8 \) brains each, \( P = 4.41 \times 10^{-14}, Q_{2,21} = 33.15 \) versus the SDS group, \( P = 2.22 \times 10^{-16}, F_{2,21} = 666, \) Tukey’s test after one-way ANOVA). Thus, the connectivity of the vascular network was preserved in SDC-cleared tissues, whereas many neocortical penetrating vessels were fractured in SDS-cleared tissues due to overstretching of the fluorescent hydrogel (Fig. 3, \( n = 6 \) and 9 brains, \( P = 8.84 \times 10^{-7}, t_{13} = 8.70, \) Student’s \( t \)-test). We refer herein to the combination of fluorescent Dex–GMA-based vascular casting and SDC-based tissue clearing as SeeNet (A method to See the cerebrovascular Network).

Molecular phenotyping compatibility of SeeNet. Next, we examined whether nucleic acids and proteins were maintained in SeeNet-treated brains. The vasculatures were cast using FITC–Dex–GMA, and the specimens were labeled using propidium iodide (PI), a DNA-staining reagent, or an antibody against Iba-1. Whole-mount imaging of the cerebral hemisphere revealed that PI and Iba-1 signals were detectable (Fig. 4a–f, \( n = 7 \) hemispheres each), suggesting that nucleic acids and proteins were retained in the tissues.

We also addressed the possible quenching of fluorescent proteins during the clearing procedure. We applied SeeNet to the brains of visually stimulated Arc-dVenus mice, in which active neurons express the modified yellow fluorescent protein dVenus\textsuperscript{26}. Whole-mount imaging revealed that SeeNet retained the fluorescence of dVenus (Fig. 4g–i, \( n = 7 \) hemispheres).

We further examined the compatibility of SeeNet with other epitopes and fluorescent proteins using a light-sheet fluorescence microscope\textsuperscript{7}. First, SeeNet-treated hemispheres were immunostained with fluorophore-conjugated antibodies against NeuN and GFAP (Supplementary Fig. 7). The optical sections of the stained samples were virtually indistinguishable from images of 2D-stained samples (Supplementary Figs. 5a, b and 7c, f), indicating that SeeNet-treated samples can be immunostained using these antibodies. Next, SeeNet protocol was applied to CX3CR1-GFP mice\textsuperscript{27}, which expresses GFP in microglia, and H-17-iCre-imCherry mice\textsuperscript{28}, which expresses mCherry in a subpopulation of olfactory sensory neurons (Supplementary Fig. 8a–f). In CX3CR1-GFP mice, GFP-positive cells were observed throughout the brain. In H-17-iCre-imCherry mice, the convergence pattern of mCherry-positive axons were clearly visualized with SeeNet protocol. These patterns were consistent with the expression patterns reported previously in these mouse lines, suggesting that the SeeNet protocol preserved the innate fluorescence of GFP and mCherry. In addition, SeeNet was able to visualize fluorescent protein expressed by intracranially injected adeno-associated virus (AAV), without apparent leakages of FITC–Dex–GMA around the injected sites (Supplementary Fig. 8g–i).

We next examined whether the fine subcellular structures of glial cells were preserved in the SeeNet protocol. GFAP-stained or CX3CR1-GFP-expressing SeeNet-treated brains were imaged by a confocal microscope. We successfully observed typical glial subcellular morphologies, i.e., astrocytic endfeet wrapping penetrating vessels\textsuperscript{30} and microglial protrusions that likely touched capillaries\textsuperscript{30} (Supplementary Fig. 9).

Then, we compared the SN ratio of 3D imaging data obtained from the SeeNet-treated samples to that obtained from samples prepared by a conventional protocol, intravenous injection of Texas Red-conjugated lectin and optical clearing with SDS/ScaleCUBIC-2 (Supplementary Fig. 10). Signals in SeeNet-treated brains were detectable at depths of at least 1000 \( \mu \)m, whereas signals in lectin/SDS/ScaleCUBIC-2-treated brains were almost indistinguishable from the background (Supplementary Fig. 10a, b, \( n = 7, 9 \) mice, respectively, \( P = 2.22 \times 10^{-16}, F_{1,140} = 5.55 \times 10^{2}\), two-way ANOVA). The mean diameter of cerebral capillaries in SeeNet-treated samples was 6.58 ± 1.21 \( \mu \)m (Supplementary Fig. 10c, mean ± SD of 90 capillaries from 9 mice). This value was slightly but not significantly larger than the previously reported in vivo diameter (6.3 ± 1.1 \( \mu \)m\textsuperscript{31}), possibly due to changes in tissue volume caused by PFA fixation, delipidation, and RI-matching.

As the capillary diameters in SeeNet-treated samples were comparable to the pixel resolution of our macro-zoom light-sheet microscope, in which one pixel measures 6.45 × 6.45 \( \mu \)m\textsuperscript{2}, we attempted whole-brain imaging of cerebral vasculature at single-capillary resolution. Because of the high SN ratios of SeeNet-treated brain, we were able to visualize blood vessels in both gray
and white matters of the brains with single-capillary resolution (Fig. 5a–d, Supplementary Movies 1 and 2). The brains were also immunostained using a FITC-conjugated antibody against α-smooth muscle actin (αSMA), a marker for arteries and arterioles. SeeNet could visualize the whole cerebral vasculature with molecularly identified arterioles and venules (Fig. 5a).

We also examined the SN ratio of 3D imaging data obtained from samples prepared with intravenous injection of an Alexa 647-conjugated anti-CD31 antibody and with optical clearing by ethanol and ethyl cinnamate (ECi)32. One sample was prepared from CX3CR1-GFP mice for the simultaneous examination of the fluorescence preservation of this method (Supplementary Fig. 11a–c). GFP fluorescence was barely visible in the gray matter of the brains and was almost invisible in the white matter (Supplementary Fig. 11d–f). The signal of endothelial cells was detectable in the gray matter but indistinguishable from the background noise in the white matter (Supplementary Fig. 11g–i). The SN ratio of the CD31 signal was significantly lower than that of casted vessels in SeeNet (Supplementary Fig. 11j, k. n = 4 and 5 optical slices from 4 or 5 mice, respectively. Gray matter; ***P = 1.32 × 10−6, t7 = 15.1, White matter; ***P = 2.53 × 10−5, t7 = 9.75, Student’s t-test).

**A microvascular path revealed by SeeNet.** Using SeeNet, we serendipitously discovered a previously unknown microvascular path. In rodents, the vascular paths of the hippocampus and the cerebral cortex were examined at the level of arterioles and venules. Once cerebral arteries are separated into the posterior cerebral artery and the longitudinal hippocampal artery, they are generally assumed to form independent networks until they converge in the internal jugular vein1,33. However, when we inspected SeeNet-treated samples at the single-capillary level, we found some microvessels that directly connected cortical and hippocampal vascular networks (Fig. 5e, Supplementary Movies 3, 4). We analyzed 54 arterioles and 57 venules of the

![Image](https://doi.org/10.1038/s41467-020-14786-z)
**Fig. 5 Large-scale molecular phenotyping-compatible 3D imaging of cerebral vasculature.**

**a** Maximum projection of light-sheet fluorescence microscopic images of the entire vasculature in a SeeNet-treated brain (1.6× zoom, ×0.63 objective lens, numerical aperture: 0.15, working distance: 87 mm) into a single stacked photo. Casted vessels are shown in red. Arterioles immunolabeled with anti-αSMA are shown in green. Scale bar = 1 mm. 

**b** The RITC-Dex-GMA signal in **a**, shown on a grayscale, was optically sectioned at $Z = 3 \text{ mm}$. Scale bar = 1 mm. 

**c, d** Magnified images of the boxed region of **b**. Vasculature in the gray matter **c** and white matter **d** was visualized with capillary resolution. Scale bar = 200 μm. 

**e** The RITC-Dex-GMA signal in a posterior part of the left hemisphere in **a** is shown on a grayscale. Magenta indicates cortico-hippocampal vascular paths observed in this area. Scale bar = 1 mm. See also Supplementary Movie 3. 

**f** The anti-αSMA immunosignal of the boxed regions in **e** is shown on a green scale. Scale bar = 100 μm. See also Supplementary Movie 6. 

**g** The percentage of the connected pairs of dorsal hippocampal transverse arterioles (red) and venules (blue) to the total number of vessels observed. Scale bar = 100 μm. See also Supplementary Movie 5. 

**h** Maximum projection of confocal images of casted vasculatures from the pia to the hippocampus. A cortico-hippocampal vascular path is shown in magenta. Scale bar = 100 μm. See also Supplementary Movie 4. 

**i** A 3D-rendered view of the cortico-hippocampal vascular path. Source data are provided as a Source Data file.
dorsal hippocampal transverse vessels from three mice (Fig. 5f, g). We found that 16.7% of the arterioles and 17.5% of the venules were connected to cortical penetrating vessels, suggesting that the flows of blood were not unidirectional. Interestingly, there were not only arteriole–venule connections (five hippocampal arteriole–cortical venule connections and four hippocampal venule–cortical arteriole connections) but also arteriole–arteriole and venule–venule connections (four and six connections, respectively). We confirmed, using higher resolution confocal imaging, that there is a cortico-hippocampal microvascular pathway connecting eSMa-immunopositive penetrating vessels and transverse vessels (Fig. 5h–j, Supplementary Movies 5, 6). These bypasses may actively exchange arterial blood and serve as a backup circulation pathway in the case of cerebral infarction.21–23.

Discussion

In this study, we developed SeeNet, a method for the structural and molecular phenotyping of the 3D cerebral vasculature. The BBB-impermeable fluorescent crosslinker RITC–Dex–GMA enabled near-complete vascular casting with high SN ratios, and SDG-enabled relatively rapid (~10 d) tissue clearing without significant affection of the synthesized fluorescent hydrogels or intrinsic nuclear acids or proteins. Several pioneering tissue-clearing studies have attempted whole-brain visualization of cerebral vasculature using conventional vascular-labeling dyes.17,35,36 However, due to the low SN ratios of the conventional dyes, these methods produce numerous false negatives. Although Di Giovanni et al. addressed this matter using temperature-dependent gelation of gelatin and FITC-conjugated albumin,10,14 the gelatin-based method has another source of false negatives because gelatin occasionally becomes clogged in microvessels. Moreover, when samples prepared by this protocol are molecularly characterized, further caution is necessary because antigenicity may be altered. In addition, the gelatin–FITC–albumin gel perfusion method costs > $200 per mouse and takes > 6 weeks, making its use for routine vascular labeling difficult. To reduce false negatives, the use of transgenic mice is an alternative option. However, because the promoter for endothelial cells is heterogeneously active, particularly in adult mice,37 Cre-based double transgenic mice are required for complete labeling of cerebral vasculatures. Therefore, the supply of double-transgenic mice is a bottleneck for research, and the use of double transgenic mice makes exploiting other transgenic mice difficult. In terms of these issues, SeeNet is quick, less costly and easily adoptable to wild-type and transgenic mice. SeeNet will enable the routinization of fine large-scale 3D tracing for molecularly characteristic cerebral vasculatures, thus advancing our understanding of the structural and functional organizations of cerebrovascular networks.

Methods

Animal ethics. Two- to five-month-old C57BL/6j (Japan SLC, Inc., Japan), CX3CR1–GFp (Stock No. 005582, The Jackson Laboratory)32, H-17-4creimCherry (Accession No. CDB03577, RIKEN LARGE)38, and Arc-dVenus transgenic mice39 were used in this study. All animals were housed under a 12:12-h light–dark light cycle (light from 07:00 to 19:00) at 22 ± 1 °C with ad libitum access to food and water. Animal experiments were performed with the approval of the Animal Experiment Ethics Committee at the University of Tokyo (approval number: 24-70) and according to the University of Tokyo guidelines for the Care and Use of Laboratory Animals. All experimental protocols were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Ministry of Education, Culture, Sports, Science and Technology, Notice No. 71 of 2006), the Standards for Breeding and Housing of and Pain Alleviation for Experimental Animals (Ministry of the Environment, Notice No. 88 of 2006), and the Guidelines on the Method of Animal Disposal (Prime Minister’s Office, Notice No. 40 of 1995).

Synthesis of RITC–Dex-GMA or FITC–Dex-GMA. The reactions are summarized in Supplementary Fig. 1. Step 1 (Supplementary Fig. 1a): A total of 4.0 g dextran 40 (4043–2231, Wako Pure Chemical Industries Ltd.), experimental protocols were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Ministry of Education, Culture, Sports, Science and Technology, Notice No. 71 of 2006), the Standards for Breeding and Housing of and Pain Alleviation for Experimental Animals (Ministry of the Environment, Notice No. 88 of 2006), and the Guidelines on the Method of Animal Disposal (Prime Minister’s Office, Notice No. 40 of 1995).

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Vascular casting with RITC–Dex-GMA or FITC–Dex-GMA. A mixture of 5% RITC–Dex–GMA, 5% acrylamide (011-08015, Wako Pure Chemical Industries Ltd.), and 0.25% VA-044 (017-19362, Wako Pure Chemical Industries Ltd.) was prepared in PBS on ice. We usually prepared 10 ml of the mixture containing dextran 40-based RITC–Dex–GMA and another 10 ml of the mixture containing dextran 200-based RITC–Dex–GMA. The mixtures were separately filtered through Whatman® qualitative filter papers (Grade 1, 1001 125, Sigma-Aldrich) to remove any precipitates and subsequently premixed with PBS. Note that elevation in the pH value, degassing, and loss of filtration may lead to insufficient casting. Then, mice were anesthetized with 25 mg/kg pentobarbital (SOM04-Y01706, Kyoritsu Seiyaku Corporation, Japan) and 10 mg/kg xylazine hydrochloride (593-12891, Wako Pure Chemical Industries Ltd.). Using a peristaltic pump (Minipuls 3, Gilson, Middleton, WI), the mice were perfused with 20 ml of the mixture containing 20 ml of 5% RITC–Dex–GMA (dextran 40 based), 5% acrylamide, and 0.25% VA-044 and 10 ml of 5% RITC–Dex–GMA (dextran 200 based), 5% acrylamide and 0.25% VA-044 at 4 °C at 4 ml/min in this order. During perfusion, all perfusate-containing equipment was placed on ice. After the perfusion was complete, the mice were transferred into 50-ml conical tubes with their head down. Then, the tube was degassed to replace the air with nitrogen because in the air impedes hydrogel formation. The tube was submerged in a 37 °C water bath and incubated for exactly 3 h (longer incubation would make delipidation slower). Vascular casting by RITC–Dex–GMA was carried out in the same manner.

Vascular labeling with fluorophore-conjugated lectin. Mice were anesthetized with 25 mg/kg pentobarbital (SOM04-Y01706, Kyoritsu Seiyaku Corporation) and 10 mg/kg xylazine hydrochloride (593-12891, Wako Pure Chemical Industries Ltd.). For labeling by intravenous injection, the tail of the mouse was placed in a beaker filled with warm water at 40 °C for 30 s so that the tail veins became dilated. One hundred microliters of Texas Red-conjugated lectin (Texas Red® labeled Lycopersicon Esculentum lectum, TL-1176, Vector Laboratories, Burlingame, CA) or DyLight594-conjugated lectin (DyLight® 594 labeled Lycopersicon Esculentum Lectum, TL-1177, Vector Laboratories) was slowly injected via a lateral tail vein with 25-G needle. More than 5 min after injection of lectin, animals were perfused with 20 ml of heparinized PBS, 20 ml of acrylamide (10 U/ml, 17513-41, Nacalai Tesque Inc.) and verapamil (0.2 mg/ml, 222-00781, Wako Pure Chemical Industries Ltd.), 20 ml of 4% PFA, and 20 ml of acrylamide and VA-044. For labeling via transcardial perfusion, anesthetized mice were perfused with 20 ml of heparinized PBS, 20 ml of PBS containing 100 μl of Texas Red-conjugated lectin, 20 ml of PFA, and 20 ml of acrylamide and VA-044.

Vascular labeling with gelatin and FITC-albumin. A gel solution containing 2% porcine skin gelatin type A (G1890, Sigma-Aldrich) was prepared in boiled PBS and cooled to < 50 °C. The solution was then combined with 1% FITC-labeled albumin (A9771, Sigma-Aldrich), maintained with stirring at 40 °C, and filtered through Whatman® qualitative filter papers (Grade 1) that had been premoistened with PBS. The filtered solution was maintained at 40 °C before the perfusion. Then, mice were anesthetized with 25 mg/kg pentobarbital and 10 mg/kg xylazine hydrochloride. Using a peristaltic pump, the mice were perfused with 60 ml of PBS containing 100 μl of Texas Red® labeled Lycopersicon Esculentum lectum, TL-1176, Vector Laboratories, Burlingame, CA) or DyLight594-conjugated lectin (DyLight® 594 labeled Lycopersicon Esculentum Lectum, TL-1177, Vector Laboratories) was slowly injected via a lateral tail vein with a 27-G needle. More than 5 min after injection of lectin, animals were perfused with 20 ml of heparinized PBS, 20 ml of acrylamide (10 U/ml, 17513-41, Nacalai Tesque Inc.) and verapamil (0.2 mg/ml, 222-00781, Wako Pure Chemical Industries Ltd.), 60 ml of 4% PFA. All solutions were maintained at 40 °C. After the perfusion of PFA, 20 ml of the
fluorescent gel perfuse was manually perfused at ~0.6 ml/s, with the body of the mouse tilted by 30° and the head down. The body was submerged in ice water to rapidly cool the gel as the final gel was casted out and then incubated at 37 °C for 48 h. The brain was carefully extracted after ~15 min of cooling and submerged in 4% PFA at 4 °C for 12–16 h.

Clearing protocol. To minimize potential damage to the pial vessels, the brains of the fixed samples were removed together with their skulls. Then, the brains were transferred to 15-ml conical tubes containing 10 ml of 0.5 M EDTA (pH 8.0) at ~0 °C and were frozen with dry ice. Sagittal sections at a 40-µm thickness were prepared with a freezing microtome (SMC-1000, Nippon Denshi Denshi, Ltd.) and were mounted on Vectashield Antifade Mounting Medium (H-1000, Vector Laboratories).

Vascular visualization with anti-CD31 and ETOH-ECI. Two- to five-month-old CX3CR1-GFP- or wild-type mice were anesthetized with 25 mg/kg pentobarbital sodium (Sigma-Aldrich), or FITC-conjugated mouse anti-GFAP (1:100; C9205, Sigma-Aldrich), or Cy3-conjugated mouse anti-GFAP (1:100; C9205, Sigma-Aldrich), or Alexa 488-conjugated mouse anti-NeuN (1:100; MAB377X, Merck-Millipore, USA). Then, the samples were delipidated and washed three times in PBS at room temperature for 2 h each and were placed in 20 ml of ScaleCUBIC-2 at 37 °C for 2 d. ScaleCUBIC-2 was prepared as a mixture of 90% sucrose (30403-55, Nacalai Tesque Inc.) and 10% mouse serum (S-1000, Vector Laboratories). The samples were decalciﬁed overnight at 37 °C while shaking. Then, the softened skulls were gently removed from the shrunken brains. The brain-hydrogel matrices were transferred into 50-ml conical tubes containing 20 ml of 10% SDC (194-08311, Wako Pure Chemical Industries Ltd.)-based buffer at pH 8.5. Then, the samples were incubated for ~7–10 d at 37 °C while shaking. The buffers were refreshed daily or every 2 d. The delipidated samples were washed three times in PBS at room temperature for 2 h each and were placed in 20 ml of ScaleCUBIC-2 at 37 °C for 3 d. ScaleCUBIC-2 was prepared as a mixture of 90% sucrose (30403-55, Nacalai Tesque Inc.) and 10% mouse serum (S-1000, Vector Laboratories). Then, the samples were decalciﬁed at 37 °C for 2.5 mm from the bregma, and ventral 0.5 mm from the surface of the brain) at a rate of 50 ml/min using a syringe pump (KD Scientiﬁc Inc., Holliston, MA, USA) connected to glass pipets (30-0034; Harvard Apparatus, Holliston, MA). After immersion in the clearing solution, the animals were perfused via intracardial injection of 1% glutaraldehyde (7080 A; TaKaRa Bio Inc., Japan), Eglycy (10424; TaKaRa Bio Inc.), or the In-Fusion HD Cloning Kit (Z0648N; TaKaRa Bio Inc.). Recombinant AAV was generated by triple transfection of the 293 AAV cell line (AAV-100; Cell Biosciences, Inc.) with AAVd2 aggregates and PCL helper from the AAV-DJ Helper Free Packaging System (PVD-6300-D); Cell Biosciences, Inc., San Diego, CA) and pAAV-CMCKilaa-MCherry-WPRE-hGH-polyA (Addgene 50476) with the In-Fusion HD Cloning Kit (Z2648N; TaKaRa Bio Inc.). Each AAV vector was puriﬁed using the AAVpro Purification Kit All Serotypes (6666; Takara Bio Inc.). Virus titers were then determined by qPCR using the primer pair AAV2 ITR2p; Luna Universal® qPCR Master Mix (M30035; New England Biolabs, Ipswich, MA), and the LightCycler® qPCR 2.0 system (Roche; Roche, Basel, Switzerland).

Virus injection. Mice anesthetized with isoflurane (Pﬁzer Inc., New York, NY) were placed in a stereotaxic frame following subcutaneous administration of ropivacaine (100 µl; Aspen Japan, Japan). rAAVd2-CMCKilaa-MCherry (1.5 × 1013 GC/ml, 250 µl) was pressure-injected into the primary somatosensory area (SI; caudal −1 mm, lateral ±2.25 mm from the bregma, and ventral 0.5 mm from the surface of the brain) and the primary visual cortex (V1; caudal −3.5 mm, lateral ±2.5 mm from the bregma, and ventral 0.5 mm from the surface of the brain) at a rate of 50 ml/min using a syringe pump (KD Scientiﬁc Inc., Holliston, MA, USA) connected to glass pipets (30-0034; Harvard Apparatus, Holliston, MA). After immersion in the clearing solution, the animals were perfused via intracardial injection of 1% glutaraldehyde (7080 A; TaKaRa Bio Inc., Japan), Eglycy (10424; TaKaRa Bio Inc.), or the In-Fusion HD Cloning Kit (Z0648N; TaKaRa Bio Inc.). Recombinant AAV was generated by triple transfection of the 293 AAV cell line (AAV-100; Cell Biosciences, Inc.) with AAVd2 aggregates and PCL helper from the AAV-DJ Helper Free Packaging System (PVD-6300-D); Cell Biosciences, Inc., San Diego, CA) and pAAV-CMCKilaa-MCherry-WPRE-hGH-polyA (Addgene 50476) with the In-Fusion HD Cloning Kit (Z0648N; TaKaRa Bio Inc.). Each AAV vector was puriﬁed using the AAVpro Purification Kit All Serotypes (6666; Takara Bio Inc.). Virus titers were then determined by qPCR using the primer pair AAV2 ITR2p; Luna Universal® qPCR Master Mix (M30035; New England Biolabs, Ipswich, MA), and the LightCycler® qPCR 2.0 system (Roche; Roche, Basel, Switzerland).

Light transmission measurements. Vascular visualization of wild-type mice was performed with DEX-GMA (nonfluorescent), acrylamide, and VA-044. Then, the samples were delipidated, cleared, decalciﬁed, and RI adjusted. Light transmission was measured from 380 to 780 nm at 5-nm intervals with an integrating sphere (Spectral Haze Meter SH 7000, Nippon Denshoku Industries, Japan). The samples were placed at the center of the optical cell (2277, Nippon Denshoku Industries). Two values were measured by the instrument: diffracted (scattered) light transmission (%, which is the value of the light transmitted through the tissue), and parallel (nondiffracted) light transmission (%), which is the value of light detected without any prevention. The transmission was deﬁned as the diffracted light transmission divided by (100–parallel light transmission)2.

Microscopy. Images were acquired at a 2-step of 10 µm and xy resolution of 0.794 µm using an inverted confocal microscope (×10, NA: 0.4; WD: 2.17 mm; Cell-Voyager CV1000, Yokogawa, Japan) and 488 and 561-nm lasers. Whole-brain images were acquired using a custom-made light-sheet ﬂuorescence microscope (developed by Olympus Corporation, Japan) using a ×0.63 objective lens (NA: 0.15; WD: 87 mm) and an sCMOS Camera (Neo 5.5, Andor Technology, Belfast, United Kingdom) with a 1.6x digital zoom (MVX10, Olympus Corporation). Lasers with wavelengths of 488, 560, and 639 nm were used for image acquisition. Images were captured at a 2-step of 7 µm with a pixel resolution of 6.45 µm.

Quantification of the SN ratio and overlap ratio. Each image was semi-automatically binarized. RTIC–Dex–GMA images were preprocessing the background subtraction algorithm and then binarized using the triple transfection method. Images of CD31 or Texas Red-conjugated lectin were preprocessed using the background subtraction algorithm, bandpass ﬁltered, and binarized using Yen’s algorithm because they could not be adequately binarized with the triangle method due to their weaker signals. Then, the binarized images were scrutinized by eye, and apparent false-positive signals were manually corrected. The overlap ratio was calculated as the quotient of the area where the CD31–positive and the RTIC–Dex–GMA or Texas Red-conjugate lectin-positive pixels were colocalized divided by the area of CD31–positive pixels. The SN ratio of the images was deﬁned as the Z-score of the mean intensities of the signals in the RTIC–Dex–GMA or Texas Red-conjugated lectin-positive areas calculated using the mean and the standard deviation of the signals in the RTIC–Dex–GMA or Texas Red-conjugated lectin-positive areas. The SN ratio of the areas in the tissue sections of the samples of the CD31–EHO–ECI–treated or SeeNet (RTIC–Dex–GMA)–treated samples was calculated in the same manner. Images were preprocessed using the background subtraction algorithm and then binarized using the triangle method. Region of interest (150 × 150 pixels) was deﬁned in the gray matter or the white matter of the optical slices at Z = 2−3150 µm.

Quantification of PL, Iba-1, and dVenus signals. For the measurement of PI and Iba-1 signal intensities, vascular casting of the samples was performed with FITC–Dex–GMA, acrylamide, and VA-044. The hemispheres of the samples were delipidated, stained, and RI adjusted. Images were taken at 200 µm deep from the pia. The signals of the casted vessels were automatically binarized using the triangle algorithm, and the 2D Euclidean distances from the nearest vessels were calculated for every pixel using the Fiji plugin. Only a few pixels were more than 40 µm away from the casted vessels, and therefore, they were
excluded from the quantification. The signals of PI or Iba-1 were automatically binarized with the triangle algorithm, and the median intensities of the signals in PI-positive or Iba-1-positive pixels within a defined range of distances from the vessels were calculated. To measure dVenus signal intensities, Arc-dVenus transgenic mice were housed under dark conditions for at least 60 h and then exposed to an environment lined with alternating black and white stripes (2 cm in width) on the walls at a light level of ~1500 lx. After 5 h of exposure to the vascular casting was performed using RITC-Dex–GMA, acrylamide, and VA-044. The whole brains were delipidated, stained, and RI adjusted. Images were taken from the deep layers of the primary visual cortex (~600 µm deep from the pia) for quantification.

Quantification of tissue swelling and opaque areas. Vascular casting of wild-type mice was performed using noncrosslinkable Dex–GMA, acrylamide, and VA-044. To measure the initial sizes of the brains, they were removed from the skull before decalcification. Then, the brains were treated with EDTA, delipidated, and RI adjusted as described above. At each time point of quantification, the samples were placed on a glass dish with the grid on the bottom and then imaged using a microscope. The area of the brain was calculated in manually segmented images, and the linear expansion value was determined by the square root of the area size changes. Opaque areas were calculated in light-transmitted images of the optically cleared samples. Specifically, the cleared samples immersed in RI-matching solution were imaged, bandpass filtered, and automatically binarized using the triangle algorithm. The original values of the pixels that made the grids on the glass dish invisible in the binarized images were calculated. The areas composed of pixels with values lower than the minimum values calculated above were defined as opaque areas (the pixels on the grid were not included). Then, the opaque areas were divided by the areas of the cleared samples that were not immersed in the RI-matching solution. The areas of the olfactory bulbs were excluded from the calculation because the olfactory bulbs were usually lost during SDS-based clearing.

Quantification of the breakage of blood vessels. Wild-type mice underwent vascular casting. The hemispheres of the samples were delipidated using SDS or SDC and were immersed in ScaleCUBIC-2. Images of the neocortex were captured using a confocal microscope. All identifiable penetrating vessels in a defined area (~50–60 vessels per sample) were inspected by eye, and breaks in the vessels were manually counted. Penetrating vessels were chosen for quantification because their large size and straight morphology allowed us to estimate original connected vessels with high confidence. Pial vessels were not analyzed because they are more vulnerable during sample handling, and a portion of pial venules could be peeled off along with the dural venous sinuses. Furthermore, quantitative comparisons of the breakage of pial vessels were impractical because the majority of pial vessels were lost in SDS-treated samples.

Statistical analysis. Student’s t-test was used for comparisons between two groups. One-way or two-way analysis of variance, Kruskal–Wallis test, Tukey’s test, and Steel–Dwass test were used for comparisons among more than two independent groups. All tests were two-sided. Statistical significance was set at P < 0.05.

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

Data availability
Source data underlying Figs. 1–5 is available as a Source Data file. Any additional data are available from the corresponding author upon reasonable request.

Code availability
Any codes used in this study are available from the corresponding author upon reasonable request.

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**Author contributions**
T.M. conceived and designed the study. T.M. performed all the experiments and the analysis, except the intracranial stereotaxic injection of AAV, which was performed by S.M. E.A.S. and H.R.U. assisted imaging by light-sheet fluorescence microscope. S.Y. provided Arc-dVenus Tg mice. A.N. and H.T. provided H-I7-iCre-imCherry mice. T.M. and Y.I. wrote the manuscript. All authors discussed the results and commented on the manuscript text.

**Competing interests**
Part of this study was performed in collaboration with the Olympus Corporation.

**Additional information**
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