A fluoro-Nissl dye identifies pericytes as distinct vascular mural cells during in vivo brain imaging

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Pericytes and smooth muscle cells are integral components of the brain microvasculature. However, no techniques exist to unambiguously identify these cell types, greatly limiting their investigation in vivo. Here we show that the fluorescent Nissl dye NeuroTrace 500/525 labels brain pericytes with specificity, allowing high-resolution optical imaging in the live mouse. We demonstrate that capillary pericytes are a population of mural cells with distinct morphological, molecular and functional features that do not overlap with precapillary or arteriolar smooth muscle actin-expressing cells. The remarkable specificity for dye uptake suggests that pericytes have molecular transport mechanisms not present in other brain cells. We demonstrate feasibility of longitudinal pericyte imaging during microvascular development and aging and in models of brain ischemia and Alzheimer’s disease. The ability to easily label pericytes in any mouse model opens the possibility of a broad range of investigations of mural cells in vascular development, neurovascular coupling and neuropathology.

Microvascular smooth muscle cells (SMCs) and pericytes play fundamental roles in the development and maintenance of the brain’s vascular network and the blood-brain barrier, signaling within the neurovascular unit, and modulation of microregional cerebral blood flow1–7 and have been implicated in many neuropathological processes2,8,9. However, precise investigation has been greatly limited by the inability to unambiguously differentiate among the different subtypes of vascular mural cells because they share many protein markers and have heterogeneous morphology, with ambiguous boundaries at the transition between precapillary arterioles and capillaries7,10–13. Thus, even though these cells can be broadly grouped into two distinct categories, one type with ring-like morphology and expression of the contractile protein α-smooth muscle actin (α-SMA) and another type with long, thin processes and no α-SMA, there is still considerable debate about the extent to which these are different cell types with different functions2,7,10–17. Development of new tools that allow unambiguous separation of these cells during in vivo imaging would greatly enhance the ability to study the physiology and pathology of the neurovascular unit.

We have discovered that a commercially available small molecule fluorescent dye, a fluoro-Nissl green18 derivative called NeuroTrace 500/525, exclusively and efficiently labels capillary pericytes in the live brain upon topical application or intracortical microinjection. Using transgenic reporter mice and dynamic imaging of spontaneous vasomotility in awake mice, we demonstrate that this dye specifically labels noncontractile vascular mural cells embedded in two layers of basal lamina that have long, thin processes spanning multiple vessel branches and lack α-SMA expression. In contrast, adjacent contractile α-SMA-expressing vascular mural cells with ring-like morphology are not labeled with this dye, strongly suggesting that these cells constitute distinct and non-overlapping populations.

We demonstrate the use of this dye for dynamic imaging in models of cerebral ischemia, aging and Alzheimer’s disease and during early postnatal brain microvascular development. Thus, we provide, to our knowledge, the first method for exclusive identification and in vivo imaging of capillary pericytes in the healthy and diseased brain. This method can be applied immediately, without the need for transgenic reporter mice, and is likely to have broad applications in many fields of research, including development and pathology of the neurovascular unit, neurovascular coupling and neurodegeneration. In addition, the robust and selective dye labeling may allow specific pericyte isolation for gene expression and proteomic studies. It also raises questions regarding the possible expression of distinct molecular transport mechanisms in pericytes that explain their selective dye uptake.

RESULTS
Specific and robust dye labeling of capillary pericytes in the live mouse brain

As we were screening various dyes for differential properties of cell labeling in the live mouse brain, we noticed that a fluorescent Nissl18 derivative with affinity for Nissl bodies, NeuroTrace 500/525 (but not other NeuroTrace variants), commonly used to label neurons in fixed tissues, had unusual properties when applied to the mouse brain in vivo. Instead of neuronal labeling as expected, topical application of this dye through a cranial window led to labeling of a distinct population of cells lining cerebral blood vessels up to 400 µm deep in the cortex (Fig. 1a and Supplementary Video 1). Labeled cells lined the smallest cerebral vessels and had the morphology of capillary pericytes7,13, with multiple slender processes extending longitudinally and spanning several vessel branches (Fig. 1). Dye labeling was very bright and concentrated both in cell soma and throughout the processes, where it displayed a punctate pattern (Fig. 1a,b). Pericyte labeling remained

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strong over 2–3 d and by day 4 had mostly disappeared. Notably, we did not observe any signs of cellular toxicity as a result of the dye administration and subsequent imaging as evidenced by the lack of changes in cellular morphology over intervals up to 3 d.

Surprisingly, no other parenchymal brain cell type was labeled at all (some meningeal and perivascular macrophages at the site of topical dye application were transiently labeled). Colabeling with the fluorescent dye sulforhodamine 101 (SR101), which labels astrocytes and oligodendrocytes\(^{19,20}\), showed no colocalization, confirming that neither of these cell types is labeled by NeuroTrace 500/525 (Fig. 1b). To precisely determine the identity of the labeled cells, we applied the dye to the cortex of transgenic mice with fluorescent protein labeling of all vascular mural cells, initiated by Cre recombinase expression driven by the promoter of the beta receptor for platelet-derived growth factor (Pdgfrb-cre:Tomato\(^{13,21}\)) mice\(^{13,22,23}\). Again, dye labeling was found specifically in fusiform capillary pericytes and not in ring-like precapillary or arteriolar SMCs nor in NG2\(^+\) glia or oligodendrocytes (Fig. 3). Discrete transition points from dye-negative SMCs to dye-labeled pericytes were clearly observed (Fig. 3a). In the NG2-cre mouse line not all pericytes are labeled due to incomplete Cre recombination\(^{13,22}\), in contrast to NeuroTrace, which provided complete labeling of all pericytes in the dye-labeled brain region (Fig. 3c,d).

To further confirm that the NeuroTrace-labeled cells were indeed pericytes embedded in the basal lamina\(^2\), we performed high-resolution confocal imaging with deconvolution, observing collagen IV basal lamina antibody staining surrounding cells labeled with NeuroTrace and Pdgfrb-cre:Tomato (Fig. 4 and Supplementary Fig. 2).
This approach allowed direct visualization of the two layers of the basal lamina with molecular specificity. All Pdgfrb-cre:Tomato cells with capillary pericycle morphology were embedded in the collagen IV-labeled basal lamina (Fig. 4a,b). During immunostaining, NeuroTrace dye diffuses out of pericytes that are labeled through in vivo cortical dye application. Thus, we had to perform sequential imaging of the same cell before and after immunostaining to directly detect dual basal lamina surrounding cells that had previously been labeled with NeuroTrace. All cells that were labeled with NeuroTrace and sequentially imaged in this manner displayed robust two-layer collagen IV-labeled basal lamina (Fig. 4c and Supplementary Fig. 2, n = 11 cells). To provide further evidence of this cellular localization, we attempted to photo-oxidize NeuroTrace for diaminobenzidine (DAB) detection at the ultrastructural level (Fig. 4d). Photo-oxidation is known to have heterogeneous success rates for different dyes24,25, and we had some limited success (Fig. 4d) at detecting the DAB signal in pericytes using transmission electron microscopy. Reasons for the heterogeneity could be poor photo-oxidative reactivity of the dye, as has been shown for other dyes24,25. Nonetheless, this combination of approaches allowed us to conclusively demonstrate that NeuroTrace-labeled cells are indeed embedded in two layers of basal lamina, further confirming that they are indeed capillary pericytes (Fig. 4 and Supplementary Fig. 2).

There are two potential routes for the NeuroTrace dye to enter the brain: directly through the glia limitans into the interstitial space26 or via diffusion through the perivascular Virchow–Robbins space. The second route has the potential to result in predominant labeling of pericytes given their perivascular location. Thus, to bypass these barriers and test whether the dye specifically labels pericytes regardless of entry route, we directly microinjected NeuroTrace into the parenchyma through a pulled glass capillary (Supplementary Fig. 3a). Two-photon time-lapse imaging during dye injection revealed exclusive pericycle labeling within minutes, with no labeling of other cell types (Supplementary Fig. 3a–c and Supplementary Video 2). Thus, the specificity of labeling is likely due to an intrinsic property of capillary pericytes that is absent from any other brain cell, including the immediately adjacent perivascular SMCs. This specificity, combined with the robust labeling brightness and persistence over days, makes the use of this dye, to our knowledge, the first method to unambiguously image and distinguish capillary pericytes from all other brain cells in vivo.

NeuroTrace-labeled pericytes do not exhibit spontaneous contractility

Neurovascular coupling is a critical brain homeostatic function that is controlled by complex interactions between cells of the neurovascular unit9. There is ongoing debate as to which subsets of mural cells are ultimately responsible for vascular dilation and constriction and whether there are subsets of capillary pericytes capable of mediating vasomotility7,10,11. Resolving this controversy is critical for our understanding of neurovascular coupling and blood flow control. To determine whether NeuroTrace-labeled pericytes are present on vessels that exhibit active vasomotility, we performed in vivo imaging in transgenic mice with mCherry labeling of SMC-expressing smooth muscle actin (Acta2 gene promoter, designated here as SMA) (SMA-mCherry)7,27 (Fig. 5). NeuroTrace exclusively labeled mural cells that did not express mCherry on vessels ranging in diameter from 2.3 to 13.7 µm (average 5.3 ± 2.2 µm, 104 vessel locations from n = 5 mice) (Fig. 5c). SMA-mCherry-covered vessels that were not labeled with NeuroTrace had a range of diameters from 2.9 to 29.9 µm (average 9.6 ± 4.7 µm, 115 vessel locations from n = 5 mice) (Fig. 5c). Consistent with the results in Pdgfrb-cre:Tomato and NG2-cre:Tomato mice (Fig. 2 and Fig. 3), NeuroTrace was able to precisely highlight transition points from completely unlabeled SMCs to strongly labeled pericytes (Fig. 5b and Fig. 6a). Furthermore, consistent with SMA-mCherry labeling, the arteriole-specific dye 633-hydrazide28 exclusively labeled vessels that were not labeled with NeuroTrace (Fig. 5d), providing further evidence of the existence of two distinct populations of mural cells, α-SMA-expressing SMCs on arterioles and NeuroTrace-labeled pericytes on capillaries.

To determine whether these two populations of cells exhibit different functions, we performed in vivo time lapse imaging of spontaneous vasomotion in awake and anesthetized mice. As was recently shown7,14, spontaneous vasomotility was found exclusively in SMC- but not pericyte-covered vessels as based on a measure that accounts for amplitude, frequency and duration of diameter changes, termed vasomotility index7 (see Online Methods) (Fig. 6). Thus, NeuroTrace specifically labels noncontractile capillary pericytes and does not label contractile precapillary and arteriolar α-SMA-expressing mural cells. Therefore, NeuroTrace provides a new method for in vivo investigations of differential mural cell physiology during neurovascular coupling and potentially during pathological processes.

Intravital imaging of pericytes during development and aging, in cerebral ischemia, and in Alzheimer’s models

A major challenge for determination of capillary pericyte function in aging and neuropathological conditions has been the lack of methods for precise identification and visualization during in vivo experimentation. Therefore, we explored whether NeuroTrace labeling could
be used in a range of conditions in which pericyte dysfunction has been implicated. First, NeuroTrace labeling in early postnatal stages, adult and aged 29-month-old wild-type mice revealed brightly labeled pericytes with no significant differences in dye labeling quality or distribution among postnatal day (P) 10, P60 and P870 mice (Fig. 7a,b) (P10: 3,388 ± 769 cells/mm³, P60: 2,860 ± 166 cells/mm³, P870: 3,245 ± 317 cells/mm³, n = 3 mice per group, two-way ANOVA, Bonferroni post-test). In addition to quantifying cell density, we also observed dye-labeled pericyte cell bodies and processes not associated with perfused vessels (Fig. 7c,d). These cells were more prevalent in P10 mice than in P60 and P870, likely due to pericyte association with unperfused vascular sprouts 29–31 (P10: 173 ± 91 cells/mm³, P60: 24 ± 28 cells/mm³, P870: 28 ± 36 cells/mm³, n = 3 mice per group, two-way ANOVA, Bonferroni post-test). Notably, however, pericytes bridging regions with no evident perfused vessels were also found in adult and aged mice, when most vascular structural plasticity has ended (Fig. 7d,e), suggesting potential signaling functions of these bridging cells and/or permanent remnants of developmental vascular pruning.
Thus, NeuroTrace can be used for pericyte labeling to determine \textit{in vivo} cell–cell interactions during development of the neurovascular unit and to test age-related mural cell-associated vascular dysfunction in any wild-type or transgenic mouse model.

Next we visualized pericytes in a model of transient ischemia via reversible bilateral common carotid artery occlusion (Supplementary Fig. 4a). This model, which has been previously characterized, allows dynamic imaging during the initial stages of ischemia. This is a time when cortical spreading depolarization, a known modulator of SMC contractility, is highly prevalent and early changes in vascular and neuronal pathology occur\textsuperscript{32}. Five minutes of bilateral carotid occlusion caused marked reduction in cerebral blood flow and resulted in reversible collapse or constriction of vessels specifically covered by \(\alpha\)-SMA-expressing SMCs (\(-56 \pm 14.7\%\) change in diameter, 23 vessel locations from \(n = 3\) mice) (Supplementary Fig. 4b–e). In contrast, NeuroTrace-labeled pericyte-covered capillaries displayed decreased or stalled blood flow but did not exhibit significant changes in diameter (\(-3 \pm 1.4\%\) change in diameter, 28 vessel locations from \(n = 3\) mice (Supplementary Fig. 4b–e). Thus, it appears that only \(\alpha\)-SMA-positive but not NeuroTrace-labeled cells contract appreciably during

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\caption{NeuroTrace-labeled pericytes are embedded in the basal lamina. (a) Confocal fluorescence image after deconvolution captured from the cortex of a \textit{Pdgfrb-cre:Tomato} transgenic mouse stained with collagen IV antibody, showing detection of the double layer of the basal lamina (arrowheads) completely surrounding a capillary pericyte labeled with Tomato. (b) Confocal projection (left) and single \(z\) plane (right) of a single pericyte cell body (white arrowheads) and proximal processes (yellow arrowheads) showing complete envelopment in the collagen IV-labeled basal lamina layers. (c) Images of single pericytes (arrowheads) labeled with NeuroTrace and Tomato and then reimaged after immunostaining for collagen IV, further confirming that NeuroTrace labels capillary pericytes (\(n = 11\) cells). (d) Transmission electron micrographs taken from tissue that has undergone photo-oxidation of NeuroTrace-labeled pericytes for detection of electron-dense DAB products. Red arrowheads indicate putative photo-oxidized DAB products found in the pericyte cell body and processes. Yellow arrowheads indicate double basal lamina surrounding the pericytes. Asterisk indicates likely ‘peg and socket’ pericyte process. Each image is representative of at least three locations in at least three animals.}
\end{figure}
transient ischemia. This dye could thus be used to identify pericytes for a variety of studies during ischemic pathology.

Finally, as smooth muscle cells and pericytes have been implicated in the pathogenesis of Alzheimer’s disease, in a mouse model of this disease (5xFAD mice). Amyloid plaques and cerebral amyloid angiopathy were labeled with the amyloid-binding dye thioflavin S (Fig. 8a,b). Consistent with our finding in aged wild-type mice, NeuroTrace labeling in 5xFAD mice was similar to that in wild-type aged matched controls (Fig. 8c) (wild-type: 2,999 ± 254 cells/mm³, 5xFAD: 2,982 ± 162 cells/mm³, n = 3 mice per group, P = 0.947, t-test). Furthermore, we did not detect NeuroTrace labeling on vessels that exhibited cerebral amyloid angiopathy (Fig. 8a,b), suggesting that overt amyloid aggregation may be exclusive to α-SMC-covered arterioles in these mice.
DISCUSSION

Current approaches for pericyte identification in live animals rely on transgenic promoter expression based on NG2 or Pdgfrb2,7,10,13,14. However, both of these markers label other cell types, and there are no known exclusive genetic markers of pericytes, prohibiting their unambiguous separation from other vascular mural cells. Even currently available dual-reporter, triple-transgenic mice cannot exclusively label pericytes7. Thus the identification of a capillary-pericyte-specific marker is crucial for future studies. To our knowledge, NeuroTrace labeling provides the first method for unambiguous in vivo identification of brain capillary pericytes as distinguished from all other brain cells. NeuroTrace is easy to use during intravitral imaging, it robustly and brightly labels capillary pericytes embedded in the vascular basal lamina, and it can be used for repeated imaging of these cells without acute evidence of toxicity. This method can now be implemented in vivo for broad interrogation of pericyte function throughout development and into advanced aging and in numerous models of brain pathology.

NeuroTrace exclusively labels capillary pericytes, which are cells with long processes that do not fully wrap around vessels, in contrast to immediately upstream precapillary and arteriolar ring-like mural cells. The specific nature of dye labeling to capillary pericytes, combined with the fact that these cells have a unique morphology, do not express α-SMA and are not contractile, provides a strong line of evidence that pericytes are a discrete cell population that is distinct from immediately adjacent SMCs and that there are no transitional precapillary cells with hybrid properties. Furthermore, the exclusivity of dye uptake suggests a distinct molecular mechanism unique to brain capillary pericytes, opening the possibility of future specific molecular targeting of pericytes for therapeutic purposes.

The precise role of capillary pericytes in neurovascular coupling is debated7,10,11,14. Under anesthetized and awake conditions, we found no evidence of spontaneous changes in vessel diameter on vessels covered by NeuroTrace labeled pericytes or mCherry-labeled SMCs (anesthetized: NeuroTrace-labeled, 31 vessels; mCherry-labeled, 30 vessels from n = 5 mice; awake: NeuroTrace-labeled, 26 vessels; mCherry-labeled, 28 vessels from n = 4 mice). Each image is representative of at least three locations in at least three animals. I.V., intravenous.
These findings do not exclude a signaling role for pericytes in neurovascular coupling, as the dynamic calcium fluctuations and gap junction coupling between these cells and others in the neurovascular unit suggest distinct cell-to-cell signaling mechanisms under various situations. NeuroTrace now allows investigation of the roles of these cells in the intact in vivo environment, with...
investigation of distinct contributions of different cell types to these diseases. The differences in our results compared to previous studies regarding pericyte changes in aging and Alzheimer's disease models are unclear. However, one possibility is that previous studies have generally pooled both SMCs and pericytes in their quantifications. Given the known presence of amyloid accumulation in arterioles, SMCs might be more vulnerable to age-related changes than capillary pericytes.

NeuroTrace labeling now provides a rapidly adoptable method to exclusively label capillary pericytes in aged wild-type and transgenic mouse models and potentially in other species. This tool will facilitate future investigations of pericycle contributions to normal brain function and pathology.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Figure 8 Pericycle imaging in Alzheimer's mouse models in vivo. (a) Pericycle labeling in a mouse model of Alzheimer's disease (5xFAD) showing cerebral amyloid (white) angiopathy on arterioles and precapillary arteries (arrows) but not pericycle-covered capillaries. Arrowheads indicate amyloid plaques found throughout the parenchyma. (b) In vivo images captured from the cortex of aged matched wild-type and 5xFAD mice. Arrows indicate cerebral amyloid angiopathy and arrowheads indicate amyloid plaques. (c) Pericyte labeling density is not different between 9-month-old wild-type (WT) and 5xFAD transgenic mice (n = 3 mice per group, mean ± s.e.m., unpaired two-tailed Student's t-test P-value as indicated, t = 0.07051, d.f. = 4). Each image is representative of at least three locations in at least three animals. I.V., intravenous.

fundamental implications for understanding the cellular mechanisms of cerebral blood flow control.

Pericycle dysfunction has been implicated in several neuropathological states, including brain ischemia and Alzheimer’s disease. The inability to precisely identify pericytes in previous studies has limited the in vivo investigation of distinct contributions of different mural cell types to these diseases. The differences in our results compared to previous studies regarding pericycle changes in aging and Alzheimer’s disease models are unclear. However, one possibility is that previous studies have generally pooled both SMCs and pericytes in their quantifications. Given the known presence of amyloid accumulation in arterioles, SMCs might be more vulnerable to age-related changes than capillary pericytes.

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Figure 8 Pericycle imaging in Alzheimer’s mouse models in vivo. (a) Pericycle labeling in a mouse model of Alzheimer’s disease (5xFAD) showing cerebral amyloid (white) angiopathy on arterioles and precapillary arteries (arrows) but not pericycle-covered capillaries. Arrowheads indicate amyloid plaques found throughout the parenchyma. (b) In vivo images captured from the cortex of aged matched wild-type and 5xFAD mice. Arrows indicate cerebral amyloid angiopathy and arrowheads indicate amyloid plaques. (c) Pericycle labeling density is not different between 9-month-old wild-type (WT) and 5xFAD transgenic mice (n = 3 mice per group, mean ± s.e.m., unpaired two-tailed Student’s t-test P-value as indicated, t = 0.07051, d.f. = 4). Each image is representative of at least three locations in at least three animals. I.V., intravenous.

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ONLINE METHODS

Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee at Yale University (IAUCU). Male and female mice aged P8–P870 housed in a 12-h light/dark cycle with 3–5 animals per cage were used. Animals were assigned randomly to groups and no animals were excluded from analysis. The following transgenic lines were used for visualization of defined cell populations: wild-type C57BL/6, Ng2-cre (ref. 22; Jax 008533), Tomato reporter Ai14 (ref. 38; Jax 007914), SMA-mCherry (ref. 27), Pdgfrb-cre (ref. 21) and Alzheimer’s mouse model 5xFAD (ref. 39).

Cranial window preparation and in vivo imaging. Mouse cranial windows were prepared as previously described40. Briefly, animals were anesthetized via intraperitoneal injections of 100 mg/kg ketamine and 10 mg/kg xylazine. A 3–4 mm craniotomy was prepared over the somatosensory cortex and the underlying dura was removed. After dye labeling (see below), a 40 cover glass was placed over the exposed area and sealed with dental cement. For all experiments except awake imaging, mice were imaged while anesthetized with a mixture of ketamine and xylazine as stated above. For awake imaging experiments (Fig. 6), mice were habituated to head fixation and imaged through an acute cranial window during quiet waking. For repeated in vivo imaging a nut was affixed to the skull and embedded in dental cement for head immobilization and window orientation. Cerebral vessels were visualized by intravenous injection of 70,000-Da Texas Red dextran, 10,000-Da Cascade Blue dextran or Evans blue. To visualize the endothelium in vivo we intravenously injected PECAM-Alexa Fluor 647-conjugated antibody. In vivo images were acquired using a two-photon microscope (Prairie Technologies) equipped with a mode-locked MaiTai two-photon laser (Spectra Physics) and 20× water immersion objective (Zeiss, 1.0 NA). The two-photon laser was tuned to the following wavelengths for optimal excitation of particular fluorophores: 1,000 nm for NeuroTrace 500/525, SMA-mCherry, Evans blue and Texas Red dextran. In some cases, an upright Leica SP5 confocal microscope with a 20× water immersion objective (1.0 NA, Leica) was used for optimal fluorophore excitation and emission separation. For confocal imaging the following wavelengths were used: 405 nm for Cascade Blue dextran, 488 nm for GFP, 561 nm for mCherry, tdTomato and Texas Red dextran and 633 nm for Evans blue and PECAM-647.

Fluorescent dye labeling. NeuroTrace 500/525 dye was either applied topically (1:25 dilution in PBS) to the cortical surface for 5 min and then the surface was thoroughly washed, or 300 nL was injected into the brain using a pulled glass pipette attached to a Nanoject II (Drummond Scientific). For topical application, bright labeling was evident 3 h after labeling and remained for at least 48 h. For amyloid labeling in 3xFAD mice, thioflavin S (0.002% in PBS) was applied topically to the cortical surface for 30 min then the surface was washed thoroughly with PBS.

Bilateral common carotid artery occlusion. Acute brain ischemia was achieved by temporary bilateral occlusion of the common carotid arteries as previously described32. Briefly, animals were anesthetized, the common carotid arteries were dissected and exposed, and suture loops were placed around each vessel. The animal’s skin was stitched while allowing the suture loops to extend through the incision for access during in vivo imaging. Animals were imaged in vivo with a confocal microscope to allow sufficient three color fluorophore separation. z-stack time-lapse sequences were acquired for 20 min and ischemia was induced by tightening the suture loops with a micromanipulator to transiently block blood perfusion for 5 min during the 20-min time lapse-sequence (Supplementary Fig. 4).

Tissue collection and immunohistochemistry. Mice were anesthetized and perfused with 4% paraformaldehyde and brains were post-fixed overnight at 4 °C. Brain tissue sections 50 μm thick were cut on a vibratome and processed for immunohistochemistry. Tissue sections were blocked in PBS containing 5% normal goat serum, 0.5% bovine serum albumin (BSA) and 0.3% Triton-X-100 at room temperature. All primary and secondary antibodies were diluted in PBS containing 0.5% BSA and 0.3% Triton-X-100. Tissue sections were incubated in primary antibodies overnight at 4 °C and secondary antibodies for 4 h at room temperature. The following primary antibodies were used: collagen IV (Abcam cat. no. AB19808; 1:250 dilution)41, Pdgfrb (eBioscience cat. no. 14–1402–82; 1:200)42 and NG2 (Millipore cat. no. MAB5384; 1:100)23,43. Antigen retrieval was performed for collagen IV staining by incubating slices at 85 °C for 20 min in PBS before blocking. Images were captured on a Leica SP5 laser scanning confocal microscope.

For sequential imaging of NeuroTrace labeled cells with collagen IV immunostaining, PFA-perfused cerebral cortex tissues that had been labeled with NeuroTrace in vivo after pial surface dye application were cut into 50-μm vibratome sections. Sections were mounted on slides in PBS and single NeuroTrace and Pdgfrb-cre:Tomato double-labeled pericytes were imaged at high resolution on a confocal microscope. Cell locations within the slice were mapped and recorded. Tissue sections were then processed for immunohistochemistry as described above. After staining, the sections were remounted, previously imaged cells were relocated and high resolution confocal images were acquired of the same cells to determine whether they were embedded in a dual layer of the basal lamina. This procedure was repeated on 4 brain tissue sections and sequential images from 11 cells were acquired as indicated in the text.

For photo-oxidation experiments, cerebral cortex tissues labeled with NeuroTrace in vivo after pial surface dye application were cut into 100-μm sections. Tissue sections were washed with 0.1 M Tris HCl buffer (pH 8.0), then incubated with 2 mg/ml DAB in 0.1 M Tris buffer (pH 8.0, freshly prepared, ice-cold) for 10 min. Photo bleaching of NeuroTrace labeled region was achieved by using a 100 W mercury lamp (488 nm) and a 10× (0.25 NA) objective, and lasted for 40–60 min. During the bleaching, ice-cold DAB solutions were replaced every 10 min. The illumination was stopped when the fluorescence of NeuroTrace had faded completely. After photo bleaching, the DAB precipitate could be seen with bright field microscopy. Then the tissue sections were rinsed with PBS thoroughly and further processed for electron microscopy.

Quantification and statistics. Images were processed and analyzed using ImageJ software. For image deconvolution (Fig. 4 and Supplementary Fig. 2), images were deconvolved with Huygens Professional version 16.05 (Scientific Volume Imaging, the Netherlands) using the CMLE algorithm, with SNR:20 and 40 iterations. Quantification of NeuroTrace labeling and Pdgfrb-cre:Tomato cell colocalization in vivo was acquired at 4 locations per animal from 3 animals. Quantification of Pdgfrb antibody and Pdgfrb-cre:Tomato cell colocalization was performed at 4 locations of the cortex, across 3 brain sections per animal from 3 animals. For spontaneous vasomotion (Fig. 6), a vasomotion index was calculated on vessels with diameters less than 15 μm. Briefly, the vasomotion index was defined as the area under the curve for percent spontaneous changes in vessel diameter over 60 s time-lapse sequences with a 5% cutoff threshold. Baseline values were determined as the average diameter of all time points for spontaneous diameter changes during each time-lapse sequence. Vessel measurements were derived from the gradient in light intensity (dIdt/ds) along a designated line selected perpendicular to the single vessels from frame scan images collected at a frequency of 1 Hz. The resulting images were thresholded and the width of the vessel was automatically determined using a custom script in ImageJ. For changes in vessel diameter during common carotid artery occlusion, single vessel locations were randomly selected from time-lapse sequences and vessel diameters were manually measured before occlusion, 5 min after the start of occlusion (designated ‘during’ in Supplementary Fig. 4) and 10 min after reperfusion (designated ‘after’ in Supplementary Fig. 4) in ImageJ. Percent changes in vessel diameter were determined for vessels covered by SMA-mCherry-expressing SMCs and vessels covered by NeuroTrace-labeled pericytes as indicated.

All data were assumed to have a normal distribution for each statistical test and all data are displayed as mean ± s.d. in the text and mean ± s.e.m. in graphs unless otherwise indicated. No data were excluded from analysis, no randomization was used to assign experimental subjects and experimenter blinding was not necessary. No statistical methods were used for predetermined sample size determination, but our sample sizes are similar to those reported in previous publications13,14,31. For each experiment at least 3 animals were used, with animal and cell numbers indicated in the text. Each representative image was successfully repeated in at least three image locations for each animal with sample sizes (n) designates as single cells followed over multiple days or single animals imaged, as indicated in the text and figure legends. To determine statistical significance for cell density quantifications and for vessel diameter changes (Figs. 7 and 8 and Supplementary Fig. 4), unpaired two-tailed Student’s t-tests or two-way ANOVAs with Bonferroni post-tests were used as indicated. Statistical analyses

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were performed with Microsoft Excel and GraphPad Prism. A P value of <0.05 was considered significant. Investigator blinding and animal randomization were not required for these experiments. A Supplementary Methods Checklist is available.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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