Non-invasive deep-tissue three-dimensional optical imaging of live mammals with high spatiotemporal resolution is challenging owing to light scattering. We developed near-infrared II (1,000–1,700 nm) light-sheet microscopy with excitation and emission of up to approximately 1,320 nm and 1,700 nm, respectively, for optical sectioning at a penetration depth of approximately 750 μm through live tissues without invasive surgery and at a depth of approximately 2 mm in glycerol-cleared brain tissues. Near-infrared II light-sheet microscopy in normal and oblique configurations enabled in vivo imaging of live mice through intact tissue, revealing abnormal blood flow and T-cell motion in tumor microcirculation and mapping out programmed-death ligand 1 and programmed cell death protein 1 in tumors with cellular resolution. Three-dimensional imaging through the intact mouse head resolved vascular channels between the skull and brain cortex, and allowed monitoring of recruitment of macrophages and microglia to the traumatic brain injury site.

**Results**

Light scattering suppression in NIR-II LSM. Our home-built light-sheet microscope employed multiple switchable lasers with Gaussian beams (658 nm, 785 nm and 1,319 nm) cylindrically focused into static light sheets for optical sectioning and an InGaAs camera for orthogonally detecting 900- to 1,700-nm fluorescence (Fig. 1a and setup details in Supplementary Fig. 1). We adjusted the effective numerical aperture (NA) of the illumination objectives to produce light sheets with balanced waist thickness (approximately 10–20 μm) and Rayleigh length (approximately 0.5–2.0 mm) (Methods), suitable for large-scale volumetric imaging with single-cell resolution. We employed models of cardiovascular and brain diseases and cancer.

Non-invasive imaging through the skin, skull and body tissues was achieved, with large penetration depths and high signal-to-background ratio (SBR)

Here we describe NIR-II LSM using organic dyes and PbS/CdS core/shell quantum dot (CSQD) probes, extending excitation and emission up to approximately 785–1,320 nm and 1,000–1,700 nm, respectively. Suppressed light scattering of both excitation and emission allowed ex vivo volumetric imaging of glycerol-cleared mouse brain of up to 10 mm with a penetration depth of approximately 2 mm. Importantly, NIR-II LSM readily afforded in vivo imaging of mouse tumor and traumatic brain injury (TBI) models non-invasively, enabling real-time observation of unusual tumor microcirculation, 3D tracking of the recruitment of macrophages and microglia to the injury region and 3D molecular imaging of immune checkpoint proteins at a cellular scale in live mammals.
several biocompatible NIR-II probes, including the organic nanofluorophore p-FE (excitation and emission of 650–850 nm and 1,000–1,300 nm, respectively; dynamic light scattering size of approximately 12 nm; Fig. 1b and Supplementary Fig. 2) and PEGylated PbS/CdS CSQDs (excitation and emission of ultraviolet to 1,500 nm and 1,500–1,700 nm, respectively; size of approximately 6.9 nm; Fig. 1b and Supplementary Fig. 2). The two probes were sequentially injected intravenously into a mouse through the tail vein at an interval of 5 min. We killed the mouse 30 min after injection while the probes were still circulating in the vasculature, fixed the brain and placed it in glycerol for ex vivo LSM imaging (Methods). Under the same 785-nm light-sheet excitation, we were able to clearly image the cerebral vasculatures as a function of tissue depth $z$ in three fluorescence emission windows of 850–1,000 nm (NIR-I, p-FE emission), 1,100–1,200 nm (NIR-IIa, p-FE emission) and 1,500–1,700 nm (NIR-IIb, CSQD emission) (Fig. 1c and Supplementary Video 1). This allowed side-by-side comparison (Fig. 1c and Supplementary Fig. 3) of fluorescence LSM imaging in three subregions of 850–1,700 nm under the same 785-nm light-sheet excitation. Refractive index mismatch during scanning was compensated for by using a linearly moving detection objective (Supplementary Figs. 4 and 5).
and no photobleaching was observed through this work, owing to the highly photo-stable NIR-II probes and minimal photo-damage inherent to LSM10,11.

With a 785-nm light sheet, we observed that during imaging of glycerol-cleared brain tissue the depth limit increased (Fig. 1c and Supplementary Video 1), the background signal decreased (Fig. 1d) and the SBR increased (Fig. 1e) at longer detection wavelengths from 850–1,000 nm to 1,100–1,200 nm and 1,500–1,700 nm (Supplementary Video 1). The imaging depth limit (defined as the tissue depth at which SBR decreased to approximately 2) increased from \( z_{SBR=2} \) approximately 1.0 mm to approximately 2.0 mm and 2.5 mm as emission wavelength increased from approximately 850 nm to approximately 1,100 nm and 1,700 nm, respectively (Fig. 1e and Supplementary Video 1). These were direct results of suppressed scattering of emission photons (scattering \( \propto \lambda^{-k} \), where \( \lambda \) is wavelength and \( k \) is in the range of 0.2–4.0 for biological tissues20) under the same 785 nm excitation. Background signal caused by scattering was the lowest in

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**Fig. 2 | Propagation of light-sheet excitation with progressively longer wavelengths up to 1,319 nm in glycerol-cleared brain tissues.**

a. \( xy \) images of quantum dot fluorescence at 1,500–1,700 nm in the vasculatures of fixed brain tissue at depth \( z \approx 200 \mu m \) under 658-nm (0.22 mW), 785-nm (0.33 mW) and 1,319-nm (1.4 mW) light-sheet illumination as shown in the inset. Six images were taken along the \( x \) direction and stitched together for each light sheet.

b. Normalized sum intensity along the \( y \) direction of images in a as a function of propagation distance (\( x \)). Similar results for \( n = 2 \) individuals (C57BL/6, female, 6 weeks old).

c, d. Monte Carlo simulations (c) and experimental results (d) showing the \( xz \) propagation of light sheets of different wavelength in 2.5% intralipid tissue phantom (mimicking the brain) containing PEGylated PbS/CdS CSQDs. Similar results for \( n = 3 \) independent experiments. Scattering coefficients are summarized in Supplementary Table 1.

e. Left, \( xy \) images of quantum dot fluorescence at 1,500–1,700 nm in brain vasculatures taken at \( z = 925 \mu m \) under excitations by 658-nm, 785-nm and 1,319-nm light sheets (Supplementary Video 2). Right, images along the \( xz \) plane at a fixed \( y \), reconstructed from \( xy \) images at various depths \( z \) (Supplementary Video 3).

f. Comparison of SBR for \( xy \) images recorded at different depths for 658-nm, 785-nm and 1,319-nm excitation. About ten randomly selected vasculatures and ten areas without vasculatures were analyzed to calculate SBR at each depth. Data are shown as the mean ± s.d.
Fig. 3 | Non-invasive in vivo NIR-II LSM of tumors on mice. a, Time-course LSM (Supplementary Video 5) of tumor vasculatures at a fixed illumination plane below the top of a xenograft MC38 tumor on a mouse ear at \( z \approx 300 \mu m \) after intravenous injection of p-FE (excitation, 785 nm; emission, 1,000–1,200 nm). b, A 4x detection objective and a 5x illumination objective in a normal non-oblique configuration were used. c, Abnormal blood flows in tumor vessels, showing on-off intermittency and direction reversal in the rectangular highlighted region in a and gradual extravasation into tumor space (Supplementary Video 5). Similar results for \( n = 3 \) individuals (CS7BL/6, female, 6 weeks old). Black arrows represent flow direction. d, A BST map showing highly heterogeneous blood perfusion in tumor vessels and slow, inhomogeneous extravasation behavior into tumor space. Similar results for \( n = 2 \) individuals (CS7BL/6, female, 6 weeks old). e, Schematic of in vivo oblique NIR-II LSM with illumination and detection at 45° to the mouse body (Supplementary Fig. 1b). f, Time-course recording (Supplementary Video 6) of PD-1+ cells (white circles) in a CT26 tumor labeled by anti-PD-1 CSQDs 2 h after intravenous injection of anti-PD-1 CSQDs at 20 frames per second by oblique LSM. g, Wide-field imaging of cells labeled with anti-PD-L1 ErNP (magenta), anti-PD-1 PEGylated PbS/CdS CSQD (green) and p-FE filling vessels (blue) in a CT26 tumor. h, Non-invasive in vivo three-plex 3D LSM of anti-PD-L1 ErNP, anti-PD-1 PEGylated PbS/CdS CSQD and vasculatures (p-FE), approximately 120 μm beneath the surface in a CT26 tumor. i, A yz cross-section of the tumor in g, j, A local zoom three-plex 3D LSM of the tumor in g (Supplementary Video 7). In f–j, similar results were obtained for \( n = 2 \) individuals (BALB/c, female, 6 weeks old). In a, c, tumors were imaged by NIR-II LSM as in b. In f, h–j, images were obtained using oblique NIR-II LSM as in e.
the emission range from 1,500–1,700 nm at all imaging depths (z up to 3 mm; Fig. 1d and Supplementary Video 1). For emission in the range from 850 to 1,200 nm, background signal increased with tissue imaging depth up to $z \approx 1$ mm owing to increased scattering by thicker tissue and decreased upon further increases in depth for $z > 1$ mm (Fig. 1d). The latter was attributed to increased light absorption by thicker tissue that attenuated the background signal.

**Fig. 4 | Non-invasive in vivo light-sheet imaging of mouse head using oblique NIR-II LSM.**

- **a**, A 3D reconstructed image of blood vessels in an intact mouse visualized through the scalp, skull, meninges and brain cortex obtained 2 h after intravenous injection of PEGylated PbS/CdS CSQDs by oblique NIR-II LSM as shown in Fig. 3e. The white triangles point to vascular channels connecting the brain cortex and skull in the meninges.
- **b**, An expanded 3D view of the scalp layer showing follicle structures.
- **c**, Top, a schematic showing the definitions of penetration depth, detection depth and imaging depth in oblique NIR-II LSM. The illumination direction was approximately 45° to the mouse head. Bottom, an original image recording a cross-section along the illumination direction in a crossing the full FOV of the camera when a 10x imaging objective was used. In a–c, similar imaging was performed at three positions on the heads of two mice (BALB/c, female, 4 weeks old), for total n = 6.
- **d**, A 3D reconstructed image of vascular channels (indicated by triangles) in the meninges obtained at a later time point (12 h after injection of PEGylated PbS/CdS CSQDs) with a 4-μm scan increment along the x axis and 10-ms exposure. Similar results for n = 2 individuals (BALB/c, female, 4 weeks old).
- **e**, Wide-field TBI imaging of a mouse head 26 h after injury and 24 h after intravenous injection of anti-CD11b PEGylated PbS/CdS CSQDs.
- **f**, 3D time-course light-sheet imaging and monitoring of the dynamics of meningeal macrophages and microglia after brain injury 24 h after injection of anti-CD11b PEGylated PbS/CdS CSQDs at the boundary of the TBI region (rectangular marked area in e). Recruitment of labeled CD11b$^+$ macrophages and microglia to the injury was monitored (Supplementary Video 8). Similar results for n = 2 individuals (BALB/c, female, 4 weeks old).
The lateral full width at half maximum (FWHM) values of the smallest cerebral vasculatures imaged in the three emission regions (850–1,000 nm, 1,100–1,200 nm and 1,500–1,700 nm; Fig. 1f) at their tissue imaging depth limits of z_{max} = 1.0 mm, 2.0 mm and 2.5 mm were approximately 7.2 μm, 9.0 μm and 8.3 μm, respectively. Using an imaging objective with high magnification and NA, lateral FWHM values of less than 5.0 μm were achieved by NIR-II LSM imaging by detecting emission at 1,500–1,700 nm under a 785-nm light-sheet illumination (Supplementary Fig. 6).

Studies of light sheets propagated in a scattering medium such as an intralipid phantom mimicking glycerol-cleared brain tissue, Monte Carlo simulations and experiments showed light sheets decaying in intensity in the x–y plane and spreading in the z direction owing to tissue scattering (Fig. 2a–d and Supplementary Figs. 7–10), which could hinder optical-sectioning capability with reduced imaging field of view (FOV) in x and y lower spatial resolution in the z direction. To circumvent this and maximize the benefit of reduced photon scattering at long wavelengths, we constructed a 1,320-nm light sheet to obtain the lowest degree of intensity decay and the least light-sheet thickness broadening relative to the 785-nm and 658-nm light sheets (Fig. 2a–d and Supplementary Figs. 8–10). In the glycerol-cleared brain tissue, 658-nm, 785-nm and 1,319-nm light sheets propagated over a distance of approximately 1.3 mm, 1.7 mm and 4.0 mm, respectively (Fig. 2a), within which imaging of cerebral vasculatures by detecting fluorescence at 1,500–1,700 nm from PbS/CdS CSQDs could still resolve small vessels (FWHM < 10 μm).

Excitingly, the 1,319-nm light sheet could propagate more than 6 mm to allow imaging of large blood vessels in glycerol-cleared mouse brain over large fields of view (Fig. 2a). Suppressed scattering of longer-wavelength light sheets was also confirmed from x-y, x-z or y-z cross-sectional images (Fig. 2e, Supplementary Fig. 11 and Supplementary Videos 2 and 3), with improved SBR (Fig. 2f) and reduced FWHM of feature sizes along the depth (z) direction, corresponding to higher vertical resolution and better sectioning capability along the z direction (Supplementary Fig. 11).

LSM with both excitation and emission in the NIR-II window at 1,000–1,700 nm minimized scattering and maximized the penetration depth and FOV. High-resolution 3D NIR-II LSM sectioning (Supplementary Fig. 12a and Supplementary Video 4; volume = 810 μm × 648 μm × 3,000 μm, z increment for depth of 3 μm, maximum z of approximately 2.5 mm) afforded volumetric resolution below 10 μm × 10 μm × 15 μm (FWHM) in glycerol-cleared brain tissue (Fig. 1f and Supplementary Fig. 11).

Under 785-nm light-sheet excitation, the maximum fluorescence signal at 1,500–1,700 nm detected in the cerebral vasculatures in the cortex layer of glycerol-cleared mouse brain as a function of depth z showed two attenuation regions (Supplementary Fig. 12c). There was an initial exponential attenuation due to a reduction in the ballistic and nearly ballistic photons (slightly deflected) emerging through the tissue following the Beer–Lambert law ln(Iz) = ln(I0) − μz, (where ln is the intensity at z, I is imaging depth, I0 is initial intensity at z = 0 mm and I is scattering MFP). This was followed by a slower-decay region at greater z from which multiply scattered photons diffusing through the brain tissue (diffusive region) were dominant. The MFP value (Supplementary Fig. 12e) extracted for glycerol-cleared brain tissue was approximately 305 μm, 440 μm and 639 μm in the windows at 850–1,000 nm, 1,100–1,200 nm and 1,500–1,700 nm, respectively, about twice the values in non-cleared brain tissue. The imaging penetration depth limits (see Supplementary Table 1 for detailed scattering coefficients and MFP comparison) were approximately 2.5I (emission at 850–1,000 nm), 2.6I (emission at 1,100–1,200 nm) and 3.3I (emission at 1,500–1,700 nm).

Non-invasive in vivo optical sectioning of live mice by NIR-II LSM. NIR-II LSM enabled non-invasive in vivo 3D imaging of disease models in live mice, facilitating cellular-resolution light-sheet sectioning through intact tissues for mammals (Fig. 3). To demonstrate non-invasive hemodynamic imaging in a tumor model, we imaged murine colorectal MC38 tumors (a tumor model used for immunotherapy) on the ear or flank of C57BL/6 mice with the NIR-II light sheet fixed at z = 2.5 mm, where the fluorescence signal was first detected (approximately 8 mm in diameter; Fig. 3a,b). Time-course imaging of the p-PE nanofluorophore immediately after intravenous injection into the mouse tail vein (785-nm excitation, 1,000 nm detection at exposure times of approximately 100–800 ms) revealed abnormal microcirculation in the tumor. Blood flows in tumor vasculatures were irregular and intermittent (Fig. 3a,c and Supplementary Video 5), with turning-on and shutting-off behavior, oscillatory/ fluctuating flowing patterns and even reversal of flow direction in the same vasculature (Fig. 3c, arrows, and Supplementary Video 5). These observations suggest unstable pressure within a tumor due to uncontrolled tumor angiogenesis. Further, analysis blood-supply time (BST; defined as the time at which the pixel intensity reached its maximum value relative to when the fluorescence flow was first detected) revealed that the p-PE nanofluorophore gradually leaked from some vasculatures (red region in Fig. 3d) but not from others (black region in Fig. 3d). Detailed experimental conditions are summarized in Supplementary Table 2.

Programmed-death ligand 1 (PD-L1) is an important immune checkpoint protein expressed by certain tumors to evade immune surveillance by binding of PD-L1 (on tumor cells) to programmed cell death protein 1 (PD-1) on T cells. PD-L1 blocking by antibody immunotherapy is a breakthrough approach to activate the immune system and treat cancer. In vivo PD-L1 imaging and mapping are important to fundamental understanding of cancer immunity and the mechanism of immunotherapy. To this end, we developed 45° oblique NIR-II LSM (Fig. 3e and Supplementary Fig. 1b) and performed non-invasive in vivo three-plex molecular imaging of a PD-L1-expressing CT26 tumor. Erbium-based rare-earth nanoparticles (ErNPs) (excitation, 975 nm; emission, 1,500–1,700 nm; Methods) conjugated to PD-L1 antibodies were injected intravenously and 24 h later, PEyGlyated PbS/CdS CSQDs (excitation, 1,319 nm; emission, 1,500–1,700 nm) conjugated to anti-PD-1 were injected. Two hours after injection, dynamic imaging into the tumor at a fixed illumination plane by oblique NIR-II LSM (Fig. 3e) detected a single PD-1+ T cell (labeled by PEyGlyated PbS/CdS CSQD) irregularly circulating in tumor vasculatures and following reversal of blood flow direction (Fig. 3f and Supplementary Video 6). In another 27 h, p-PE was injected intravenously for labeling of tumor vessels. Wide-field imaging was first used to image cells labeled for PD-L1 and PD-1 by anti-PD-L1 ErNPs and anti-PD-1 CSQD, respectively (Fig. 3g), and the fluorescence signals of ErNP and PbS/CdS CSQD (both in the range of 1,500–1,700 nm) were differentiated by resolving the vastly different lifetimes of the two probes (approximately 5 ms for ErNP and 20 μs for PbS; Methods). We then performed three-plex oblique NIR-II LSM to profile the 3D spatial distribution of PD-L1, PD-1 and vasculatures (labeled by circulating p-PE in the channel for 1,100–1,300 nm) in the tumor (Fig. 3h) through the intact skin on the tumor without installing an invasive window chamber (Fig. 3i). We observed PD-1-expressing T cells extravasated from vessels (Fig. 3h) and surrounding PD-L1-expressing cells in the tumor, representing an important step in the initiation of cancer-cell-killing immunotherapeutic effect (Fig. 3h, j and Supplementary Video 7). These results demonstrate multiplexed molecular imaging in 3D using oblique NIR-II LSM, which could be particularly useful for longitudinal imaging over long periods of time owing to its non-invasive nature. With this modality, sub-6-μm FWHM in the lateral x–y plane and sub-15-μm FWHM in the z direction using a 50× detection objective can be realized.
(Supplementary Figs. 13 and 14), which is useful for cellular-scale molecular imaging of multiple targets in vivo.

Strong scattering of light by the scalp and skull typically necessitates scalp removal \(^{3,4}\). To demonstrate non-invasive oblique NIR-II LSM in non-protruding tissues, we imaged intact mouse heads through the layers of scalp, skull, meninges and brain cortex, 2 h after injection of PEGylated PbS/CdS QSDQ probes (Fig. 4a, b). The 1,319-nm illumination and fluorescence detection at 1,500–1,700 nm afforded a penetration depth of approximately 774 µm along the tilted light-sheet direction into the head using a 10× imaging objective (Fig. 4c) with a FWHM of 7–15 µm in the xy plane and 7–17 µm FWHM along the z direction, gleaned by measuring the smallest vessels (Supplementary Fig. 15). In the meninges, interesting vascular-channel-like structures connecting the skull bone and brain cortex (marked by triangles in Fig. 4a, d) were observed. These channels could be important to the immune system of the brain that has been revealed recently \(^{42}\) and were resolved here by non-invasive LSM imaging in vivo.

Further, in a mouse model of TBI \(^{41}\) (Fig. 4c), meningeal macrophages and microglia were labeled by intravenously injected anti-CD11b PEGylated PbS/CdS CSQDs 2 h after injury. At 24 h after injection, non-invasive 3D time-course LSM monitored the recruitment of meningeal macrophages and microglia to the injured site as an inflammatory response \(^{41}\) (Fig. 4f and Supplementary Video 8).

Discussion

This work developed NIR-II LSM for in vivo and ex vivo deep-tissue volumetric imaging through highly scattering biological tissues. LSM with both excitation and emission in the NIR-II window avoided shadows or stripes along the illumination direction by suppressing tissue scattering and adsorption effects encountered by LSM in the visible region \(^{5}\). Non-invasive NIR-II LSM enabled in vivo observation in wide-field detection mode with suppressed background, facilitating dynamic process tracking and molecular imaging at cellular resolution over the millimeter scale. NIR-II LSM provides a complementary method to two-photon microscopy at a lower cost and under less invasive conditions (Supplementary Table 3). NIR-II LSM imaging can be further advanced by developing brighter fluorophores with longer excitation and emission wavelengths, and genetically encoded fluorophores with various capabilities and functionalities. It is also important to apply new configurations of LSM \(^{39}\) and use an InGaAs camera with lower readout noise and dark current \(^{46}\). Recent developments such as noncoherent structured illumination \(^{46}\) and optical-lattice illumination \(^{47}\) can be introduced to improve the resolution and contrast of NIR-II LSM. Real-time molecular imaging of multiple targets by rapid sectioning of 3D tissues of live mammals should become possible.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41592-019-0398-7.

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Author contributions

H. Dai and F.W. conceived and designed the experiments. H. Dai and F.W. designed the optical system. F.W. set up the optical system. F.W., H.W., Z.M. and Y.Z. performed the experiments. Q.S. did the Monte Carlo simulations. F.W., H.W., Z.M., Y.Z., Q.S., Y.T., L.Q., H. Du, H.M., Y.L., W.J.L., G.H., L. Liu and H. Dai analyzed the data. M.Z. developed the PbS/CdS CSQD dyes. F.W., H.W., Z.M. and H. Dai wrote the manuscript. L. Li and J.L. performed TBI. All authors contributed to the general discussion and revision of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

NIR-II fluorescent probes. This work used the organic nanophosphor p-FE dye, PE–Glyated PbS/CdS CSQDs (Supplementary Fig. 2), ERNPs, and the organic renaturatable ET dye, p-FE. PE–Glyated PbS/CdS CSQDs and 50 µl streptavidin (1 mg ml⁻¹ PBS, ProSpec) were added to 450 µl MES solution (10 mM, pH 6.5). EDC (60 µl, 65 mM in water) was freshly prepared and added dropwise to the solution. The solution was stirred at room temperature for 3 h. Unreacted streptavidin was removed by a centrifugal filter (cutoff = 100,000). The final product (referred to as PbS/Gd streptavidin) was suspended in 100 µl PBS. Anti-mouse PD-1 (1 lgg, BioXCell, catalog number BE0007, clone RMP1-14) was dissolved in 300 µl SEZ II, 100 µl Sulfo-NHS-LC-Biotin (6 µg, 1.7 mg ml⁻¹ in DMSO) was added to the solution, and the solution was stirred at room temperature for 1.5 h. Unreacted biotin was removed by a centrifugal filter (cutoff = 100,000). The final product (referred to as anti-mouse PD-1–biotin) was suspended in 100 µl PBS buffer. As prepared PbS/CdS streptavidin and anti-mouse PD-1–biotin was mixed, and stirred at room temperature for 2 h. Excess antibody was removed by a centrifugal filter (cutoff = 300,000). A similar method was used to conjugate anti-mouse/human–CD11b (BioXCell, catalog number BE0007, clone M1/70) to PbS/CdS CSQD. The TT dye exhibit similar spectroscopic properties as the p-FE dye with an excitation maximum of 785 nm and emission maximum of approximately 1,200 nm. The TT dye contains an azide group that can be used for conjugation to PD-1 antibodies by click chemistry for molecular imaging. Purified anti-mouse PD-1 antibody was purchased from Selleckchem (catalog number A2004, clone SP142). Conjugation was done with copper-free click chemistry using linker DBCO–PEG4–NHS purchased from Click Chemistry Tools. Detailed information on the antibodies is included in the Life Sciences Reporting Summary.

NIR-II light-sheet microscope setup. As shown in Supplementary Fig. 1a, lasers with wavelengths of 658 nm, 785 nm or 1,319 nm were directed into a spatial filter consisting of two achromatic lenses (L5 and L4) and a 50-µm pinhole, generating illumination with maximum excitation power of 1.7 mW, 11.9 mW or 8.2 mW on the front side of the objective, respectively. This spatial filter was introduced to improve the circularity and quality of the illumination beam and to generate a uniform light sheet across the FOV. The excitations could be selected by removable neutral-density filters. The measured light-sheet waist and Rayleigh length were consistent with those from theoretical estimations based on the effective NA values (see Supplementary Fig. 7).

Ex vivo NIR-II LSM of mouse brains. For ex vivo LSM of mouse brains in various NIR-I and NIR-II regions (data shown in Figs. 1c and 7c, and Supplementary Figs. 3–6 and 11), C57BL/6 mice were first injected with 200 µl p-FE dye with optical density (OD) = 4 at 808 nm, followed by injection with 200 µl PE–Glyated PbS/CdS CSQD (OD = 4 at 808 nm) 5 min after injection with p-FE. Mice were killed 30 min after injection under anesthesia, and brain tissues were taken out and fixed with 10X illumination objective and 50X detection objective were used, the effective NA of illumination was adjusted to be approximately 0.17. For the 5X illumination objective and the 40X or 10X detection objective, the effective NA of illumination was adjusted to be approximately 0.10 (ref. 25).

The effective NA was estimated using NA = n sin(θ = n sin(α/2πf)), where n is the refractive index, α is half of the aperture angle, D is the illumination width of the light sheet (adjusted by slit S1 for LSM imaging) as it is exiting the illumination objective, and f is the focal length of the illumination objective. At a given width of slit S1, D was measured experimentally by measuring the diameter of light close to the apertures of the illumination objective. D was adjusted by S2 when the cylindrical lens was rotated by 90° for imaging the side view of the light sheet (Supplementary Fig. 7). The measured light-sheet waist and Rayleigh length were consistent with those from theoretical estimations based on the effective NA values (see Supplementary Fig. 7).

The resolution of NIR-II LSM is limited by the diffraction limit and scattering that still exists in NIR-II (Fig. 1b). The diffraction-limited resolution along z for 10X, 0.25–NA detection objectives was 14.8 µm (850–1,000 nm), 18.4 µm (1,100–1,200 nm) and 25.6 µm (1,500–1,700 nm) estimated by nl/NA (refs. 2, 53). The value of 25.6 µm was larger than the measured light-sheet thickness (Supplementary Fig. 7). Therefore, resolution along the z direction was set by light-sheet thickness under this condition, which was approximately 10 µm (Supplementary Fig. 7).

The 0.5–NA, 50X detection objective, the diffraction-limited resolution along the z direction was 2.6 µm (850–1,000 nm), 3.2 µm (1,100–1,200 nm) and 4.4 µm (1,500–1,700 nm). These analyses suggest that the z resolution of our current NIR-II LSM, suitable for 10X–100X magnification, was approximately 10 µm, that is, z-NA = 0.25, suitable for single-cell detection along z. z-NA = 0.25–NA detection objective, and 0.9 µm (850–1,000 nm), 1.2 µm (1,100–1,200 nm) and 1.6 µm (1,500–1,700 nm) using the 50X, 0.6–NA detection objective (estimated by Rayleigh criteria, 0.61/NA). The light-sheet waist and Rayleigh length are contradicting factors and optimizing one means degrading performance in the other. The selection of the actual NA for each experiment was a tradeoff between these two parameters to obtain uniform light sheets that were as thin as possible across a large enough FOV. The actual NA and corresponding waist and confocal length for each dataset are summarized in Supplementary Table 2.

LSM 3D volumetric imaging and scanning. For 3D normal NIR-II LSM imaging (Supplementary Fig. 1a), as the imaging depth changed, an obvious misalignment of the light sheet and the working plane of the imaging objective appeared owing to the refractive index mismatch between air and tissue. This was compensated for by linear movement of the detection objective (Supplementary Figs. 4 and 5) concurrent with a change in the z position of the sample. Sample scanning in z and the z compensation through movement of the detection objective was performed using a 3D translation stage (KMT500, Thorlabs) and a single-axis translation stage (M5T50, Thorlabs, respectively (Supplementary Fig. 4). Different detection windows were selected by using corresponding long-pass and short-pass filters. Synchronous control of 3D translation-stage movement and image recording was realized using LabView software through a data acquisition card (NI USB-6088, National Instruments).

For oblique LSM (Supplementary Fig. 1b), lateral movement for 3D imaging was performed using a translation stage (MI-VP25XL, Newport) and another acquisition card (NI USB-6210) was used. The original images recorded by the camera in oblique LSM were 45° to the lateral direction (Fig. 3c). We transformed the original data to xy axes in ImageJ/Fiji by using the function affine transform (shear, scaling and rotation).

Maximum-intensity projection (Supplementary Fig. 12b,c) and 3D rendering was performed using ImageJ. Multicolor fluorescence images were also merged in ImageJ.

Mouse handling and tumor xenograft. Mouse handling was approved by Stanford University’s administrative panel on laboratory animal care. All experiments were performed in accordance to the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. C57BL/6 and BALB/c female mice were purchased from Charles River. Bedding, nesting material, food and water were provided. Four- to six-week-old C57BL/6 mice were anesthetized with isoflurane (1.5% for induction and 1% to maintain anesthesia) and then underwent intraperitoneal injection of 0.2 ml 25% melanoma cell suspension (approximately 2 × 10⁶ cells/ml) on the back flank of the mice in each group. The mice were housed in 2 mice per cage with a 12h:12h light–dark cycle. The mice were maintained in a pathogen-free environment and allowed free access to food and water. Mice were killed 30 min after injection under anesthesia, and brain tissues were taken out and fixed with 10% neutral phosphate buffered formalin. Based on the results, representative images were selected for presentation and analysis. All animals were maintained in accordance with the institutional guidelines for animal care and use (Dawes et al., 2017).
10% neutral-buffered formalin at room temperature. After washing in PBS twice, the fixed mouse brain was preserved in glycerol at 4°C for further imaging. We note that fixating and glycerol treatment induced a partial tissue-clearing effect with the imaging depth increased to about twice of that in a non-clearing tissue.

For NIR-II imaging of mouse brains (data shown in Fig. 2a and Supplementary Fig. 12a), C57BL/6 mice were injected with 200 µl PEGylated PbS/CdS QDQD (OD = 4 at 808 nm) and killed 30 min after injection. The brain tissues were taken out and fixed with 10% neutral-buffered formalin at room temperature. After being washed in PBS twice, the fixed mouse brain was preserved in glycerol at 4°C for ex vivo imaging. For the 5x illumination objective and the 4x (data shown in Fig. 2a) or 10x (data shown in Figs. 1c and 2e, and Supplementary Figs. 4, 5, and 11) detection objective, the actual NA of illumination was adjusted to be approximately 0.039 or 0.051. When the 10x illumination objective and the 50x detection objective were used, the actual NA of illumination was adjusted to be approximately 0.17 (Supplementary Fig. 6). The corresponding waits for different wavelengths are shown in Supplementary Fig. 7. Other detailed experimental conditions, such as z scanning increment, exposure time, and excitation and emission wavelengths, are summarized in Supplementary Table 2.

In vivo wide-field NIR-II fluorescence imaging. The NIR-II wide-field fluorescence images in Figs. 3g and 4e and Supplementary Fig. 13 were recorded using a two-dimensional liquid-nitrogen-cooled InGaAs camera (Princeton Instruments, 2D-OMA V) or Nikon 640 (Raptor Photonic). An 808-nm fiber-coupled laser (Koheras Lasers) was used as the excitation source. A set (850- and 1,000-nm short-pass filters) was applied to filter the excitation light. The actual excitation intensity after passing through filters was approximately 70 mW cm⁻². The fluorescence signal was collected by two achromatic lenses to the InGaAs camera with different magnifications after filtering by corresponding low-pass and long-pass filters. Two-channel fluorescence images were merged in InImage.

For three-color wide-field imaging of PD-L1- and PD-1-expressing cells and vessels in CT26 tumor-bearing BALB/c mice, 200 µl anti-PD-L1 ErNP (10 mg ml⁻¹) was injected intravenously. After 24 h, 200 µl anti-PD-1 PEGylated PbS/CdS QDQD (OD = 0.5 at 808 nm) was injected and, in another 29 h, 250 µl p-FE (OD = 5 at 808 nm) was injected into the tail vein. Wide-field imaging (Fig. 3g) was performed immediately after injection of p-FE. Fluorescence signals of ErNPs and PbS/CdS CSQD were recorded under continuous-wave 808-nm excitation with an exposure time of 20 ms. Finally the p-FE probes circulating in vessels were excited by an 808-nm laser with modifications. In brief, a bench stereotactic impactor was mounted on a stereotaxic frame at 45° (David Kopf Instruments). Anesthetized mice were placed on a customized foam mold in a prone position. To induce p-FE, the tip was driven towards the mouse head at a speed of 4.0–4.5 mm s⁻¹, by a maximal distance of 0.2 s by the electronic control box and an impact depth of 3 mm adjusted by the stereotaxic device. After recovery for 2 h, 4- to 6-week-old C57BL/6 mice were intravenously injected with 200 µl anti-CD11b PEGylated PbS/CdS QDQD (OD = 0.5 at 808 nm) and monitored by wide-field system and observation of NIR-II LSM with a 5x illumination objective and a 10x imaging objective 24 h after injection. The z scanning increment, exposure time and excitation and emission wavelengths are summarized in Supplementary Table 2.

Statistics and data analysis. Data analysis was performed in MATLAB 2017 or Origin 9.0. The s and mean values shown in Figs. 1 and 2, and Supplementary Figs. 4, 6 and 7 were calculated by Origin 9.0. The fitting lines in Supplementary Fig. 12e were derived by the weighted least-square method in Origin 9.0. In Fig. 1d,e, background was measured from a randomly selected area without vasculatures. SBR is the ratio of fluorescence signals in randomly selected vasculatures over the background. For each representative experimental result, the number of successful independent experiments performed on different mice is indicated in the corresponding figure legend.

Study of light-sheet propagation in multiple media. We experimentally compared light-sheet propagation in glycerol solutions using light sheets with different NAs and excitation wavelengths. These experiments were performed in glycerol containing uniformly dispersed PEGylated PbS/CdS QDQD. The emission was collected in a 1,500- to 1,700-nm window excited by 658-nm, 785-nm and 1,319-nm light-sheet illuminations. To directly observe light transmission in glycerol, we rotated the cylindrical lens by 90° and used mechanical slits to control the actual NA and the spanning range along the direction (Supplementary Fig. 1a). By so doing, the illumination plane was rotated by 90° and the light sheet shape could be imaged along the y direction for its z-plane for the side view (Supplementary Fig. 7a). In a transparent medium (Supplementary Fig. 7a), our experimentally measured waist and the double Rayleigh range of the light sheet were consistent with theoretical estimations (Supplementary Fig. 7b,c).

To study the propagation of the light sheet with wavelengths of 658 nm, 785 nm and 1,319 nm in a scattering medium, we performed experimental imaging of light-sheet propagation in intralipid solutions of different concentrations (Supplementary Fig. 8a). Light scattering was apparent as the intralipid concentration increased from 0% to 5.00% when 658-nm or 785-nm excitation was used. Impressively, 1.319-nm light-sheet excitation retained its shape over the longest distance. We further simulated light-sheet propagation in the intralipid phantom by a Monte Carlo method that is based on the method developed by Wang et al. Using the scattering coefficient µs and anisotropy g estimated by µs/µa, we simulated the light sheet propagation using a Monte Carlo method that is based on the method developed by Wang et al. Using the scattering coefficient µs and anisotropy g estimated by µs/µa
where \( \lambda \) is the wavelength, \( \mu_s^\prime = \mu_s (1-g) \) is the reduced scattering coefficient and concentration is the concentration of intralipid. Equations (1) and (3) were only used for the 10.00% intralipid solution. The available spectral range of equation (1) is between 750 nm and 830 nm (ref. 3). We first calculated the \( \mu_s \) of 10% intralipid solution at different wavelengths using equation (1). Then, we obtained \( \mu_s \) for 1.25%, 2.50% and 5.00% intralipid solutions on the basis of the linear relationship between \( \mu_s \) and concentration in equation (2) and \( \mu_s^\prime = \mu_s (1-g) \). These parameters are also summarized in Supplementary Table 1.

The illumination waist measured in water at NA = 0.039 was used as the initial FWHM of incident light in Monte Carlo simulations. The simulated results were consistent with the experimental observations (Supplementary Figs. 8 and 9).

Generally, the length over which the light sheet transmits by less than \( \sqrt{2} \) times the initial waist (\( w_0 \)) is regarded as the distance useful for imaging\(^5\). Under this definition, the critical length was larger than 1,000 \( \mu \)m for the 1,319-nm excitation in 1.25%, 2.50% and 5.00% intralipid solutions, much larger than that of 658-nm and 785-nm excitation. Because the scattering coefficient of intralipid solutions can be conveniently adjusted by controlling concentration, this is a widely used phantom to study photon–material interactions. To study the light-sheet propagation in a uniformly scattering medium with scattering characteristics similar to the mouse brain, we performed simulations using the scattering coefficient of 2.5% intralipid solution and the anisotropy factor of the brain as measured by Shi et al\(^6\). We compared the simulation results with our experimental observations in glycerol-cleared mouse brain in Supplementary Fig. 10. The simulated light propagation in the brain was consistent with experimental results for 658-nm, 785-nm and 1,319-nm excitation (Supplementary Fig. 10f–h). The critical distances for uniform illumination were approximately 210 \( \mu \)m, 320 \( \mu \)m and 1,000 \( \mu \)m for excitation using 658-nm, 785-nm and 1,319-nm light sheets in mouse brain, respectively (Supplementary Fig. 10f–h).

The light-sheet excitation intensity along the incident direction is another important parameter for imaging in scattering tissue, as it affects the transmission distance of excitation in the tissue and determines the illumination field. As the anisotropic concentration increased, the intensity along the propagation direction attenuated more quickly, but the 1,319-nm excitation decayed the slowest compared with the 658-nm and 785-nm excitations (Supplementary Fig. 9–l). Intensity attenuation was influenced by scattering, absorption and anisotropy of the tissue. As the brain had larger anisotropy than intralipid solution, the light sheet transmitted longer in the brain (Supplementary Fig. 10a,b,e). To study the illumination field of 658-nm, 785-nm and 1,319-nm light sheets in mouse brain, we performed LSM imaging of brain tissue at a depth \( z \approx 200 \mu \)m along the light-sheet incident \( x \) direction for up to 1 cm (data shown in Fig. 2a,b). Although photons in the light sheet could propagate as far as approximately 6,000 \( \mu \)m to excite fluorescence in large-diameter vasculatures, only in the initial limited distance could small blood vessels (FWHM < 10 \( \mu \)m) be observed (Fig. 2a). These limited distances were approximately 1,380 \( \mu \)m, 1,676 \( \mu \)m and 3,900 \( \mu \)m for 658-nm, 785-nm and 1,319-nm excitation, respectively as longer excitation attenuated more slowly in mouse brain.

For high-quality optical sectioning in LSM, both uniform light-sheet waist and available illumination field need to be ensured across the FOV.

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

**Data availability**

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

**References**

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Labview 8.0, Labview 9.0 were used to synchronously control 3D translation stage movement and image record.

Data analysis

Matlab 2018a was used to process the original data obtained by the liquid-nitrogen-cooled InGaAs camera (2D-OMA V, Princeton Instruments).
ImageJ 1.51j8 was used to perform maximum intensity projections, 3D rendering and affine transform (shear, scaling and rotation) of the data obtained by oblique NIR light sheet microscopy.
Origin 9.0 was used to draw the curves and analyze the standard deviation.

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Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
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The data that support the findings of this study are available from the corresponding author upon request.
### Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes were chosen according to prior experience with similar models and determined by the reproducibility of molecular imaging. The sample sizes were specified in corresponding figure legends. |
|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded from the analyses. |
| Replication | All attempts at replication were successful. |
| Randomization | No group was allocated, and any experiment involving comparison of imaging performance in different wavelength windows was done on the same animal or tissues. |
| Blinding | Not applicable, since no group was allocated. |

### Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

#### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| x   | Antibodies            |
|     | Eukaryotic cell lines |
| x   | Palaeontology         |
| x   | Animals and other organisms |
| x   | Human research participants |
|     | Clinical data         |

#### Methods

| n/a | Involved in the study |
|-----|-----------------------|
|     | ChIP-seq               |
|     | Flow cytometry         |
| x   | MRI-based neuroimaging |

#### Antibodies

**Antibodies used**

1. anti-mouse PD-1 (BioXCell; Cat. #: BE0146; Clone Name: RMP1-14; Lot #: 659318AZ; Dilution: 150 ug/ 200 uL);
2. anti-mouse/human-CD11b (BioXCell; Cat. #: BE0007; Clone Name: M1/70; Lot #: 655017M2; Dilution: 150 ug/ 200 uL); 
3. anti-mouse-PD-L1 (Selleckchem; Cat. #: A2004; Clone Name: SP142; Lot #: 02; Dilution: 150 ug/ 200 uL)

**Validation**

1. anti-mouse PD-1 (BioXCell, clone RMP1-14):
   - Website: https://bxcell.com/product/invivomab-anti-m-pd-1/
   - Relevant citations from website:
     - [1] Grasselly, C., et al. (2018). "The Antitumor Activity of Combinations of Cytotoxic Chemotherapy and Immune Checkpoint Inhibitors Is Model-Dependent." Front Immunol 9: 2100.
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2. anti-mouse/human-CD11b (BioXCell; Cat. #: BE0007; Clone Name: M1/70)
   - Website: https://bxcell.com/product/m-cd11b/
   - Relevant citations from website:
     - [1] Becker, A. M., et al. (2015). "ADAM17 limits the expression of CSF1R on murine hematopoietic progenitors." Exp Hematol 43(1): 44-52 e41-43.
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     - [3] Yokota, N., et al. (2014). "Contributions of thrombin targets to tissue factor-dependent metastasis in hyperthrombotic mice." J Thromb Haemost 12(1): 71-81.
3. anti-mouse-PD-L1 (Selleckchem; Cat. #: A2004; Clone Name: SP142):
Website: https://www.selleckchem.com/products/Atezolizumab.html?
gclid=EAIaIQobChMlgbvP9M6E4AI/AcpkCh2nwgkEAAYASAEgLSyvQ_BwE
Relevant citations from website:
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Animals and other organisms
Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | 4-6-week-old C57BL/6 and BALB/c female mice were purchased from Charles River |
| Wild animals       | This study did not involve wild animals. |
| Field-collected samples | This study did not involve samples collected from the field |
| Ethics oversight   | Mouse handling was approved by Stanford University’s administrative panel on Laboratory Animal Care. All experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.