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Traumatic Brain Injury Imaging in the Second Near-Infrared Window with a Molecular Fluorophore

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ToC figure

Traumatic brain injury (TBI) is a leading cause of death and disability worldwide, therefore important to understanding pathogenesis. Here, we designed a bright, renal-excreted and biocompatible near infrared II molecular fluorophore for *in vivo* imaging of TBI. We observed a transient hypoperfusion in the injured cerebral region, followed by fluorophore leakage. NIR-II fluorophores can provide a non-invasive assessment to TBI.

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Author contributions

H.D., J.L., Y.L., X.Z., and A.A. conceived and designed the experiments. X.Z., A.A., S.D., and J. L. performed the all experiments. L.J., L.L., and A.N., contributed to staining. Y.L., H.W., and R.W. contributed to the design and synthesis of molecules. H.D., J.L., Y.L., X.D., A.A., H.W., S.D., T.W.C, G.H., Z.M., and J. M. C. analyzed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Keywords

Traumatic Brain Injury; NIR-II Imaging; Molecular Fluorophore

Traumatic brain injury (TBI) is a major global health problem with millions of people suffering each year from traumatic events in accidents, sports and military conflicts around the world.^[1] The pathophysiology of TBI is complex and can be divided into primary and secondary damage. The primary damage is severity-dependent and results from mechanical forces applied to the skull and brain at the time of impact. Studies utilizing various experimental models of moderate to severe TBI indicate that the primary brain damage triggers a cascade of molecular and biochemical events, leading to long-lasting secondary neuronal and glial damage including neuro-inflammation, brain edema, and delayed neuronal death.^[2] Cerebral blood vessels are sensitive and vulnerable to the bio-mechanical damages caused by TBI, and subtle vascular changes could alter the hemodynamic response necessary for normal cellular function, leading to neural dysregulation.^[3] *In vivo* imaging of cerebral microvasculature has been a valuable approach to understand the cerebrovascular response following TBI and brain imaging data suggest that vascular pathology could serve as a TBI biomarker.^[2, 4] A variety of *in vivo* imaging techniques have been developed. Among these, two-photon microscopy provides structural imaging from fluorophores inside living tissue, with excellent spatial resolution.^[5] However, the imaging process requires invasive craniotomy that causes neuroinflammation and is typically limited to low temporal resolutions and a small field of view.^[6] Laser Doppler flowmetry has the ability to measure microcirculatory flow *in vivo*, and to visualize relative changes in blood perfusion in the cortex with a large field of view,^[7] but is limited in spatial resolution. Magnetic resonance imaging (MRI) is powerful for noninvasive, longitudinal assessment of hemodynamics in individual animals,^[8] but faces technical challenges concerning scanning protocols (e.g., limited spatial and temporal resolution), sensitivity and analysis techniques.^[9]

As a recent development, fluorescence imaging in the second near infrared window (NIR-II) detects light emission from fluorophores in the 1,000–1,700 nm range, useful for non-invasive *in vivo* imaging at sub-centimeter tissue depth with sub-10 micron spatial resolution.^[10] Deeper imaging is achieved compared to conventional NIR imaging in the

NIR-I (750–900 nm) range due to reduced photon scattering by biological tissues at longer wavelengths.^[10a–e, 11] We recently reported that epifluorescence imaging in the NIR-IIa window (1,300–1,400 nm) and NIR-IIb window (1,500–1,700 nm) afforded an imaging depth of 2–3 mm (compared to ~0.2 mm imaging depth in NIR-I) in the brain of mice capable of sub-10 microns and sub-5 microns cerebrovascular imaging resolutions, respectively.^[12] This was further aided with the capability of video rate dynamic imaging to follow vascular hemodynamics.^[12] Thus far, carbon nanotubes and inorganic nanoparticles are the most commonly used NIR-II fluorescent agents for *in vivo* imaging with the caveat of slow excretion of nanomaterials from the body and side effects concerns.^[10c–g] To improve safety in preclinical models and potential clinical use, NIR-II fluorescent agents based on organic conjugated polymers or dyes wrapped in aqueous stable micelles were developed to impart biocompatibility, yet they still demonstrated suboptimal excretion kinetics.^[13] Recently, we developed a NIR-II emitting molecular fluorophore with high aqueous solubility, useful for deep-tissue imaging of mouse tumor models but with a drawback of low fluorescence quantum yield.^[14]

Here, we designed a novel NIR-II molecular fluorophore with excellent aqueous and photostability. Importantly, the new fluorophore exhibited 2-fold higher quantum yield than carbon nanotubes and previous NIR-II molecular dye CH1055 (~0.3 %) and PEG coated IR1061 nanoparticles (0.5 %), high biocompatibility and fast renal excretion of ~83% within 24 hours post injection. Through tail vein intravenous injection of the NIR-II dye into mice, we performed noninvasive NIR-II imaging of the mouse brain to investigate cerebrovascular injury in a mouse model of TBI.^[15] The *in vivo* NIR-II imaging enabled us to directly visualize cerebral hypoperfusion, fluorophore leakage and trapping in the injured brain tissue, with high-spatial and temporal resolution to characterize the dynamics of neurovascular changes following TBI. NIR-II imaging with advanced fluorophores presents a unique tool for investigating vascular pathology for TBI models.

We employed molecular engineering approaches to design fluorophores with bright emission in the NIR-II window in aqueous solution (See detailed synthesis, modeling and characterization in Supplementary Information, and Supplementary Figure S1 and S2). The molecular fluorophore (named **IR-E1**) contained a donor-acceptor-donor structure, with benzo[1,2-c:4,5-c']bis([1,2,5]thiadiazole) (BBTD) as the acceptor with thiophene-based units as the donor to afford a fluorophore with a low band gap (Figure 1a). A novel design was the use of bulky 3,4-ethoxylene dioxothiophene (EDOT) as a bridging group to protect the conjugated backbone from intermolecular and intramolecular interactions (see supplementary scheme 1 for low quantum yield without the EDOT bridge).^[16] Polyethylene glycol (PEG) chains were attached to the molecule to impart aqueous solubility (Figure 1a). The UV-Vis-NIR absorption spectrum of **IR-E1** in water exhibited an absorption peak at 830 nm, while the fluorescence emission spectrum showed a main emission peak at 1071 nm under 808 nm excitation (Figure 1b). The quantum yield (QY) of **IR-E1** was ~0.7 % under 808 nm excitation (measured against an IR-26 reference with a nominal quantum yield of 0.5%,^[17] see Figure S3), which was ~2X of carbon nanotubes (Figure 1c). The weight averaged molecular weight (MW) of IR-E1 was ~4.5 kDa (Figure S2c) with a hydrodynamic size of ~3.6 nm in water (Figure 1d). The molecular weight and

hydrodynamic size were small, allowing for renal excretion with a size cutoff of ~ 5.5 nm.^[18] The fluorescence intensity of IR-E1 in water, phosphate buffer saline (PBS) and fetal bovine serum (FBS) showed no decay over time, indicating high aqueous stability (Figure 1e). The dye also showed high photostability in PBS, water, and FBS with negligible fluorescence decay under continuous 808 nm laser at a power density of 0.33 W/cm² for 2 hours (Figure 1f).

A traumatic brain injury was induced in the right side of the head of wild type C57BL/6J mice by a controlled cortical impactor mounted on a stereotaxic frame with a ~ 3 mm probe tip^[15] (Figure 2a and see Methods). Two hours after TBI, IR-E1 fluorophores were intravenously injected for dynamic *in vivo* fluorescence imaging (> 1300 nm emission were collected under an 808 nm excitation; imaging rate = 3 frames per second) of the brain without craniotomy (Figure 2b–d). NIR-II fluorescence signals in the inferior cerebral vein, transverse sinus and middle cerebral vessels in the contralateral hemisphere showed up immediately within 1 s post injection (p.i.), rapidly increased and plateaued at ~ 10 s p.i. In contrast, little signal was seen in the right (ipsilateral) hemisphere (Figure 2b, Supplementary Movie 1) at 10 s p.i. (Figure 2c) and was only partially visible (signal in the injured region of interest ROI/signal in the corresponding left ROI ~ 41%) even at 1 min p.i., well beyond the signal plateauing time point of the left side of the brain (Figure 2d). Analysis of the dynamic fluorescence signal change found that the cerebral blood perfusion rate^[12b] in the injured brain region was about 1/6 of that in the corresponding left side of ROI (Figure 2e), confirming a much reduced blood flow in the injured region of the brain (Figure 2e). With a total of 4 mice, we observed similar hypoperfusion in the injured brain region, with perfusion rates in the range of 1/6 and 1/4 of the contralateral side of the brain.

In healthy control mice with a sham injury, the NIR-II fluorescence rapidly increased and plateaued in both cerebral hemispheres (Figure 2g) with identical perfusion rates (Figure 2h).^[12b] Principal component analysis (PCA) of 100 frames of the dynamic images differentiated the arterial (red) and venous vessels (blue) in the mouse brain (Figure 2i). For the TBI mouse, PCA showed hypoperfusion in both arterial and venous vessels in the injured brain region (Figure 2f).

We compared NIR-II imaging of our TBI mouse model with conventional fluorescence imaging using the FDA-approved ICG dye emitting in the NIR-I window (850–900 nm). Blurred images of the mouse brain were observed due to stronger photon scattering in the NIR-I (Figure S4). Also, ICG exhibited a short circulation time, with little signal observed in the cerebral circulation at 5 min p.i. (Figure S4d, e) due to its rapid hepatic clearance. These two factors limit the use of ICG for TBI imaging.

In sham control mouse brain, NIR-II fluorescence signal continuously decayed after ~ 10 s p.i., and nearly disappeared in ~ 2 h p.i. (Figure 3 a,e) as the fluorophores were cleared from blood circulation without any leakage through the intact blood-brain barrier. In strong contrast, following the initial hypoperfusion phase (Figure 2) in the injured region of the right brain, NIR-II fluorescence signal was much higher than the left brain at 2 h p.i. (Figure 3b), continuously increased at 4 h p.i. and became more spread out up to ~ 8 h p.i. (Figure 3c), suggesting leakage through damaged BBB and accumulation and trapping of

fluorophores within brain parenchyma. Imaging at 24 h p.i. demonstrated that the NIR-II fluorescence signal in the injured region decreased with a reduction in the area of NIR-II signal spread (Figure 3d). The trapped signal greatly decreased and nearly vanished in 1–2 weeks post injection (Figure S5a–c), suggesting clearance of IR-E1 from the injured brain. The accumulated NIR-II signal in the injury region 2–4 h p.i. was the highest in a region of ~ 3–4 mm based on the trapped NIR-II signal width (Figure 3f), spreading over to as large an area as 5 mm at 8 h p.i. (Figure 3c), suggesting a core region of primary injury with BBB damage and blood leakage in the vicinity.

Ex vivo imaging of whole mouse brain removed from sacrificed TBI mice observed fluorophores retained in the injured brain region (Figure S5d). Imaging of brain tissue slices from the injured region confirmed neurovascular damage, as evidenced by aquaporin-4 (expressed in astrocytic end-feet to form a continuous perivascular sheath)^[19] immunostaining (Figure 3g–i). We observed blood vessel rupture in the lesion epicenter and increased aquaporin-4 immunofluorescence intensity in the penumbra (Figure S6), and overlay images demonstrated the presence of IR-E1 outside of the blood vessels (Figure 3g). Also, extensive leakage of plasma IgG into brain tissue, a standard marker for BBB impairment,^[9] was observed in and around the site of injury in the brains of TBI animals collected at 24 h post TBI (Figure 3h,i), but not in the sham control animals.

BBB damage and recovery were further investigated by *in vivo* NIR-II imaging and *ex vivo* staining. We injected IR-E1 at different time points up to 7 days post TBI and imaged the dynamic changes of NIR-II fluorescence signal in the mouse brain (Figure S7). When IR-E1 was injected at day 1 and 3 post TBI, we still observed accumulation of NIR-II fluorescence signal in the injured right side of the cerebral hemisphere, suggesting persistent BBB leakage (Figure S7d–f). No significant IR-E1 accumulation was found when IR-E1 was injected at day 7 post TBI, indicating BBB recovery to a large extent. These results were consistent with *ex vivo* vessel staining and IgG extravasation/trapping data (Figure S7g–i).

Higher magnification NIR-II dynamic imaging was done to glean the details of blood hypoperfusion and fluorophore leakage in injured brain regions of mice (Supplementary Movie 2). We zoomed into a region near the impact site of the brain and tracked NIR-II fluorescence corresponding to the slowly moving blood perfusion front (Figure 4). We observed that in the injured region, the blood perfusion front moved in an ultra-slow manner at a speed of ~ 0.002 mm s⁻¹ (Figure 4). We were able to observe blood perfusion into single vessels (Figure 4f–i). Perfusion into a vessel at ~ 25–30 min post injection of the NIR-II dye was observed (Figure 4f–h and red arrows), which was a remarkably long time for blood circulation to reach the vessel. The measured blood flow speed in the single vessel was measured to be as low as ~ 0.02 mm s⁻¹.

We resolved a slowly appearing ‘spotty’ pattern (Figure 5a–e, Supplementary Movie 3) of the initial fluorophore leakage under the skull of TBI mice (*n* = 3) after ~ 30 min p.i., and the leaked signal increased and expanded slowly over a period of 40–60 min (Figure 5f–h), suggesting slow venous subdural hematoma (see also Supplementary Movie 3).^[20] The spotty leaking sites were attributed to individual damaged venous vessels between the skull and brain tissue caused by shear forces during the probe impact in our TBI model.^[21]

We performed principal component analysis (PCA) of the video-rate recorded dynamic NIR-II image frames of TBI. PCA was able to group regions in images recorded in a dynamic sequence into spatially defined components according to the time variance of the signal in these regions, allowing differentiation of arterial and venous vessels (Figure 2f,2i).^[10a, 12b] In the TBI cases, PCA analysis of 100 frames of vascular leakage images recorded 5–60 min p.i. revealed components corresponding to arterial and venous vessels that were more rapidly perfused with NIR-II signals (red and blue in Figure 5i, respectively), and a component with the slowest variance in time (green in Figure 5i) corresponding to venous leakage of NIR-II fluorophores slowly into the subdural space.

To investigate the pharmacokinetics, excretion and toxicity of IR-E1 *in vivo*, we intravenously injected IR-E1 into healthy C57BL/6 mice ($n = 6$) at a dose of 10 mg/kg (same dose to TBI), and performed whole-body imaging to follow the fluorophores *in vivo*. We observed that IR-E1 accumulated in the bladder within hours p.i. (bright spot in Figure S8a). We collected urine samples at different time points and detected strong NIR-II fluorescence signals in all urine samples with the NIR-II signal peaking at 3 h p.i. (Figure S9a,b). The total urine excretion was estimated to be about 83 % at 15 hour p.i. (Figure S8c). Analysis of blood concentration of the fluorophore showed a blood circulation half-time of IR-E1 of ~ 1.3 h (Figure S8b), consistent with the ~ 1.2 h half-time of fluorescence decay observed in NIR-II imaging of cerebrovasculature in healthy mice (Figure S10).

At day 1, 7 and 30 post intravenous injection of IR-E1 into healthy mice at 10 mg/kg dose, we examined overall toxicity parameters and found that the mice injected with IR-E1 showed no body weight loss (Figure S11a) compared with control mice without dye injection. Hematology indicators, such as white blood cells (WBC), red blood cells (RBC), did not show any significant differences from the control group, suggesting no obvious infection or inflammation (Figure S11b). In addition, clinical biochemical indicators, such as creatinine (CREA), total protein (TP), and other indicators were unchanged, suggesting no significant adverse effects of IR-E1 to basic liver and kidney function (Figure S11c). Postmortem pathological analysis showed no histological damage in kidney, heart, liver, spleen and lung tissues at up to 30 day p.i. (Figure S12).

In this study, we synthesized a novel molecular NIR-II fluorophore IR-E1 with a higher QY than carbon nanotubes^[10c] (the most commonly used NIR-II contrast agent thus far) and previously reported organic fluorophores emitting above 1000 nm in aqueous solutions.^[13a, 14] In vivo imaging of IR-E1 fluorescence in the long wavelength NIR-II window afforded significantly reduced tissue scattering over traditional fluorescence imaging, allowing for the optical imaging with sub-centimeter imaging depth,^[12b] high spatial (sub-10 microns) and temporal resolution over the entire TBI mouse brain without craniotomy.^[12b] Using IR-E1, we performed spatially-resolved, longitudinal *in vivo* imaging to investigate TBI-induced hemodynamic abnormalities and cerebrovascular damages in the injured mouse hemi-brain. The mechanisms underlying hypoperfusion are largely unclear and may be related to physical damage to the blood vessels or local increases in intracranial pressure.^[22] The enhanced spatial and temporal resolution achieved by our method has allowed us to describe cerebral vascular responses to TBI on an unprecedented scale, including hypoperfusion and subdural hematoma at the single vessel level, with the

capability of monitoring ultra-slow cerebral hemodynamics. For example, we observed an immediate hypoperfusion (Figure 2, 4) in the ipsilateral cortex of TBI-injured mice, followed by fluorophore leakage and accumulation (Figure 3). The dynamics of fluorophore leakage and accumulation provided a direct assessment of BBB damage and leakage confirmed by *ex vivo* imaging of extravasated fluorophores and IgG. The acute hypoperfusion (Figure 2) we observed by NIR-II imaging is consistent with other studies of rodent TBI models using autoradiography, radiolabeled microspheres, laser Doppler flowmetry or magnetic resonance imaging.^[7–8, 23] In humans, single-photon emission computed tomography (SPECT) using radioactive gamma rays also revealed regions of hypoperfusion in frontal, pre-frontal and temporal cortices, and sub-cortical structures in TBI patients.^[24] Whole brain imaging revealed that the perfusion rate of the injured hemisphere after 2 h decreased to 1/6–1/4 of that seen in sham mice (Figure 2e), and imaging of injured brain regions revealed ultra-slow perfusion with single-vessel resolution (Figure 4). Low flow velocity in single vessels and slow blood perfusion kinetics in our model—in some cases as slow as 25 min p.i.—make these dynamics difficult to assess using laser Doppler or more conventional techniques. Over a longer time course, we observed fluorescent signal accumulation in the damaged hemisphere (Figure 3e), suggesting leakage into the brain parenchyma indicative of BBB disruption, which we confirmed *ex vivo* (Figure 3g–i). The accumulated NIR-II signal in the TBI-injured region diffused and expanded in size (Figure 3b,c) up to ~ 8 h p.i. before withdrawing towards recovery in the subsequent days and weeks. We also observed a slowly appearing ‘spotty’ fluorophore leakage out of single vessels (Figure 5b–e, Supplementary Movie 3) under the skull (Figure 5f–h), suggesting slow venous subdural hematoma^[25] (see also Supplementary Movie 3) caused by shear force damage of the veins^[25] between the skull and brain tissue in our TBI impact model. This finding represents the first dynamic imaging of subdural hematoma in TBI with single-vessel resolution. One advance that enabled us to achieve such resolution was the differentiation of fluorescence signals by principal component analysis of the signal time-variant in an image sequence, resolving vessels rapidly perfused with NIR-II signals (Figure 5i) and slowly varying NIR-fluorescence corresponding to venous leakage through impaired BBB (green in Figure 5i).

Animal studies have demonstrated that BBB disruption is one of the major factors that contributes to delayed neurological dysfunction and degeneration associated with TBI.^[4, 26] Many of the various techniques developed for imaging BBB leakage in animal injury and disease models^[9] are non-invasive and are suitable for longitudinal studies, yet have other limitations. For example, contrast-enhanced MRI with gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA)^[27] and single photon emission computed tomography (SPECT)^[28] provide quantitative assessment of BBB impairment for both research and in clinical settings. These techniques are limited by low contrast, poor spatial and temporal resolution^[9] and the inconvenient practical use of radiotracers. Fluorescence imaging holds the potential to measure BBB permeability non-invasively with high sensitivity. So far only a few studies have demonstrated feasibility in animal models, using traditional NIR dyes with emission peaks below 850 nm.^[29] Imaging is limited to small penetration depth of ~ 0.2 mm and requires invasive and potentially confounding craniotomy. Two-photon imaging has also been applied to TBI investigations,^[5, 30] achieving excellent spatial resolution but with

limited penetration depth, small field-of-view and relying on craniotomy. NIR-II fluorescence imaging is a simple epi-fluorescence technique with deeper imaging depth (sub-centimeter) than traditional NIR imaging at ~ 800 nm that has broad potential preclinical applications. It allows for a flexible field-of-view in the range of microns to whole mouse-body,^[10] affording down to sub-10 micron spatial resolution and high temporal resolution at up to ~ 20 frames per second.^[13b] The key to NIR-II imaging is the development of bright, biocompatible fluorophores emitting in the 1000–1700 nm spectral range. To attain fluorescence emission in the NIR-II range, molecular fluorophores are required to have conjugated backbones exhibiting small bandgaps (<1.2 eV). However, extended conjugation generally leads to higher susceptibility to intermolecular interactions and thus nonradiative decay processes^[31] responsible for low fluorescence quantum yield. This problem is made worse in aqueous solution as water is a poor solvent to shield the conjugated backbones. Here we employed a strategy of enveloping groups and EDOT functionalization for the donor-acceptor-donor core to reduce intermolecular and intramolecular interactions. The enveloping group is composed of 1,2,6-tri-substituted benzene with extended side chains in the 2,6 positions, which can sheath the conjugated backbone. A bulkier EDOT bridging unit between thiophene and the BBTD acceptor (Figure 1a) was critical to enhance fluorescence QY, as EDOT introduced a conformational distortion of the conjugated backbone^[32] (i.e., a non-planar EDOT-BBTD-EDOT configuration, see Figure S2d) that reduced both intermolecular and intramolecular interactions. Experimentally, removing EDOT to form thiophene-BBTD-thiophene structure led to a planar structure and reduced quantum yield (**IR-T1**, see Figure S1, QY = 0.06% in water) due to higher intramolecular or intermolecular interactions.^[33] Also, the polar 3,4-ethylenedioxy group on EDOT favored the dispersity of the fluorophore in water,^[34] desired for biocompatible fluorophores. PEG chains were introduced on the enveloping groups of **IR-E1**, wrapping the conjugated backbone for high aqueous solubility. All of these contributed to the enhanced emission quantum yield and biocompatibility of **IR-E1**, affording the brightest molecular fluorophore in NIR-II window thus far.

In summary, we developed a novel NIR-II molecular fluorophore that allowed us to characterize dynamic vascular changes in a mouse TBI model, including initial transient hypoperfusion that resolved as fluorophore leakage and accumulation caused by damage to the cerebrovasculature. Brain imaging studies suggest that such pathology could be used as a biomarker in drug trials or the clinic and as a target for therapeutic invention.^[26] Since BBB disruption plays a key role in the pathogenesis of TBI,^[26] our study opens up new opportunities to probe TBI pathogenesis at the morphological and cellular level to better devise therapeutic approaches to TBI.

Experimental Section

A detailed description of experimental procedures can be found in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. A novel NIR-II fluorophore IR-E1 with bright fluorescence above 1000 nm

(a) Molecular structure of IR-E1. (b) UV-vis absorption and fluorescence emission spectra of an aqueous solution of IR-E1 under an excitation of 808 nm. (c) Fluorescence imaging of a typical Hipco carbon nanotube suspension and an IR-E1 solution at the same optical absorbance under an 808 nm laser excitation demonstrates that fluorescence intensity of IR-E1 is ~ double that of the nanotube. (d) Hydrodynamic size of IR-E1 measured by dynamic light scattering is ~ 3.6 nm, which is below the renal cut-off of 5.5 nm. (e) Fluorescence intensity of IR-E1 in water, PBS, and FBS measured over 1 week, showing excellent aqueous stability. Error bars represent standard deviation of fluorescence intensities of the pixels over the entire sample. (f) Photostability test curves of IR-E1 in water, PBS, and FBS under continuous 808-nm radiation up to 2 hours. IR-E1 in all media exhibited negligible photo-bleaching.

Figure 2. Dynamic *in vivo* cerebral NIR-II imaging of hypoperfusion in a TBI mouse model

(a) Photo of a mouse with scalp shaved following TBI given by controlled cortical impact injury at the site indicated in the right side of the head. Inset: schematic of angles employed by metal tip impact to mouse cranium at a speed of 5.3 mm/s. (b–d) Dynamic NIR-II image frames (300 ms, 1,300 LP) recorded at indicated time points after IR-E1 post injection (10 mg/kg) showing reduced blood flow in the injured right hemisphere. Imaging was performed through the skull and scalp of mouse without craniotomy. The red box shows region of interest (ROI) for perfusion analysis in “e”. Imaging of IR-E1 NIR-II fluorescence in mouse brain was performed 2 hours post-TBI. (e) NIR-II signal in the left (contralateral) and right (ipsilateral) cerebral hemispheres (normalized by signal in the left side at 10 s p.i. when the signal began to plateau) of the TBI mouse versus time, showing significantly reduced blood perfusion in the injured hemisphere. (f) Principal component analysis of 100 frames from dynamic imaging of the TBI mouse, resolving arterial (red) and venous cerebral vessels (blue). (g) NIR-II image (300 ms, 1,300 LP) of a sham mouse injected with the IR-E1 fluorophore (10 mg/kg) at 10 s p.i. (h) Normalized NIR-II signals using signal at 10 s p.i. in the left and right cerebral hemispheres of a sham mouse versus time. (i) Principal component analysis of 100 frames from dynamic imaging of the TBI mouse, resolving arterial (red) and venous cerebral vessels (blue).

Figure 3. NIR-II fluorophore leakage and accumulation in TBI mouse brain

(a) NIR-II-imaged brain of a sham mouse at 2 h IR-E1 (10 mg/kg) p.i. in NIR-II window (1,300 LP) with 300 ms exposure time, showing no significant fluorescence retained in either brain hemisphere. (b–d) NIR-II-imaged brain of mouse given TBI (same as in Fig. 2) at **between** 2 and 24 h p.i. of IR-E1 (10 mg/kg) in NIR-II window (1,300 LP, 300 ms), showing (b) accumulation, (c) spreading and (d) the commencement of IR-E1 clearance in the injured brain hemisphere. Red box in “d” shows the ROI for the time course signal analysis in “e”. (e) Time course of NIR-II signal in sham and TBI mouse in the healthy and injured ROI. Bars represent mean $\pm$ standard deviation ($n = 3$ mice/group). (f) The cross-sectional intensity profile along the blue dashed line in “b” at different time points post-injection. (g) Ex vivo imaging of a brain slice from the TBI mouse (24 h after TBI) showing IR-E1 fluorophores (red) outside of blood vessels immunolabeled with Alexa Fluor 647-labeled aquaporin 4 (Aq4, green). (h, i) Brain slices 24 h post-TBI were stained with antibodies against aquaporin 4 (green) and mouse IgG (red). 3-dimensional reconstruction of the fluorescent images obtained in serial optical sections showing IgG extravasation in the mice with TBI in “h” but not in the sham control in “i”.

Figure 4. NIR-II dynamic imaging of hypoperfusion in a TBI mouse at single-vessel resolution (a–f) Video rate dynamic image frames recorded in the injured region of a TBI mouse (2 h after TBI) in the hypoperfusion phase with 300 ms exposure time in NIR-II window (>1300 nm) after intravenous injection (10 mg/kg), showing ultra-slow blood perfusion from the transverse sinus region (bright lower-left region) into the injured region. Blue arrow points to the perfusion front over which the signal traveled. (f–h) A single vessel was observed to be perfused over ~ 2.9 minutes. Red arrows point to the blood flow in front of the vessel.

Figure 5. High resolution dynamic imaging of NIR-II fluorophore leakage into injured mouse brain

(a–h) Dynamic images (500 ms, 1300 LP) of fluorophore leakage and accumulation in the injured region of a mouse 2 h following TBI at time points 10 min IR-E1 p.i. (10 mg/kg).

(i) Principal component analysis of image frames recorded between 10 and 60 min p.i. showing arterial (red) and venous cerebral vessels (blue) and fluorophore leakage (green).