Multifunctional in vivo vascular imaging using near-infrared II fluorescence

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In vivo real-time epifluorescence imaging of mouse hind limb vasculatures in the second near-infrared region (NIR-II) is performed using single-walled carbon nanotubes as fluorophores. Both high spatial (~30 μm) and temporal (<200 ms per frame) resolution for small-vessel imaging are achieved at 1–3 mm deep in the hind limb owing to the beneficial NIR-II optical window that affords deep anatomical penetration and low scattering. This spatial resolution is unattainable by traditional NIR imaging (NIR-I) or microscopic computed tomography, and the temporal resolution far exceeds scanning microscopic imaging techniques. Arterial and venous vessels are unambiguously differentiated using a dynamic contrast-enhanced NIR-II imaging technique on the basis of their distinct hemodynamics. Further, the deep tissue penetration and high spatial and temporal resolution of NIR-II imaging allow for precise quantifications of blood velocity in both normal and ischemic femoral arteries, which are beyond the capabilities of ultrasonography at lower blood velocities.

Development of new therapies for peripheral arterial diseases (PADs) may be facilitated by imaging that provides anatomic and hemodynamic information with high spatial and temporal resolution. However, current methods for assessing vasculature and hemodynamics in small vessels in vivo are suboptimal.¹ For imaging vascular structures, microscopic computed tomography (micro-CT) and magnetic resonance imaging (MRI) can resolve features down to ~100 μm with deep penetration but are limited by long scanning and post-processing times, and difficulties in assessing vascular hemodynamics.²⁻³. Vascular hemodynamics are usually obtained by Doppler measurements of micro-ultrasonography with high temporal resolution of up to 1,000 Hz, but spatial resolution attenuates with increased depth of penetration.⁴

In vivo fluorescence-based optical imaging has inherent advantages over tomographic imaging owing to high temporal and spatial resolution.⁵⁻⁶. Single-walled carbon nanotubes (SWNTs), nanoscale cylinders of rolled-up graphite sheets composed of carbon, are an emerging nanomaterial with unique optical properties for in vivo anatomical imaging,⁷⁻⁸, tumor detection,⁹,¹⁰ and photothermal treatment.¹⁰⁻¹². One unique feature of SWNTs is their intrinsic fluorescence in the second near-infrared window (NIR-II, 1.1–1.4 μm) upon excitation in the traditional near-infrared region (NIR-I, 0.75–0.9 μm), with large Stokes shift up to ~400 nm. Compared to the NIR-I window, which has been extensively explored for in vitro and in vivo imaging,¹³⁻¹⁸, the longer wavelength emission in NIR-II makes SWNTs advantageous for imaging owing to reduced photon absorption,¹⁹,²⁰ reduced scattering by tissues,²¹, negligible tissue autofluorescence and, thus, deeper tissue penetration.⁷,⁸,²². This allows for unprecedented fluorescence-based imaging resolution of anatomical features deep to the skin.

Here we report the use of biocompatible, brightly fluorescent SWNTs as NIR-II imaging agents for imaging vascular structures down to ~30 μm in mouse hind limb using an epifluorescence imaging method with an indium-gallium-arsenide (InGaAs) imaging system. Compared to micro-CT, NIR-II fluorescence imaging attains an ~3-fold improvement in spatial resolution. Further, NIR-II imaging allows for differentiation of arteries from veins through principal component analysis (PCA),²³ to obtain dynamic contrast-enhanced imaging. We can also quantify femoral artery blood flows in both normal and ischemic hind limbs and reveal the degree of occlusion due to ischemia. Thus, a single NIR-II imaging modality enables multifunctional imaging capable of accomplishing what is typically done by several traditional techniques, including micro-CT, ultrasonography and MRI, and incorporates many desirable features such as high spatial resolution (~30 μm), fast acquisition (<200 ms), good tissue penetration (1–3 mm), vessel specification and blood flow quantification for hind limb vessel imaging.

RESULTS

NIR-I and NIR-II fluorescence imaging of vasculatures

First, to glean the differences between in vivo NIR-I and NIR-II fluorescence imaging, we made biocompatible SWNT-IRDye-800 conjugates as dual-color imaging agents, where IRDye-800 is a commercial NIR-I fluorophore. High-pressure carbon monoxide conversion SWNTs were stably suspended by biocompatible surfactants of 75% DSPE-PEG (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)], 5 kDa) and 25% DSPE-PEG-NH₂ (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)],

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5 kDa) with amine groups covalently functionalized with IRDye-800 (Fig. 1a and Supplementary Fig. 1a–c). Both the SWNT and IRDye-800 label could be excited by a 785 nm laser, but they had different emissions. The IRDye-800 dye emitted at ~800 nm in the NIR-I, whereas the SWNT emitted in the 1.1–1.4 µm NIR-II region (Fig. 1b). The dual-color emission of SWNT–IRDye-800 conjugate ensured co-localization of SWNTs and IRDye-800 dyes, and enabled us to image the same tissue in two distinct spectral windows so as to evaluate photons in different wavelengths for live animal imaging.

For parenteral administration and live animal imaging, we prepared and intravenously injected a solution (200 µl) of 0.10 mg ml⁻¹ (1.0 mg per kg body weight) SWNT-IRDye-800 conjugates (Fig. 1c) into a nude mouse. We estimated the maximum SWNT concentration in the blood would be 17 times lower than the half-maximal inhibitory concentration (IC₅₀) of vascular endothelial cells (Supplementary Fig. 1d). The circulation half-time of DSPE-mPEG–functionalized SWNTs was ~5 h (ref. 8), and our previous studies had shown the lack of acute or long-term toxicity of such PEGylated SWNTs in vivo. For parenteral administration and live animal imaging, we prepared a solution (200 µl) of 0.10 mg ml⁻¹ of SWNT-IRDye-800 conjugates (Fig. 1c) into a nude mouse. We estimated the maximum SWNT concentration in the blood would be 17 times lower than the half-maximal inhibitory concentration (IC₅₀) of vascular endothelial cells (Supplementary Fig. 1d). The circulation half-time of DSPE-mPEG–functionalized SWNTs was ~5 h (ref. 8), and our previous studies had shown the lack of acute or long-term toxicity of such PEGylated SWNTs in vivo. In contrast, when the same mouse was imaged in the NIR-II region by detecting SWNT fluorescence, there was a substantially improved spatial resolution of vessels at all magnifications. Moreover, the NIR-II window clearly visualized smaller, higher-order branches of blood vessels at higher magnifications. Cross-sectional intensity profiles all showed sharp peaks, with the calculated vessel diameter values consistent with expected values (Fig. 1h–j, bottom). In contrast, it was impossible to calculate vessel diameters on the basis of the NIR-I images, in which we observed an approximately two- to threefold broadening of the cross-sectional profiles on average.

NIR-II and micro-CT for vessel imaging

Micro-CT is a commonly used three-dimensional X-ray imaging technique based on tomographic reconstruction, with a spatial resolution down to ~100 µm and excellent penetration depth. Accordingly, we compared the spatial resolution of the proximal femoral artery and vein achieved by NIR-II and micro-CT methods in the same mouse (Fig. 2a,b). We performed a cross-sectional analysis across the blood vessels in each image, and the corresponding intensity profiles are shown for NIR-II (Fig. 2c) and micro-CT (Fig. 2d). We identified two peaks corresponding to femoral artery and vein in each plot and fitted them into two Gaussian functions to extract the widths. The vessel widths extracted from the NIR-II image (0.284 mm and 0.255 mm) agreed with those from the micro-CT image (0.292 mm and 0.247 mm). The analysis of micro-CT images validated the vessel diameters measured by NIR-II imaging, suggesting the two techniques are comparable in imaging vascular structures several hundred microns in diameter. The depths of femoral vessels and their collateral localization of SWNTs and IRDye-800 dyes, and enabled us to image the same tissue in two distinct spectral windows so as to evaluate photons in different wavelengths for live animal imaging.

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Figure 1  NIR-I and NIR-II fluorescence imaging of blood vessels in the mouse. (a) A schematic showing that upon excitation by a 785-nm laser, the SWNT-IRDye-800 conjugate emits at the ~800 nm NIR-I region from IRDye-800 dye and the 1.1–1.4 µm NIR-II region from the SWNT backbone. (b) Absorption spectrum of the SWNT–IRDye-800 conjugate (black dashed line), emission spectrum of IRDye-800 dye (green line) and SWNTs (red line). (c) A digital camera photograph (left) and a NIR-II fluorescence image of injected solution containing 0.10 mg ml⁻¹ SWNT-IRDye-800 conjugates. (d) Schematic of the imaging setup for simultaneous detection of both NIR-I and NIR-II photons using silicon and InGaAs cameras. A zoomable lens set was used for adjustable magnifications. (e–g) NIR-I fluorescence images (top) and cross-sectional fluorescence intensity profiles (bottom) along red-dashed lines of a mouse injected with the SWNT-IRDye-800 conjugates. Gaussian fits to the profiles are shown in red dashed curves. (h–j) NIR-II fluorescence images (top) and cross-sectional fluorescence intensity profiles (bottom) along red-dashed bars of a mouse injected with the SWNT-IRDye-800 conjugates. Gaussian fits to the profiles are shown in red dashed curves.
vessels ranged from 1–3 mm on the basis of micro-CT images in combination with dissection measurements.

To compare the resolution limits between NIR-II and micro-CT, we determined the smallest vessels these two techniques were able to discern. In the distal hind limb of the mouse, the NIR-II image showed greater numbers of small vessels compared to the micro-CT image at the same location (Fig. 2f). The smallest measurable vessel by NIR-II had a Gaussian-fit diameter of only 35.4 μm (Fig. 2g), whereas micro-CT could not discern any vessel smaller than ~100 μm in diameter (Fig. 2h). Furthermore, the NIR-II method generated images much faster than micro-CT (300 ms for NIR-II and 2 h for micro-CT).

**Differentiation of arterial and venous vessels**

To determine whether NIR-II fluorescence imaging could distinguish arterial from venous circulation, we monitored the blood flow inside the vessels by recording movies in the NIR-II window immediately upon tail-vein injection of SWNT fluorophores. To enable greater temporal resolution (that is, shorter exposure time) for dynamic recordings, we used an 808-nm laser at 140 mW cm\(^{-2}\) for more efficient excitation of SWNT fluorophores.

When we injected a 200-μl solution containing 0.10 mg ml\(^{-1}\) bio-compatible SWNTs into the tail vein of a nude mouse (mouse C1), we observed a NIR-II signal from the SWNTs in the proximal femoral artery within 5 s and the entire femoral artery and some of the proximal musculature after ~8 s (Fig. 3a,b). Outflow of SWNTs into the femoral vein was reflected by the increased feature width (vascular bundle in the femoral sheath) at later time points but was difficult to distinguish from the femoral artery by visual inspection due to the proximity of the artery and the vein (Fig. 3c). However, owing to the time delay between the first appearance of the signal in the artery and its later appearance in the vein (Supplementary Movie 1), it was possible to employ PCA to differentiate the two types of vessels.\(^8\),\(^23\) Essentially, the PCA approach assigns image pixels to groups (components) on the basis of their variance, that is, pixels that vary similarly in time. We applied PCA to the video-rate images and clearly discriminated the arterial component from the venous component. By convention, we color-coded the arterial PCA component in red and the venous component in blue (Fig. 3d). We obtained similar results with an additional three mice (Supplementary Fig. 2a–f).

We repeated these studies in mice after surgically inducing unilateral hind limb ischemia (mice C1–C3). In this model, ligation and excision of the proximal superficial femoral artery and ligation of the deep femoral artery reduced limb perfusion by ~80% in the immediate postoperative period.\(^2\) On the first postoperative day, we studied mice with SWNT-assisted NIR-II fluorescence imaging. After tail vein injection of SWNTs, we observed a marked delay in the appearance of fluorescent signals in ischemic limbs (Fig. 3e–g) compared to healthy limbs. As in normal mice, we performed PCA on the first 200 frames (~37.5 s) of video-rate recording (Supplementary Movie 2) of the ischemic mice. However, owing to the markedly reduced perfusion,
we did not observe venous return in the ischemic limb at this time point (Fig. 3h and Supplementary Fig. 2k–j). It was necessary to continue imaging for an extended period (>2 min) to visualize the femoral vein (Supplementary Fig. 3 and Supplementary Movie 3). This delay in venous return was consistent with severe limb ischemia.

We also applied the dynamic contrast PCA approach to demonstrate that arteries and veins subserving a larger region of tissue could be distinguished. In these studies, we imaged a larger field of view including both the abdominal and femoral regions. Within 5 s of tail vein injection (Supplementary Movie 4), we observed signals in the aorta as well as the iliac, femoral and epigastric arteries and their branches in the abdomen and pelvis (Fig. 4a,b). The NIR-II signal from these vessels peaked and then subsided by ~10 s after injection with increasing intensity in the tissue (Fig. 4c). Subsequently, we observed signals in veins draining these regions, including the previously identified femoral vein and inferior epigastric veins (Fig. 4d). As before, we used PCA to assign pixels to arterial or venous conduits on the basis of their time variance (Fig. 4e), where arteries and veins were resolved. Notably, even the aorta can be seen in supine position of the mouse, indicating a penetration depth of >5 mm in the abdomen for NIR-II imaging (Fig. 4f). This result confirmed the capacity of NIR-II dynamic contrast-enhanced imaging to distinguish arteries from veins.

Blood flow quantification in ischemic and control limbs
NIR-II imaging revealed a marked delay in the appearance of fluorescence signal in ischemic hind limbs versus control healthy limbs. We then assessed blood flow in the ischemic hind limb of mouse 11 quantitatively. From 12 s to 19.5 s after injection of SWNTs, propagation of the signal could be visualized from the proximal site of arterial occlusion to the distal femoral artery (Fig. 5a), presumably filling through collateral channels. The position of the signal front at each time point was then extracted from each frame and plotted against time, showing a linear relationship of flow distance versus time with a blood velocity of 0.163 cm s⁻¹ (Fig. 5b). To calculate blood flow, we measured the mean diameter of the femoral artery (174 µm). The measurement of blood flow velocity and arterial diameter permitted the calculation of femoral artery blood flow (2.33 × 10⁻⁳ ml min⁻¹).

We found the progression of NIR-II signal through the femoral artery in normal mice to be so rapid that our current video imaging rate lacked sufficient temporal resolution. Alternatively, to assess blood velocity in these mice, we measured the increase in signal intensity in a region of interest (ROI) (a predefined segment of the femoral artery from PCA). We normalized the signal intensity to compensate for differences in actual injected dose or fluorescence quantum yield and plotted the average ROI intensity versus time (see Online Methods). In the mouse with hind limb ischemia, we observed a NIR-II intensity increase of 2.18% s⁻¹ (Fig. 5c). We then plotted blood flow and NIR-II intensity against each other (Fig. 5d) to yield a linear slope of ~0.0737 cm %⁻¹ that correlated velocity with intensity change. We used this coefficient to convert the NIR-II intensity increase rate to blood velocity in the normal mice where blood velocity exceeded the temporal resolution of our current imaging device. To further establish this translation coefficient, we reproduced the blood velocity quantification with two other ischemic mice, 12 and 13, and obtained an average value of 0.0747 ± 0.0019 cm %⁻¹ (Supplementary Fig. 4 and Supplementary Table 1).

The intensity-to-velocity conversion coefficient was applied to measurements of femoral artery blood flow in a healthy, control mouse where the blood velocity was so rapid that our current NIR-II imaging device was unable to track a discrete flow front over time (Fig. 5e). Alternatively, we measured the NIR-II intensity change in an ROI of the femoral artery as a function of time (Fig. 5f), revealing a normalized NIR-II intensity increase of 68.7 ± 5.2% s⁻¹ before it peaked. By using the conversion coefficient of 0.0747 cm %⁻¹, we were able to translate the intensity increase to a blood velocity of 5.13 ± 0.39 cm s⁻¹. To validate this measure of blood velocity, we used ultrasonography to obtain a Doppler-derived velocity in the same mouse at the same ROI of the femoral artery by an operator blinded to the SWNT imaging values. In the same animal, the ultrasound measurement provided a Doppler-derived velocity of 4.97 ± 0.17 cm s⁻¹ (Fig. 5g), which was in good agreement with the NIR-II video-imaging result (a deviation of ~3%).

To further validate our intensity-to-velocity conversion coefficient and to understand the physics behind it, we used a simplified fluid dynamic system, in which SWNT solution was pumped into catheter tubing filled with purified water (Supplementary Fig. 5k), and derived the coefficient on the basis of NIR-II intensity increase (see Online Methods). By changing the experimental settings one at a time, we found the coefficient remained invariant (0.0764 ± 0.0025 cm %⁻¹) as we varied the injected SWNT concentration, SWNT fluorescence quantum yield, tubing diameter, ROI length and velocity. It did vary with the distance between the injection site and ROI (pre-ROI length) (Supplementary Fig. 5a–j and Supplementary Table 2). This finding was also confirmed by numerical simulations (Supplementary Fig. 5l–s and Supplementary Table 2) based on a linear flow model with axial mixing²⁹. In the context of in vivo blood velocity quantification, because the pre-ROI length simply reflects the length of blood vessels in which the injected SWNTs had to travel before reaching the femoral artery, the coefficient should be invariant given the same type of animals with roughly same blood volume and therefore applicable from ischemic to control mice.

To demonstrate the reproducibility of the NIR-II fluorescence- based blood velocity quantification, we compared the Doppler-derived velocities to those of the NIR-II method in three additional control mice (Supplementary Fig. 6). A summarized plot suggested excellent agreement of results between the two methods (Fig. 5h). Subsequently, we performed these studies in three ischemic mice.
DISCUSSION

Current methodologies for physiological imaging of PADs are sub-optimal in that no single modality provides adequate spatial and temporal resolution to accurately assess all essential parameters, that is, vascular structure, arterial inflow, venous outflow and tissue perfusion. NIR-II imaging technique simultaneously provides anatomical and hemodynamic information and surpasses the need to use multiple imaging modalities to obtain equivalent data, owing to reduced tissue scattering and deeper anatomical penetration of NIR-II over shorter wavelengths. This is due to the inverse wavelength dependence (−1.9, α = 0.22–1.68) of photon scattering as they travel through subcutaneous tissue and skin (see ref. 8 and references therein).

NIR-II imaging compares favorably to micro-CT. Even with voxel dimensions of 40 μm, micro-CT was only capable of resolving vessels of ~100 μm, whereas NIR-II could distinguish vessels approximately three times smaller, without obvious background signal from soft tissue. In terms of imaging depth, NIR-II can achieve a penetration depth of 1–3 mm inside the hind limb in vivo without losing fidelity of the vessel structures, whereas micro-CT is able to reconstruct the whole-body structure in three dimensions owing to the unlimited penetration of X-rays. The time required for micro-CT (hours) as opposed to NIR-II imaging (subseconds) also means longer anesthesia time and high radiation doses, carrying risks of nephrotoxicity, anaphylaxis and tissue injury.

NIR-II imaging has three salient advantages over ultrasonography. First, NIR-II is able to image smaller vessels. Even with a high-frequency (40 MHz) transducer, the diameter of mouse vessels could not be accurately determined with ultrasound, owing to poor spatial resolution and low contrast. Second, NIR-II imaging is able to resolve both arterial and venous vessels anatomically and hemodynamically using PCA. Third, NIR-II imaging can be used to acquire hemodynamic data in conditions of reduced flow (for example, ischemic hind limb), below the detection limit of ultrasonography.

Given the many benefits of NIR-II fluorescence imaging over other pre-clinical imaging modalities, this dual-modality method may be useful in a variety of cardiovascular models. Although our current work focused on assessing blood flow of small vessels, NIR-II imaging could be used to characterize in vivo the degree of stenosis or aneurysmal dilation of vessels. Because of its temporal resolution, it might also be useful for imaging dynamic changes in vessel diameter caused by active vasodilation or vasoconstriction. It is conceivable that NIR-II fluorescence imaging with SWNTs and other novel NIR-II fluorophores, including quantum dots and synthetic organic dyes, could lead to many translational and clinical applications.
METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.D., J.P.C., N.F.H., G.H. and J.C.L. conceived of and designed the experiments. G.H., J.C.L., J.T.R., U.R., L.X. and N.F.H. performed the experiments. G.H., J.C.L., U.R., L.X., N.F.H., J.P.C. and H.D. analyzed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Preparation of water-soluble SWNT-IRDye-800 bioconjugate. The preparation of water-soluble and biocompatible SWNTs can be found in detail in a previously published paper with some modifications. In general, raw high-pressure carbon monoxide conversion SWNTs (Unidym) were suspended in 1% wt/wt sodium deoxycholate aqueous solution by 1 h of bath sonication. This suspension was ultracentrifuged at 300,000 g to remove the bundles and other large aggregates, the supernatant was retained and 0.75 mg mL⁻¹ of DSPE-PEI [poly(2,4-dihydroxy-6-aminomethyltrimethylammonium)-N-[2-amino(polyethyleneglycol, 5000)], Laysan Bio] along with 0.25 mg mL⁻¹ of DSPE-PEG (5 kDa)-NH₂-[poly(2,4-dihydroxy-6-aminomethyltrimethylammonium)-N-[2-amino(polyethyleneglycol, 5000)], Sunbright) was added. The resulting suspension was sonicated briefly for 5 min and then dialyzed at pH 7.4 in a 3,500-Da membrane (Fisher) with a minimum of six water changes and a minimum of 2 h between water changes. To remove aggregates, the suspension was ultracentrifuged again for 1 h at 300,000 g. This surfactant-exchanged SWNT sample has lengths ranging from 100 nm up to 2.0 µm with the average length of ~500 nm. These amino-functionalized SWNTs were further conjugated with IRDye-800 (IRDye 800CW, LI-COR) dye molecule according to the protocol that has been used in our group. Briefly, an SWNT solution with amine functionality at 300 mM after removal of excess surfactant was mixed with 0.1 mM IRDye-800 NHS ester (LI-COR) in PBS at pH 7.4. The reaction was allowed to proceed for 1 h before purification to remove excess IRDye-800 by filtration through 10-kDa filters. The as-made SWNT-IRDye-800 conjugate solution was kept at 4 °C and away from light to avoid photobleaching of IRDye-800 fluorescence.

UV-Vis-NIR absorption measurements. The UV-Vis-NIR absorption spectrum of the as-made SWNT-IRDye-800 bioconjugate was measured by a Cary 6000i UV-Vis-NIR spectrophotometer, background-corrected for contribution from water. The measured range was 500–820 nm.

NIR fluorescence spectroscopy of SWNT-IRDye-800 bioconjugate. The NIR fluorescence spectrum was taken using a home-built NIR spectroscopy setup. The excitation source was a 200-W ozone-free mercury/xenon lamp (Oriel), which was dispersed by a monochromator (Oriel) to generate an excitation line with a central wavelength of 785 nm and a bandwidth of 15 nm. The excitation light was allowed to pass through the solution sample in a 1-mm-path cuvette (Starna Cells, Inc.), and the emission was collected in a transmission geometry. The excitation light was rejected using a 790-nm long-pass filter (Semrock) so that the fluorescence of both SWNT-800 and SWNTs could be collected in the 790–1,500 nm emission range. The emitted light was directed into a spectrometer (Acton SP2300i) equipped with a liquid-nitrogen–cooled InGaAs linear array detector (Princeton OMA-V). Spectra were corrected post-collection to account for the sensitivity of the detector and extinction feature of the filter using the MATLAB software.

Determination of cytotoxicity of SWNTs. We determined the SWNT toxicity in vitro by MTS assay using a CellTiter96 kit (Promega) on human dermal microvascular endothelial cells (Lonza). Approximately 5,000 cells were incubated per well with 100 µl of EGM2MV growth medium (Lonza) and serially diluted SWNT solution (n = 3 for each concentration). The cells were kept at 37 °C in a humidified atmosphere containing 5% CO₂ for 24 h in the presence of SWNTs at different concentrations. Immediately before addition of 15 µl of CellTiter96, a colorimetric indicator of cell viability, the SWNT-spiked medium was removed from each well plate and replaced with fresh medium. This prevented any interference in the absorbance readings from SWNTs. After 1 h, the color change was quantified using a plate reader and taking absorbance readings at 490 nm. Cell viability was plotted as a fraction of the absorbance of control wells incubated without SWNTs.

Mouse handling, surgery and injection. Six-week-old female athymic nude mice were obtained from Charles River. All mouse studies were approved by Stanford University’s Administrative Panel on Laboratory Animal Care. Induction of unilateral hind limb ischemia was performed according to our previous studies. Control, unsurgerized mice (n = 4) and mice with induced ischemia (n = 3) were used in the study. For the injection of nanotube solution, a 28-gauge syringe needle was inserted into the lateral tail vein, allowing for bolus injection with the room lights turned off during the first frames of imaging. All mice were initially anesthetized before imaging in a knockdown box with 21 min⁻¹ O₂ gas flow mixed with 3% isoflurane. A nose cone delivered 1.5 min⁻¹ O₂ gas and 3% isoflurane throughout imaging.

In vivo NIR fluorescence imaging with tunable magnifications. Mice were mounted on a heated stage in the supine position beneath the laser at 10 min after injection. NIR fluorescence images were collected using a 1,344 × 1,024 pixel silicon charge-coupled device camera (Hamamatsu) for detecting photons in NIR-I and a liquid-nitrogen-cooled, 320 × 256 pixel two-dimensional InGaAs array (Princeton Instruments) for collecting photons in NIR-II. A flip mirror was used to switch photon collection between the two cameras (Fig. 1d). The excitation light was provided by a 785-nm diode laser (Renishaw) coupled to a 4.5-mm focal length collimator (Thorlabs) and filtered by a 790-nm band-pass filter with a bandwidth of 10 nm (Thorlabs). The excitation power density at the imaging plane was 8 mW cm⁻², much lower than the safe exposure limit of 296 mW cm⁻² at 785 nm determined by the International Commission on Non-ionizing Radiation Protection. The emitted light from the mouse was filtered through a 790-nm long-pass filter (Semrock) and an 850-nm short-pass filter (Thorlabs) coupled with the silicon camera for the NIR-I imaging window, or through a 900-nm long-pass filter (Thorlabs) and an 1,100-nm long-pass filter (Thorlabs) coupled with the InGaAs camera for NIR-II imaging. A lens set was used for obtaining tunable magnifications, ranging from ×1 to ×2.5 magnification by changing the relative position of two NIR achromats (200 mm and 75 mm, Thorlabs) and from ×2.5 to ×7 magnification by changing the relative position of two other NIR achromats (150 mm and 200 mm, Thorlabs). A binning of 4 and an exposure time of 300 ms were used for the silicon camera (1,344 × 1,024 pixels) to capture images in the NIR-I window, and a binning of 1 and an exposure time of 300 ms were used for the InGaAs camera (320 × 256 pixel) to capture images in the NIR-II window. Different binning values were used to compensate the difference of array size of the two cameras.

Microscopic computed tomography for vascular imaging. Micro-CT scans were performed using a MicroCAT II micro-CT scanner (Siemens Preclinical Solutions) with the following parameters: X-ray voltage 80 kVp, anode current 50 mA and exposure time 2,000 ms per 576 frames through 360° rotation. Micro-CT scans were performed with the injected blood pool contrast agent Fenestra VC (50 mg ml⁻¹ iodine, Advanced Research Technologies) intravenously into the lateral tail vein at 0.3 ml per 20 g body weight with a 28-gauge needle. Mice (n = 2) were scanned 1 h after injection for 1 h total scan time. Three-dimensional reconstruction was performed by COBRA 1.5 and visualized using Amira 5.4. Resulting voxel dimension was 40 µm. To determine the depth of femoral vessels and their collaterals, distance between the vessels and the edge of surrounding tissue was measured in reconstructed micro-CT images using ImageJ software. Video-rate imaging in the NIR-II window. Video-rate imaging was performed on the same home-built imaging system as in the steady-state imaging case except that only an InGaAs camera was used for imaging in the NIR-II window. The excitation light was provided by an 808-nm diode laser (RMPC lasers) coupled to a 4.5-mm focal length collimator (Thorlabs) and filtered by a 850-nm short-pass filter and a 1,000-nm short-pass filter (Thorlabs). The excitation power density at the imaging plane was 140 mW cm⁻², lower than the safe exposure limit 329 mW cm⁻² at 808 nm. The emitted light from the mouse was filtered through a 900-nm long-pass filter and a 1,100-nm long-pass filter (Thorlabs) so that the intensity of each pixel in the InGaAs two-dimensional array represented light in the 1.1–1.7 µm range. A lens pair consisting of two achromats (150 mm and 200 mm, Thorlabs) was used to focus the image onto the detector with a magnification of ×2.5. The detector was a 1,100-nm long-pass filter (Semrock) and an 850-nm short-pass filter (Thorlabs) and a flip mirror was used to switch photon collection between the two cameras.
Dynamic contrast-enhanced imaging based on PCA. Dynamic contrast-enhanced images were obtained in a similar fashion to previous work by the Hillman group and our group. The first 200 consecutive frames immediately after injection were loaded into an array using MATLAB software, and the built-in princomp function was used to perform PCA. During the mathematical process, faster flowing features showing up early in the movie are grouped in the negative fourth principal component, and slower flowing features showing up later in the movie are grouped in the negative second principal component. Therefore, negative pixels for the second principal component were color-coded in blue to represent venous vessels and negative pixels for the fourth principal component were color-coded in red to represent arterial vessels.

Quantification of blood velocity and flow based on NIR-II fluorescence. Average region of interest (ROI) NIR-II fluorescence intensity was computed using MATLAB software within a given arterial region of each frame, which was determined by PCA analysis as explained in the previous paragraph. Then the NIR-II fluorescence intensity was plotted as a function of time to reveal the change over 4 min after intravenous injection. The intensity in the femoral artery increased rapidly within the first 5–10 s before residing. The NIR-II intensity values were normalized against the maximum intensity within the ROI time traces to compensate for any differences in actual injection dose and relative fluorescence quantum yield. Linear fit was then performed on the rising edge of the normalized plot, and its slope (in % s⁻¹) was translated into blood velocity in terms of cm s⁻¹ by using the intensity-to-velocity conversion coefficient. Since the NIR-II signal increase was averaged over the ROI, the velocity value was the mean blood flow velocity along the femoral artery. Blood flow (F) in ml min⁻¹ (cm³ min⁻¹) was calculated on the basis of the blood velocity (V) in cm s⁻¹ and the diameter of the vessel (d) in μm as follows:

\[ F = \pi \times (d/2)^2 \times V \times (6 \times 10^{-7}) \]

Validation of intensity-to-velocity coefficient based on tubing flow model. A solution of SWNTs with known concentration was pumped by a syringe pump into catheter tubing with known diameter filled with purified water at a preset velocity (Supplementary Fig. 5k). The following settings were used for the standard condition: SWNT concentration = 0.10 mg x ml⁻¹, SWNT fluorescence quantum yield (QY) ~2.5%, tubing diameter = 760 μm, tubing length within ROI (that is, ROI length) = 2.5 cm, fluid velocity = 1.4 cm s⁻¹ and tubing length before ROI (that is, pre-ROI length) = 8.5 cm. To screen the dependency of the coefficient on all these variables, the parameters were changed one at a time while keeping all others unchanged: SWNT concentration changed to 0.025 mg ml⁻¹, SWNT fluorescence QY to 5.0%, tubing diameter to 380 μm, ROI length to 1.25 cm, fluid velocity to 0.14 cm s⁻¹ and pre-ROI length to 19.5 cm. Under each combination of settings, the NIR-II fluorescence intensity within the selected ROI was plotted as a function of time from immediately after injection to the time when intensity plateaued. Then NIR-II intensity values were normalized against the maximum intensity (plateau intensity) within the ROI time traces. Linear fit was performed on the linear rise of each normalized plot to obtain a slope in % s⁻¹. The intensity increase rate (slope) in % s⁻¹ was then used to divide the velocity in cm s⁻¹ to obtain the intensity-to-velocity conversion coefficient in cm %⁻¹.

For numerical simulation, a tubing linear flow model with axial mixing was adapted from a previous publication. A sigmoidal function with time and velocity dependence was used to simulate NIR-II intensity distribution at the flow front upon mixing using MATLAB software. The flow front function was given analytically by the equation

\[ F(x,v,t) = \frac{1}{1 + e^{\frac{-QY}{\text{exc}} v x}} \]

where the excitation power density I, absorption coefficient ε, initial degree of mixing A₀ (~0.001 cm) and mixing constant K (~0.5) were fixed, concentration c, tubing diameter d, fluorescence quantum yield QY and velocity v were varied in the simulations to find the dependency of the coefficient and the flow front distribution of SWNT fluorophores F was expressed as a function of its location x, velocity v and time t. The normalized ROI intensity was computed numerically as follows:

\[ I_{\text{norm}}(t) = \frac{\int_{x=0}^{L_{\text{pre-ROI}}} I(x,v,t)dx}{\int_{x=L_{\text{pre-ROI}}}^{L_{\text{ROI}}} F(x,v,t)dx} \]

where L denotes the length of the tubing SWNT fluorophores passed through. I_{\text{norm}}(t) was then plotted against time (Supplementary Fig. 5l–r). The linear rise region was fit to a linear equation, and the slope was used to divide the velocity, giving the intensity-to-velocity conversion coefficient

\[ \text{Coeff.} = \frac{\partial I_{\text{norm}}(t)}{\partial t} \]

Ultrasound for quantifying blood flow in femoral artery. Ultrasound measurements were performed using a linear real-time transducer (40 MHz) connected to a Vevo 2100 ultrasound system (VisualSonics). The femoral artery was identified employing Duplex-ultrasonography (B-Mode and power Doppler). Flow velocity profiles were recorded by pw-Doppler imaging. Velocity-time integrals (VTI) and cardiac cycle length (CL) were measured using the Vevo 2100 device software. Arterial diameter (d) was known for each mouse from previous NIR-II imaging. Femoral flow (F) was calculated as:

\[ V = \pi \times (d/2)^2 \times V \times (6 \times 10^{-7}) \]

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