Near-Infrared II Fluorescence for Imaging Hindlimb Vessel Regeneration
with Dynamic Tissue Perfusion Measurement

Hong et al: NIR II Fluorescence Imaging of Hindlimb Blood Perfusion

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Abstract

Background—Real-time vascular imaging that provides both anatomic and hemodynamic information could greatly facilitate the diagnosis of vascular diseases and provide accurate assessment of therapeutic effects. Here we have developed a novel fluorescence-based all-optical method, named near-infrared II (NIR-II) fluorescence imaging, to image murine hindlimb vasculature and blood flow in an experimental model of peripheral arterial disease, by exploiting fluorescence in the NIR-II region (1000-1400 nm) of photon wavelengths.

Methods and Results—Owing to the reduced photon scattering of NIR-II fluorescence compared to traditional NIR fluorescence imaging and thus much deeper penetration depth into the body, we demonstrated that the mouse hindlimb vasculature could be imaged with higher spatial resolution than in vivo microCT. Furthermore, imaging over 26 days revealed a significant increase in hindlimb microvascular density in response to experimentally induced ischemia within the first 8 days of the surgery (P < 0.005), which was confirmed by histological analysis of microvascular density. Moreover, the tissue perfusion in the ischemic hindlimb could be quantitatively measured by the dynamic NIR-II method, revealing the temporal kinetics of blood flow recovery that resembled microbead-based blood flowmetry and laser Doppler blood spectroscopy.

Conclusions—The penetration depth of millimeters, high spatial resolution and fast acquisition rate of NIR-II imaging makes it a useful imaging tool for murine models of vascular disease.

Key Words: near-infrared II (NIR-II), fluorescent imaging, angiography, hemodynamics, acute limb ischemia, carbon nanotubes, vascular regeneration
Peripheral arterial disease (PAD) is an atherosclerotic arterial occlusive disease of the limbs that affects over 10 million people in the US.¹ Novel therapeutic approaches to stimulate vascular regeneration and improve blood perfusion are often tested in the murine model of experimentally induced hindlimb ischemia. The evaluation of new therapies in this model would be aided by non-invasive imaging approaches that provide hemodynamic and anatomical information. We have previously reported the application of near-infrared II (NIR-II) fluorescence-based imaging as a multifunctional approach for quantifying limb blood flow and vessel structural information, achieving higher spatial resolution than conventional microscopic computed tomography (µCT), while the temporal resolution matches that of ultrasonography for tracking blood dynamics in real time.²

The multifunctional imaging capabilities in the NIR-II window originate from the intrinsic fluorescence properties of single-walled carbon nanotubes (SWNTs), which are cylindrical molecules made of rolled-up graphite sheets comprised of carbon.³ Semiconducting SWNTs exhibit strong resonant absorption in the traditional near-infrared region (NIR-I, 750-900 nm) and re-emit fluorescence in the NIR-II region (1000-1400 nm) with a large Stokes shift of ~400 nm, making them novel NIR-II fluorophores for in vivo fluorescence imaging.⁴ ⁵ The benefits of using the long wavelength NIR-II fluorescence over traditional NIR-I fluorescence comes from reduced scattering and thus much deeper tissue penetration depth of photons, due to the inverse dependence (~λ⁻⁰.₄)~₁.₆₈) of photon scattering on wavelength in turbid biological tissues.⁶ Moreover, the large Stokes shift of SWNTs and the absorption and emission of SWNTs in the biologically transparent NIR window also greatly reduce background autofluorescence, which minimizes the interference from indigenous tissue signals.⁷ When compared with non-optical imaging modalities such as µCT and magnetic resonance imaging
(MRI), NIR-II maintains all the strengths of a fluorescence-based optical imaging modality, including easy implementation,
high diffraction-limited spatial resolution (microns), and fast wide-field acquisition rate (tens to hundreds of frames per second).

Here, we report the use of NIR-II based fluorescence imaging to provide multifunctional information on limb perfusion and collateral vessel formation in an experimental model of PAD, over the course of 26 days. Our results demonstrate the ability of NIR-II imaging to quantify tissue perfusion, with validation by microbead blood flowmetry and laser Doppler blood spectroscopy, as well as reveal the temporal kinetics of collateral vessel formation with a spatial resolution higher than μCT. The high spatial resolution and dynamic recording capability of NIR-II fluorescence imaging allows us to non-invasively image the temporal kinetics of vascular remodeling and formation of collateral vessels, while enabling blood flow quantification, which is not attainable by any single imaging modality as such μCT or ultrasound. Our finding of the transient vascular density increase as a response to acute hindlimb ischemia, followed by the return to normal vascular level, agrees with previous studies and provides a non-invasive and direct way of visualizing collateral formation.

Methods

Unilateral Hindlimb Ischemia

All animal studies were carried out under the approval of the Institutional Animal Care and Use Committees (IACUC) at Stanford University. Female athymic nude mice (Charles River, 13 weeks old) were anesthetized and maintained under 2% isoflurane and oxygen flow rate of 1 L/min. For the 10-day short-term study and the 26-day long-term study, 6 mice each were used for induction of unilateral hindlimb ischemia, which was performed by ligating and excising the
femoral artery, according to our previous publications.\textsuperscript{9,10} Please note that the time intervals in all experiments refer to post-operative time points.

**Laser Doppler Tissue Perfusion Measurement**

To measure tissue perfusion of mouse hindlimb using laser Doppler spectroscopy (PeriSCan PIM3, Permed AB), the animals were anesthetized and maintained under 2\% isoflurane and oxygen flow rate of 1 L/min. The animals were pre-warmed to 37.5 °C body temperature and placed in the supine position during laser Doppler blood spectroscopy. Recovery of limb blood flow over time was expressed as the relative ratio of perfusion in the ischemic over the non-ischemic limb.\textsuperscript{9}

**Preparation of Biocompatible SWNT-Based NIR-II Contrast Agents**

Raw HiPCO SWNTs (Unidym, Inc.) were suspended in an aqueous solution of sodium deoxycholate at a concentration of 1 wt\% by bath sonication for 1 h. This SWNT suspension was ultracentrifuged at 300,000 g, the supernatant was retained and 1.0 mg·ml\textsuperscript{-1} of DSPE-mPEG(5 kDa) (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol, 5000)], Laysan Bio) was added. The resulting suspension was then dialyzed against 1x PBS in a 3500 Da membrane (Fisher) with pH controlled at 7.4 to remove sodium deoxycholate, leaving the SWNTs coated with the biocompatible DSPE-mPEG only.

**Near-Infrared II Fluorescence-Based Blood Perfusion Measurement**

Near-infrared II fluorescence-based small animal \textit{in vivo} imaging was done on a customized imaging setup with optimized parameter settings according to our established protocol.\textsuperscript{2,4-6,11} For dynamic tissue perfusion imaging using NIR-II fluorescence, one mouse was mounted on the imaging stage at a time in its supine position prior to injection. The excitation was provided by an 808-nm laser diode (RMPC lasers) connected to a collimator with a focal length of 4.5 mm
(Thorlabs). The excitation light was filtered through an 850-nm short-pass filter (Thorlabs) and a 1000-nm short-pass filter (Thorlabs). The average power density of the excitation laser on the imaging stage (140 mW cm\(^{-2}\)) was significantly lower than the safe exposure limit of 329 mW cm\(^{-2}\) at 808 nm for animals. The emitted NIR-II fluorescence was filtered through a 900-nm long-pass filter and an 1100-nm long-pass filter (Thorlabs) and focused onto an indium-gallium-arsenide (InGaAs) 2D detector (Princeton Instruments) through lenses. The InGaAs camera started recording images immediately after a 200 \(\mu\)L bolus of NIR-II contrast solution containing 0.10 mg/mL biocompatible single-walled carbon nanotubes (SWNTs) was injected into the mouse tail vein, which was set to be \(t = 0\) s. An exposure time of 100 ms was used for all images in the video. The frame rate was 5.3 frames \(s^{-1}\) due to an overhead time of 87.5 ms for the acquisition of each frame.

Consecutive video rate images were then loaded into MATLAB software for perfusion analysis. In a typical procedure, same regions of interest (ROI) of both control and ischemic hindlimbs were selected from the video frames in a same way as the laser Doppler method, and the NIR-II fluorescence intensity increase within each ROI was plotted against time from 0 s to 65.625 s (350 frames) post injection (p.i.). The plot featured a linear rising edge followed by a plateau region due to blood saturation of NIR-II contrast agent. The plot was normalized against the saturation level of the control limb, and the linear rising edge (after normalization) was used to fit a line with its slope representing the perfusion level, similar to a previous publication of fluorescence-based perfusion quantification. Two slopes were generated for each mouse, one derived from the control limb, and the other derived from the ischemic limb. The slope of the ischemic limb was then normalized against the slope of the control limb to obtain the relative tissue perfusion \((RTP)\) in units of percentage.
where by definition the RTP of the control hindlimb is 100%.

To estimate the absolute blood velocity within the hindlimb, a NIR-II intensity-to-
velocity conversion coefficient of 0.0747±0.0019 cm·%⁻¹ for the hindlimb of athymic nude mice
was used to translate the percentage NIR-II intensity increase into local blood velocity, as
previously described.² The normalized slope has a unit of %/s, which is multiplied by the
conversion coefficient in units of cm/% to give the blood velocity in units of cm/s.

**Fluorescent Microbead Perfusion Assay**

To validate the NIR-II-based blood perfusion measurements, additional hindlimb ischemia
experiments were performed in which the relative fluorescence intensity of systemically-
delivered fluorescent microbeads were quantified for up to 10 days after induction of hindlimb
ischemia. Prior to euthanasia on days 0, 3, 7, and 10, the anesthetized mice received
intracardiac injections of fluorescent microbeads (blue-green FluoSpheres 15μm diameter, 0.5 X
10⁶ beads), followed by intra-cardiac injections of heparin (2000 U) and nitroglycerin (500 mg).

Microbead extraction and quantification were performed based on established methods.¹⁴ In
brief, the ischemic and control hindlimb tissues were excised and digested in KOH (0.5 M) at 52
°C to extract the microbeads. The microbeads were then dissolved in cellosolve acetate before
measuring in a fluorescence plate reader. The data is shown as relative fluorescence intensity
between the ischemic and control limb, normalized by tissue weight (n≥4 for each time point).

**Vessel Structural Imaging Using Near-Infrared II Fluorescence**

To monitor vascular regeneration/remodeling in the ischemic mouse hindlimb, NIR-II imaging
was applied at a high magnification with pixel size of ~78 μm. The same excitation source and
detector as the dynamic NIR-II tissue perfusion imaging were used except that the distance
between the two focusing achromats was adjusted to afford a ~2.5-fold higher magnification. This field of view could only cover one hindlimb at a time due to the magnification. All NIR-II images were taken at ~10-15 min p.i.

For vessel width analysis, a line was drawn on each NIR-II image, perpendicular to the femoral vessels. The NIR-II intensity values on this line were extracted using the ImageJ software and plotted against their physical locations on this line. Vessels intersected by the line were represented as peaks in the intensity profile and each peak was fitted using the Origin software into a Gaussian function. The full width half maximum (FWHM) of each peak is related to the Gaussian width \( w \) by the following relationship:

\[
\text{FWHM} = \sqrt{2\ln 2} \times 1.177w
\]

Two different methods, line cross-sectional peak counting and fast Fourier transform (FFT), were applied on all NIR-II hindlimb images to calculate microvascular density change over the period of revascularization. For the line cross-sectional peak counting method, four straight lines were drawn on the NIR-II image. In the control hindlimb, two horizontal lines were drawn medially and laterally to the femoral vessels (lines 1 and 2 in Figure S1), and two vertical lines were drawn near the proximal and distal ends of the femoral vessels (lines 3 and 4 in Figure S1). The two horizontal lines were drawn close to the femoral vessels without intersecting them in order to account for as many branch vessels as possible. In the ischemic hindlimb, the femoral artery was excised, but these lines were drawn surrounding the same region.

Then the NIR-II intensity on each line was extracted using the ImageJ software and plotted against the physical location on the line, where the peaks in the cross-sectional profile of each line were counted as existing vessels. Duplications in vessels counted by this approach were subtracted from total number of vessels of all four cross-sectional profiles. The total vessel count...
from the ischemic hindlimb was normalized against the control hindlimb to give relative vessel number (ischemic/control). An automated approach to quantify vessel density using FFT was also used to confirm the results (see SI).

**Vessel Structural Imaging Using μCT**

Vessel structural imaging using a μCT scanner (MicroCAT II, Siemens Preclinical Solutions) was performed according to our previous publication. After induction of hindlimb ischemia, the mice were injected with ion-based Fenestra VC (Advanced Research Technologies) contrast agent (0.3 ml per 20 g body weight) by tail vein injection. Fenestra was injected slowly into the tail vein of the mice over 30-60 s to prevent embolization. The μCT scans were acquired 30-45 minutes p.i. The animals were placed in prone position in the scanner, anesthetized with 2% isoflurane with a 1 L/min O₂ flow rate. Each scan time lasted 40 minutes (80 kVp, 500 μAmps, 576 views, 2 sec/view) per animal. After acquisition of CT images, three-dimensional reconstruction to a 40 μm isotopic voxel size was performed with Cobra (EXXIM Computing) and the data was analyzed using Amira 5.4 ( Visualization Sciences Group) for visualization of the vessels.

For vessel width analysis, a line was drawn on each μCT image, perpendicular to the vessels of interest, while avoiding the bone features. Then the contrast profile was analyzed in a manner similar to NIR-II images to obtain vessel widths.

**Immunofluorescence Quantification of Microvascular Density**

To validate the non-invasive measurements of vessels numbers using NIR-II, additional animal studies were performed in which the microvascular density in the ischemic hindlimbs were quantified by immunofluorescence analysis. At specified time points, the ischemic gastrocnemius tissues were excised for frozen sectioning and immunofluorescence staining for
endothelial marker, CD31 (BD Transduction), according to our previous studies (n>4 for each time point). At least three sections per animal were quantified. As a basis for comparison, gastrocnemius tissues from normal contralateral limbs were also immunofluorescently stained for CD31 (n=5). High-powered images were photographed for each section, and relative microvascular density was expressed as the average number of CD31-expressing vessels in the ischemic tissue, normalized by the average number of CD31-expressing vessels in normal limbs.

**Statistical Analysis**

Data shown are mean ± standard deviation. Statistical analysis was performed using Graphpad Prism software 5.0 (2007). For comparison of tissue perfusion and vascular density in the same animals over time, Repeated Measures Analysis of Variance (ANOVA) with a Tukey's adjustment was used. P-values less than 0.05 were considered statistically significant.

**Results**

**Recovery of Tissue Perfusion in the Ischemic Hindlimb**

Tissue perfusion was monitored on a group of athymic nude mice (n=6) up to 10 days after induction of hindlimb ischemia. NIR-II fluorescence-based dynamic perfusion imaging resembled laser Doppler-based perfusion measurement in revealing the steady recovery of perfusion in the ischemic hindlimb from day 3 to day 10 post-surgery (Figure 1), as indicated by the increase in intensity units in the ischemic hindlimb on day 10 (Figure 1B&D), compared to day 3 (Figure 1A&C). In comparing the images derived from NIR-II fluorescence microscopy and laser Doppler spectroscopy, NIR-II images provided much higher spatial resolution and discrimination of vascular structures that bear resemblance to angiograms.

To develop a quantitative method for tissue perfusion measurement based on the
fluorescence in the NIR-II, we analyzed the average NIR-II intensity in the control and ischemic hindlimbs as a function of time immediately after injection (Figure 2A-D), taking advantage of the fast dynamic recording capability of NIR-II fluorescence imaging (Videos 1&2). The rising edge of each plot was fitted into a linear function whose slope was related to blood velocity proportionally by an intensity-to-velocity conversion constant, as derived from our previous publication. Therefore, for internal comparison, the ratio between the slope derived from the ischemic hindlimb and the control hindlimb could be used as a measure of relative tissue perfusion, similar in concept to the mean perfusion ratio from laser Doppler-based and microbead-based perfusion measurements. The quantitative tissue perfusion values in Figure 2E exhibit a statistically significant increase in tissue perfusion in the ischemic hindlimb postoperatively from day 0 to day 10, as revealed by the NIR-II method (P<0.005). These results were further validated by laser Doppler blood spectroscopy (P<0.05) and microbead perfusion assay (P<0.05), in which a similar increase in blood perfusion could be quantitatively assessed over time.

Whereas restoration of blood flow over time could be documented by all three methods, we observed some differences in the magnitude in relative blood perfusion. The comparatively lower perfusion levels by NIR-II than by laser Doppler could be attributed to the difference of photon wavelength utilized for probing blood flow by these two techniques. Since laser Doppler applies red laser light with center wavelength of 650-690 nm, whereas NIR-II exploits photons in the range of 1100-1400 nm, NIR-II probes tissue perfusion within a much deeper region from the skin than laser Doppler and thus provides more volumetric perfusion assessment in thicker tissue than laser Doppler. In contrast, the microbead method is capable of measuring tissue perfusion at any depth underneath the skin by sacrificing the animal and resecting the tissue.
sample. Therefore, NIR-II is a surface-weighted technique in that the deeper tissues generally contribute a lower level of signal in the NIR-II, and that it does not adequately probe perfusion at depths > 3 mm due to severe scattering of photons. This agrees with the fact that the NIR-II tissue perfusion measurement lies between laser Doppler and the microbead method (Figure 2E). Our results show that NIR-II is a good compromise between laser Doppler and microbeads in that it can quantify blood flow at greater penetration depth than laser Doppler, while allowing serial imaging, which cannot be accomplished by microbeads.

**Structural Imaging of Angiogenesis with NIR-II and μCT**

Besides providing dynamic tissue perfusion imaging capability, NIR-II fluorescence imaging method could also be applied to quantify changes in vascular anatomy in the hindlimb region in response to ischemia over a 10-day period after surgery. Using image analysis approaches, we quantified the NIR-II intensity values that intersect the line perpendicular to the femoral vessels (Figure 3). The plots showing NIR-II intensity versus physical location were used to identify collateral vessels, represented by individual peaks. Based on the number of distinctive peaks, the NIR-II images of the mouse hindlimb vasculatures on post-operative days 3 and 7 (Figure 3B&F) revealed a large number of newly recruited collateral vessels in the ischemic hindlimb in response to the surgical excision of the femoral artery, which is further confirmed by internal comparison between the ischemic and control hindlimbs of multiple mice imaged at a higher magnification (Figure 4). In contrast, the vascular anatomy of the healthy control hindlimb remained unchanged over time, based on the similar numbers of individual peaks (Figure 3A&E). These NIR-II fluorescence images were compared with a standard vascular imaging method, μCT. Due to spatial resolution limitations (low contrast-to-noise ratio, high background signal from bone), μCT images (Figure 3C, D, G&H) showed fewer vessel-like structures in all images,
and the trend of vessel regeneration in the ischemic hindlimb between day 3 and day 7 was not as clearly revealed as in the NIR-II method.

**Quantitative Microvascular Density Analysis**

In addition to qualitatively demonstrating the increase of collateral vessel formation from day 3 to day 7 in the ischemic hindlimb (Figure 3&4), we further quantified microvascular density in the hindlimb from the NIR-II images by counting the vessels that intersect the surrounding borders of the femoral vessels and normalizing the vessel count in the ischemic hindlimb against the control hindlimb, to reveal the temporal kinetics of microvascular density (Figure S1; see Methods for more detail). The results shown in Figure 5A&B for both the short-term (10-day) and long-term (26-day) studies revealed a statistically significant temporal increase of the relative vessel numbers between days 7 and 12, reaching up to ~6-fold increase compared to immediately after the induction of hindlimb ischemia on day 0 (*P<0.005). This was followed by a return to normal relative vessel numbers by day 26. These results were confirmed by another automated approach to quantify vessel density (Figure S2). The long-term vascular density data is consistent with the histological quantification of microvascular density (Figure 5C&D), and reveals a significant recruitment of collateral vessels in response to ischemia during the first 8 days which exceeds normal values of vascular density. This phase is followed by a gradual return to normal levels of vascularity, possibly due to regression of collateral vessels or resolution of the acute inflammatory response triggered by ischemia.17-19

**Discussion**

The salient finding of this study is that NIR-II fluorescence imaging enables dynamic tissue perfusion measurement and high-resolution structural vascular imaging in an experimental model.
of PAD. This multifunctional approach combines traditional imaging modalities of laser Doppler, magnetic resonance angiography, and μCT in a single modality. Using this method, we are able to non-invasively track the recovery of limb perfusion after the induction of hindlimb ischemia by quantifying tissue perfusion and microvascular density simultaneously. This approach is an attractive alternative to performing invasive histological quantification of microvascular density and performing invasive microbead blood flowmetry for tissue perfusion measurement. NIR-II based-fluorescence imaging also revealed vascular branching and structural information that was superior to laser Doppler method, owing to a much higher spatial resolution (Figure 1) and μCT imaging owing to a much higher contrast-to-noise ratio (Figure 3&4).

NIR-II imaging revealed the temporal kinetics of collateral vessel growth. We noted that after the initial decline in vascular density induced by femoral-artery excision, there was a substantial increase in microvascular density up to 8 days after the surgery. This increase in vascular density was followed by the return to normal vascular density values by day 26. This pattern in vascular response is consistent with our own histological analysis of microvascular density as well as Hershey et al. who demonstrated enhanced vascular density in rabbit hindlimb at 5 days after induction of hindlimb ischemia, followed by the return to pre-operative levels by day 20.20 Hoefer et al. used post-mortem angiography to quantify collateral arteries in the ischemic hindlimb and reported a transient increase after 1 week, followed by the return to pre-operative levels by 3 weeks.18 It is likely that the transient enhancement in microvascular density may be associated with acute remodeling in response to ischemia, leading to the initial recruitment of collateral vessels, followed by a gradual decline due to their regression.

Dynamic tissue perfusion by MRI has been recently reported.21 Although MRI can assess tissue perfusion with deeper penetration depth into tissue, its temporal resolution is limited to ~2
s/frame, which is significantly lower than dynamic NIR-II imaging method (~0.18 s/frame). Magnetic resonance angiography has also been used in the setting of rabbit hindlimb ischemia, but its resolution was limited to the voxel size of ~0.5 mm, approximately 6 times larger than our NIR-II imaging method (78 μm).22 Based on the results from a direct comparison to μCT, as well as the reported resolution of MRI, NIR-II imaging provides superior spatial and temporal resolution.

NIR-II fluorescence compares favorably to traditional NIR-based fluorescence imaging with respect to the depth of penetration and degree of light scatter. As photons travel through a turbid medium such as the tissue and skin, the overall number of photons can be substantially attenuated due to scattering, while the scattering coefficient for different types of animal tissues scales with \( \sim \lambda^{-w} \) (\( w=0.22\text{-}1.68 \)) that decreases dramatically for emission with longer-wavelength \( \lambda \).23, 24 Therefore, the longer wavelength of fluorescence is used for imaging, the deeper penetration depth can be achieved with less loss of image fidelity. On the other hand, animal tissues generally have less autofluorescence at longer wavelengths than shorter ones. Traditional NIR-I fluorescence in the 750-900 nm region has been extensively used for vascular imaging. However, due to the immense scattering and lingering autofluorescence, the imaging depth of \textit{in vivo} NIR-I fluorescence imaging is usually ~0.5 mm.25, 26 In order to use short-wavelength NIR-I fluorescence to image features deep inside the body, usually the skin and surrounding tissue above the vessels of interest are removed to reduce scattering and improve the resolution of the vascular images.27-29 However, these invasive interventions during image acquisition increase the complexity of imaging and the morbidity of subjects. Therefore NIR-II imaging provides a solution to non-invasive through-tissue imaging of vasculatures at a depth of 1~3 mm (Figure S3) without the need of removing the surrounding tissues, while maintaining the high image fidelity.
Owing to the deep penetration depth of NIR-II photons, we envisage the use of NIR-II fluorescence in a CT scanner for fluorescence-mediated tomography, which might help resolve overlapping vessels in the z-stack that were not resolvable by a single projection in our current epifluorescence imaging setup.

NIR-II fluorophores may be more powerful than NIR-I fluorophores for quantification of tissue perfusion and vascularization, owing to higher spatial resolution even at deeper depths. Currently, indocyanine green (ICG) is one of the few NIR-based fluorescence dyes that is approved by the FDA for clinical applications in blood flow measurement.\(^3\)\(^0\) Choi \textit{et al.} reported the use of the ICG for calculation of perfusion rate in terms of \%/min for ischemic and control hindlimbs based on the dynamics of NIR-I fluorescence intensity.\(^3\)\(^1\) However, due to the high degree of scattering of photons in the NIR-I region, the reported perfusion analysis has limited spatial resolution and therefore does not provide crisp angiographic images at the same time, whereas NIR-II-based contrast dyes can provide perfusion analysis and high resolution angiography simultaneously (Figures 1\&2).

Although NIR-II fluorescence imaging method can provide dual functions of both vessel structural imaging and dynamic tissue perfusion imaging, there are limitations of this new vascular imaging technique that need to be considered. First, although NIR-II fluorescence in the long wavelength region of 1100-1400 nm can afford penetration depth of millimeters in a scattering medium,\(^2\)\(^,\)\(^6\) much deeper than the \(\sim0.5\) mm penetration depth of traditional fluorescence imaging methods,\(^2\)\(^,\)\(^5\) the penetration depth is only sufficient in small animal model experiments. The imaging depth for large experiment animal or humans would still be confined near the surface. Like in laser Doppler spectroscopy, hair interferes with NIR-II fluorescence imaging, but we have found in our experiments that simple depilation before imaging generates
images with comparable quality to athymic nude mice. Even though NIR-II imaging compares favorably to μCT in terms of higher spatial resolution and faster image acquisition time, the NIR-II fluorescence technique lacks the deep penetration depth for most human tomographic imaging where μCT and MRI are widely used. Second, the NIR-II contrast agent used in this study is SWNTs, nanoscopic materials that have a delayed clearance time, but such SWNTs have little toxicity due to their biocompatible surface coating. Efforts to produce a small molecule-based NIR-II contrast agent, comparable to other fluorescent dyes widely used in medicine such as fluorescein isothiocyanate (FITC), are under way. It is anticipated that such small molecule-based NIR-II contrast agents can make NIR-II imaging a clinically applicable imaging modality. Lastly, the use of InGaAs camera for NIR-II detection is limited compared to silicon based cameras that are commonly used to detect traditional short-wavelength photons. However, we believe that the many advantages of NIR-II fluorescence imaging in various biomedical applications could lead to more widespread use of infrared detectors and promote the development of new types of detection techniques.

**Conclusion**

In conclusion, we used a biocompatible carbon nanotube-based blood pool contrast agent with NIR-II fluorescence for dynamic tissue perfusion measurement and structural vascular imaging in murine model of PAD. NIR-II imaging is a novel technique providing simultaneous anatomic and hemodynamic information not achieved by conventional imaging modalities such as laser Doppler or μCT. The high temporal resolution of NIR-II allows us to dynamically track tissue perfusion. Moreover the high spatial resolution of NIR-II fluorescence imaging enables this platform to reveal vascular remodeling in the mouse hindlimb after acute ischemia.
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Disclosures

JPC, NFH, HD, GH and JCL are inventors on patents, owned by Stanford University, which are related to the imaging technology described in this manuscript.

References


Figure Legends

Figure 1. Corresponding NIR-II (A&B) and laser Doppler perfusion images (C&D) on day 3 (A&C) and day 10 (B&D) after surgery-induced hindlimb ischemia, where the arrow denotes the ischemic hindlimb. Note that the NIR-II images in A&B are extracted from Videos 1&2 that dynamically record the tissue perfusion labeled by NIR-II contrast, and that these two images both correspond to the time point of 20 s p.i.

Figure 2. Comparison of quantitative tissue perfusion after induction of hindlimb ischemia. (A-D) Normalized NIR-II fluorescence intensity in control and ischemic hindlimbs as a function of time p.i. on post-operative days 0, 3, 7 and 10. (E) A bar chart showing the relative tissue perfusion values (ischemic/control) on day 0, 3, 7 and 10 derived from laser Doppler technique (red bars), NIR-II fluorescence method (blue bars) and microbead measurements (green bars). Statistically significant differences of perfusion are found between days 0 and 10 for all methods (*P < 0.05 and **P < 0.005).

Figure 3. Comparison of NIR-II fluorescence (A, B, E, F) and μCT images (C, D, G, H) of mouse hindlimb vasculature (top) and cross-sectional vessel width measurements (bottom) on day 3 (A-D) and day 7 (E-H) after surgery-induced hindlimb ischemia.

Figure 4. High magnification NIR-II fluorescence images of mouse hindlimb vasculature of two different mice (one mouse is shown in A,B,E,F and the other in C,D,G,H) at day 7 after surgery-
induced hindlimb ischemia. Both medial (A-D) and lateral (E-H) aspects of control (A,C,E,G) and ischemic (B,D,F,H) hindlimbs are shown.

Figure 5. Quantification of collateral vessel formation in the ischemic limb during revascularization. NIR-II fluorescence based results of short-term (A) and long-term (B) revascularization studies are expressed relative to the vessel numbers in the control limb, and histology based results of a short-term study (C) are expressed in microvascular density. (D) Fluorescence microscopy images of ischemic hindlimb tissues resected on day 3 (left) and day 7 (right) after the induction of acute hindlimb ischemia with CD31 staining (green) and nucleus staining (blue). Statistically significant difference in comparison to day 0 is shown as *P < 0.05 and **P < 0.005.
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Supplemental Material for

Near-Infrared II Fluorescence for Imaging Hindlimb Vessel Regeneration with Dynamic Tissue Perfusion Measurement

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Supplemental Methods

Microvascular Density Quantification Based on Fast Fourier Transform (FFT)

We verified the cross-sectional peak counting method for microvascular density measurements using FFT analysis. FFT-based vessel density measurement works by applying a bandpass filter in the spatial frequency domain that selects a range of feature widths to convert an image with multiple vessels running in different directions into a quantitative measure of microvascular density. Using an image of the ischemic hindlimb (Figure S2A), we employed a bandpass filter (Figure S2B) for vessel widths between 78 μm and 468 μm. Note that most of the vessels seen in the image domain (Figure S2A) have widths in the range of 1-6 pixels with each pixel expanding 78 μm in space. Due to the limitation of pixel size of the camera under such a magnification, we selected 78 μm as the lower bound of the bandpass Fourier filter. On the other hand, the upper bound of 468 μm was chosen since this represented the size of the largest vessels observed in the thigh. Therefore, by applying a Fourier filter with a bandpass ranging from 78-468 μm, we selectively collected the signal only from collateral vessels as a measure of microvascular density, and suppressed all low-frequency background signals that did not contribute to the vascular density. Then the filtered frequency domain plot (Figure S2C) was integrated over the entire image, normalized against the integrated value corresponding to the control hindlimb, and used as a quantitative measure of microvascular density in any given image. In both short- and long-term studies, after an initial fall in vascular density immediately after the surgery, we observed a similar increase in microvascular density over the first 7 days after surgery, followed by a return to the normal levels (Figure S2D-E). This data is consistent with the results obtained from the line cross-sectional peak counting method (Figure 5).

Quantitative Assessment of the Sampling Depth of NIR-II Hindlimb Imaging

To find out the sampling depth of NIR-II fluorescence imaging in the hindlimb, a healthy nude mouse was injected with a 200 μL bolus of 0.10 mg/mL SWNTs (the same dose as other experiments in the paper) intravenously. The mouse was imaged at 90 min post injection (p.i.) of nanotubes in both hindlimbs to make sure the SWNTs were still circulating in blood vessels (see Fig. S3A&B), and then the mouse was sacrificed immediately after imaging was done. The time
of sacrifice after injection was shorter than the blood circulation half-life of SWNTs (~5 h)$^1$ and thus ensured most of the injected SWNTs were still inside blood vessels to light them up in the images.

After the mouse was sacrificed, the mouse body was frozen using liquid nitrogen and the left hindlimb was dissected for transection of the limb. The hindlimb was cut perpendicularly to the femur and the femoral vessels in the middle of the thigh and the cross-section of the cut was imaged in the NIR-II (see Fig. S3C-E). Based on the transection images, we measured the distance of the femoral vessels to the closest skin surface and found the depth of the femoral vessels as ~1.5 mm. The distance of the femur to the closest skin surface was found as ~2.9 mm. To confirm the depth measurement of the femoral vessels, we also dissected the other hindlimb (right hindlimb) of the same mouse injected with carbon nanotubes. Instead of cutting this hindlimb perpendicularly to the femur and femoral vessels, this time we cut the tissue parallel to the femur in thin, 0.5-mm thick slices and imaged the hindlimb in the NIR-II after removing every slice (Fig. S3F). We found that the femoral vessels disappeared after the fourth 0.5-mm thick slice was removed, suggesting the femoral vessels were located between 1.5 mm (3 slices of 0.5-mm thickness) and 2 mm (4 slices of 0.5-mm thickness). This result is consistent with the depth found in the transverse cutting experiment (1.5 mm).
Supplemental Figures and Figure Legends

Figure S1. NIR-II fluorescence-based microvascular quantification approach. Four lines surrounding the boundary of the femoral artery in the control limb were drawn. The vessels that intersect those 4 lines were represented as distinct peaks, which could be counted.
Figure S2. Microvascular density analysis of ischemic mouse hindlimb over time based on the FFT method. (A) A representative image showing high degree of vascular regeneration in the mouse ischemic hindlimb at post-operative day 7. (B) A Fourier bandpass filter in the spatial frequency domain. The filter maintains features in the range of 1-6 pixel widths by suppressing the low spatial frequency signal close to origin of spatial frequency domain (the black hole in the center). (C) The frequency domain plot of A after passing the Fourier bandpass filter shown in B. Note the black disk in the center of the filtered frequency domain plot is due to the Fourier bandpass filter in B. (D) Relative microvascular density change measured by FFT for a short-term, 10-day study. (E) Relative microvascular density change measured by FFT for a long-term, 26-day study.
Figure S3. Determination of penetration depth of NIR-II imaging. (A&B) In vivo NIR-II fluorescence images of right (A) and left (B) hindlimbs of a nude mouse injected with carbon nanotubes. Images were taken at 90 min post injection, immediately before the mouse was sacrificed for dissection and ex vivo tissue imaging. (C) A white-light photo showing the transverse cross-section of the left hindlimb shown in B. (D&E) NIR-II fluorescence images of the transverse cross-sectional view of the left hindlimb shown in C. The femoral vessels and femur can be seen owing to labeling of carbon nanotubes (D) and their distances to the closest skin surface (which is the distance along the direction of in vivo hindlimb vessel imaging) were measured in E. (F) NIR-II fluorescence images of the right hindlimb shown in A, with the
femoral vessels positioned parallel to the detector. These images were taken after removing 0.5-mm slices from above the femoral vessels incrementally. The depth of the femoral vessels was determined by finding after how many slices of 0.5-mm thickness the femoral vessel features disappeared.
Supplemental Video Legends

Video 1. NIR-II video-rate imaging on day 3 after induction of acute hindlimb ischemia

NIR-II video-rate imaging showing blood flow in a healthy hind limb (left to the viewer) and an ischemic hindlimb (right to the viewer) of a mouse on day 3 post surgery. Total video time: 65.6 s, frame rate: 5.3 frames/s.

Video 2. NIR-II video-rate imaging on day 10 after induction of acute hindlimb ischemia

NIR-II video-rate imaging showing blood flow in a healthy hind limb (left to the viewer) and an ischemic hindlimb (right to the viewer) of a mouse on day 10 post surgery. Total video time: 65.6 s, frame rate: 5.3 frames/s.


Supplemental References