NANOTECHNOLOGY ENABLED BIOMEDICAL FLUORESCENCE IMAGING
IN THE SECOND NEAR-INFRARED WINDOW

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DOCTOR OF PHILOSOPHY

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Abstract

Fluorescence imaging in the second near-infrared window (NIR-II, 1.0-1.7 μm) has many salient advantages over the visible (400-750 nm) and the traditional near-infrared (NIR-I, 750-900 nm) windows owing to the reduced photon scattering and negligible tissue autofluorescence. However, NIR-II fluorescence imaging has been limited by the scarcity of materials with sufficient NIR-II fluorescence quantum efficiency, and single-walled carbon nanotube (SWNT) had been the only fluorophore for biological imaging in the NIR-II window. This work aims to enhance the intrinsic NIR-II fluorescence of SWNTs, apply SWNTs for \textit{in vivo} imaging of real-world medical problems in animal models and develop new NIR-II fluorophores other than SWNTs. First, a plasmonic gold substrate is used to enhance the intrinsically low NIR-II fluorescence of SWNTs and to improve the sensitivity of cancer cell imaging using SWNTs as molecular targeting probes. The sensitive distance dependence of fluorescence enhancement of SWNTs is then exploited to probe the trans-membrane motion of single nanotube molecules and reveal the internalization pathway as receptor-mediated endocytosis. The biocompatible SWNTs are further applied to an \textit{in vivo} animal model of lower limb ischemia, where we demonstrate microvascular imaging and hemodynamic measurement using NIR-II fluorescence, with improved spatial resolution over X-ray computer tomography (CT) and broader dynamic range of blood flowmetry than ultrasound. In a rationally chosen sub-region of NIR-II in the 1.3-1.4 μm range, chemically separated SWNTs allow for non-invasive brain vascular imaging through intact scalp and skull with sub-10 μm resolution at millimeter depth of penetration. Lastly, two new materials, Ag_2S quantum dots (QDs) and conjugated
copolymers are developed to expand the toolbox of NIR-II fluorophores. The Ag$_2$S QDs afford *in vitro* targeted cancer cell imaging and *in vivo* mouse imaging with high tumor uptake. The high fluorescence quantum yield of the conjugated copolymer allows for ultrafast dynamic NIR-II imaging of the arterial blood flow with waveform cardiac cycles revealed in hemodynamic analysis. The many benefits of NIR-II fluorescence imaging demonstrated in this work based on the development of a handful of biocompatible NIR-II nanomaterials bode well for future biological research and clinical applications with this new imaging technique.
I would like to first thank Professor Hongjie Dai for his invaluable advice and guidance throughout my PhD study and research. I also need to express my thanks to Dr. Kevin Welsher for training on optics and programming, and to Dr. Scott Tabakman, Dr. Sarah Sherlock, Dr. Joshua Robinson and Dr. Zhuo Chen for various training on animal handling and bioconjugation.

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# Nanotechnology Enabled Biomedical Fluorescence Imaging in the Second Near-Infrared Window

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Chapter 1. Introduction

1.1 Imaging in Biology and Medicine

To see is to believe. For human and any other living creature on this planet, ‘seeing’ is the most important and straightforward way, if not the only way, to perceive the environment and thus interact with the world. In science, the objects of ‘seeing’ vary vastly in size and visibility. For scientists, ‘seeing’ means a more interrogative process equipped with sophisticated tools to probe hidden information from our naked eye, which we call ‘imaging’. To biological scientists and medical doctors, imaging reveals both structural and functional information from subcellular organelles to interconnected organs on a whole body level, which empowers the user to uncover the molecular mechanism of biological systems and to diagnose various diseases with sufficient sensitivity and accuracy.\textsuperscript{1,2}

All current imaging techniques involve the use of some types of the electromagnetic radiation to probe the structures and functions of the object. Based on the type of electromagnetic wave being used and the method of image formation, most of today’s biological imaging techniques and medical imaging modalities can be divided into three major categories: tomographic, coherent and optical (Fig. 1.1). Tomographic imaging modalities, such as X-ray computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and single-photon emission computed tomography (SPECT), have enjoyed their widespread use in the clinic since the 1970s.\textsuperscript{3-6} These imaging techniques exploit X-ray, radioactive isotopes, or radiowaves in a magnetic field to achieve very deep penetration through
the human body. However, major limitations of the tomographic imaging modalities include sub-optical imaging resolution (sub-millimeter spatial resolution), long scanning and post-processing time, and health concerns due to the exposure to radiation and magnetic fields. Coherent imaging methods, such as ultrasonography, optical coherence tomography (OCT) and photoacoustic imaging, visualize the structural difference within biological tissues by detecting the phase shift of the returning acoustic or optical waves reflected from the object. The Doppler functionality also adds the fast dynamic measurement capability to ultrasound and OCT with very high temporal resolution (up to kHz). However, the intrinsically long wavelengths of acoustic waves (0.1 mm ~ 10 m) and the inherent speckle artifacts of all coherent imaging techniques limit their spatial resolution. Moreover, coherent imaging predominantly offers anatomical imaging rather than molecular imaging due to the relatively large size of the contrast agents needed to apply as molecular reporters.

Compared to the aforementioned two categories of imaging modalities, optical imaging has the benefits of high spatial resolution and fast acquisition rate. Being an important component of optical imaging, fluorescence imaging has the unique capabilities of resolving different structures with higher sensitivity in multiple color channels and specific targeted imaging of certain molecules of interests. Fluorescence imaging relies on the emission of a photon with a different wavelength from the excitation photon, which is absorbed by a light-sensitive molecule called the fluorophore. A large number of fluorescence-based optical imaging techniques have been developed for imaging in vitro and in vivo samples, including widefield
fluorescence microscopy, confocal fluorescence microscopy, two-photon and multi-photon fluorescence microscopy (TPFM and MPFM), super-resolution fluorescence microscopy, and fluorescence-mediated tomography (FMT). Although fluorescence imaging techniques have shown to achieve diffraction-limited spatial resolution (<1 μm) and even sub-diffraction imaging resolution (~10 nm for super-resolution microscopy), one of the major limitations remains the very superficial penetration depth of imaging, which dictates that traditional fluorescence imaging technique is most useful for histological assessment of ex vivo thin tissue slices or imaging in vitro cell cultures.

1.2 The Challenges of Deep Tissue Fluorescence Imaging

The penetration depth of fluorescence-based optical imaging in an in vivo setting for live animals and in an ex vivo setting for a thick slab of tissue is usually limited by the intrinsic optical properties of the turbid biological tissue. There are usually three major factors that contribute to the poor contrast-to-noise ratio (CNR) of fluorescence imaging at increased depths: the scattering of emitted fluorescence photons when traveling inside the tissue, the absorption of emitted fluorescence photons by the tissue, and the autofluorescence photons emitted from the tissue itself when being excited. Each one of these three contributing factors will be discussed in detail as follows.
The scattering of photons inside the biological tissue is an elastic process of photon absorption of a scattering particle, followed by re-emission of another photon without any loss of energy. Although energy is conserved in the scattering process, this re-emitted photon is usually associated with a change of direction, which causes the loss of spatial information that is crucial to image formation. Consequently, the energy of scattered photons moves from the contrast to the noise, resulting in a decreased CNR. Scattering of photons is usually modeled with the random-walk theory, despite that fact that in biological tissues the change of photon direction after each scattering event is not completely randomized, but has higher probability in the forward direction (that is, with little change of direction). The scattering coefficient ($\mu_s$) of a turbid medium is the reciprocal of the mean free path ($l_s$) of a traveling photon, but due to the forward scattering property, such a photon would take multiple scattering events (or travel multiple mean free paths) before it becomes random-walk-like and completely ‘forgets’ its original forward direction. Therefore, the reduced scattering coefficient ($\mu'_s$) is considered a better measure of photon scattering in biological tissue and is defined as: $\mu'_s = \mu_s(1 - g)$, where $g$ denotes the degree of the forward scattering and is usually in the range of 0.8-1. One can also see that the effective mean free path ($l'_s$) can be expressed as $l'_s = l_s/(1 - g)$, where the higher the degree of forward scattering (higher $g$), the longer distance the photon can travel before it becomes random-walk-like (longer $l'_s$).

For biological tissues, the reduced scattering coefficient has an inversely proportional relationship with wavelength as $\mu'_s \propto \lambda^{-w}$ while the inverse exponent $w$ is
dependent on the size and concentration of scattering particles in the tissue and ranges from 0.22 to 4 for different tissue types. Here the reduced scattering coefficients for typical biological tissues related to this work are given as follows as functions of wavelength and also plotted in Fig. 1.2, where one can see that scattering decreases at longer wavelengths for all tissue types: 

\[
\mu'_s (\text{skin})/\text{mm}^{-1} = 0.11(\lambda/\mu\text{m})^{-4} + 1.61(\lambda/\mu\text{m})^{-0.22} \quad \text{(where the two terms are attributed to Rayleigh scattering and Mie scattering, respectively)}
\]

\[
\mu'_s (\text{muscle})/\text{mm}^{-1} = 0.56(\lambda/\mu\text{m})^{-1.045}
\]

\[
\mu'_s (\text{skull})/\text{mm}^{-1} = 1.72(\lambda/\mu\text{m})^{-0.65}
\]

\[
\mu'_s (\text{brain tissue})/\text{mm}^{-1} = 4.72(\lambda/\mu\text{m})^{-2.07}
\]

Compared to the monotonically decaying trend of photon scattering inside different biological tissues versus wavelength, the absorption of photons is a combined effect of many chromophores existing in the tissue and is thus more complicated. First of all, water makes up 60% of the total body weight of mammals and any absorption by water can be significant in the context of imaging. Fig. 1.3 shows the major absorption peaks of water molecules in the 1400-1500 nm and >1700 nm regions, which should be avoided when performing fluorescence imaging in vivo. Second, there are certain biomolecule chromophores that absorb strongly in the visible window, with oxygenated and deoxygenated hemoglobin contributing the most in the 300-650 nm region (Fig. 1.4). Since the absorbance of most tissues is dominated by water and hemoglobins, we can identify two optically transparent windows suitable for in vivo
fluorescence imaging, the 650-1400 nm range, and the 1500-1700 nm range, when only photon absorption is considered.

While scattering and absorption reduce CNR by reducing the contrast signal, autofluorescence reduces CNR by increasing the noise level. When photons are absorbed by certain chromophores in the tissue, there is a chance for the light absorbers to re-emit fluorescence, which is termed ‘autofluorescence’ and interferes with the ‘true signal’ from the fluorescent reporters. It has been reported that the tissue autofluorescence, which is most prominent in major internal organs and bodily fluids, also depends on wavelength and decreases as wavelength increases.\textsuperscript{32} Chlorophyll from external food intake,\textsuperscript{39} as well as the endogenous NAD(P)H, flavins and collagens\textsuperscript{40,41} are known as the strongly autofluorescent biomolecules in the 300-750 nm wavelength region.

Due to scattering and absorption of emitted photons as well as the interference from autofluorescent photons, it remains highly challenging for traditional fluorescence imaging to reach penetration depths > 150 μm.\textsuperscript{42} Therefore novel fluorescence imaging techniques to overcome this problem are highly desired.

1.3 Current Deep Tissue Fluorescence Imaging Techniques

To overcome the penetration problem of traditional fluorescence imaging, various imaging methods have been developed in the past decade, by exciting the fluorophores in different ways [such as TPFM, MPFM and selective plane
illumination microscopy (SPIM)] and detecting fluorescence in different regions [such as near-infrared (NIR) fluorescence microscopy].

Unlike traditional one-photon confocal microscopy, TPFM and MPFM resolve the axial focal plane at increased depth owing to the highly localized, non-linear excitation. The probability of exciting non-linear fluorescence scales to the second and third power with the photon flux, ensuring that only the fluorophores at the focus (the beam waist) of the excitation beam can be excited and leaving all out-of-focus planes unexcited. Moreover, in a scattering medium, the scattered photons from the excitation beam deviate from the original path and become unable to excite any fluorescence due to reduced photon flux, partly eliminating the scattering-induced image blur. Furthermore, the anti-Stokes shift of non-linear fluorescence is different from the Stokes autofluorescence and reduces interference from the endogenous fluorophores in the tissue.43 State-of-the-art TPFM and MPFM have achieved penetration depths of >1 mm for living brain imaging.26,27,44,45 However, the photon absorption issue remains unsolved and becomes even more problematic for non-linear microscopy due to significant loss of excitation power in the non-focused volume, and as a result a much higher power density of pulsed laser is needed for TPFM and MPFM.43,46

In traditional fluorescence microscopy, both excitation and emission (detection) are aligned coaxially along the depth axis. In contrast, a thin sheet of light is utilized in SPIM to excite a single plane inside the sample at a time, forming an orthogonal geometry between the excitation and detection.47 This unique design of microscopy allows one to illuminate and image a selected thin slice of a thick sample, where the
photon scattering is reduced compared to traditional confocal microscope where the entire thick sample is excited. However, SPIM is still susceptible to photon scattering and absorption, which limit the penetration depth to ~500 μm.\(^{30}\)

NIR fluorescence imaging detects emitted photons in a longer wavelength, NIR region (750-900 nm) than its counterpart in the visible region (400-750 nm). The aforementioned discussion on photon scattering, absorption and autofluorescence dictates that the NIR region has lower scattering (due to the inversely proportional relationship of scattering versus wavelength), less absorption (away from the absorption bands of hemoglobin) and reduced autofluorescence. With NIR fluorescence, it has been reported that much deeper penetration depths of up to 800 μm can be achieved \textit{in vivo} with confocal fluorescence microscope.\(^{48,49}\) However, with the lingering scattering and autofluorescence in the NIR region, \textit{in vivo} high resolution fluorescence imaging at depths of >1 mm still remains highly challenging.\(^{31,32}\)

1.4 Fluorescence Imaging in the Second Near-Infrared Window

The scattering of photons in turbid medium and the autofluorescence of biological tissue both scale inversely proportional to wavelength, as discussed in Section 1.2. The arguments on the absorption of photons in tissues and bodily fluids determine the boundaries of the optically transparent windows (650-1400 nm and 1500-1700 nm) suitable for fluorescence imaging. Taken together, a new window ranging from 1000 nm to 1700 nm should give even deeper penetration depth with
crisper image resolution than the traditional NIR region (750-900 nm). To draw a distinction between this two NIR windows, we call this longer NIR window as the second near-infrared window (NIR-II window) while the traditional NIR window is termed the first near-infrared window (NIR-I window).

Fluorescence photons in the NIR-II window have significantly reduced scattering and autofluorescence interference compared to NIR-I, while the tissue absorption is comparable for both NIR-I and NIR-II windows. Owing to these benefits, our lab and others have shown that NIR-II in vivo fluorescence imaging can generate crisp images at an increased penetration depth.

Single-walled carbon nanotubes (SWNTs) are the first NIR-II fluorophore applied for in vivo NIR-II fluorescence imaging, owing to their intrinsic photoluminescence in the NIR-II window upon excitation in the visible and NIR-I windows. Due to the quantum confinement along the transverse direction of a single carbon nanotube, which can be considered as a quasi-one-dimensional nanomaterial, SWNTs feature very sharp maxima of electronic density of states called van Hove singularities in their energy band diagrams. Absorption of photons typically occur across the bandgap of the second van Hove singularities (E22) with energy in the visible and NIR-I windows, followed by fluorescence emission across the bandgap of the first van Hove singularities (E11), which is usually in the NIR-II window.

The energies of van Hove maxima of SWNTs in a band diagram are mainly dependent on the diameter of the nanotube and the chiral angle at which the specific
nanotube is rolled up from a single graphene sheet (Fig. 1.5). Rolling up the graphene sheets with different chiral angles results in nanotubes with different chiralities, which are determined by two indices \( m \) and \( n \) that define the roll-up vector as follows:

\[
\text{Roll-up vector } (n,m) = n\overline{a_1} + m\overline{a_2}
\]

where the length of the roll-up vector is the circumference of the nanotube. Based on simple geometry, one can derive the diameter of a nanotube based on its chirality \((n,m)\):

\[
d = \frac{\sqrt{3}d_{c-c}}{\pi} \cdot \sqrt{n^2 + nm + m^2}
\]

Different chiralities also result in semiconducting \([(n - m) \mod 3 = 0]\) and metallic SWNTs \([(n - m) \mod 3 = 1, 2]\) while only semiconducting SWNTs have photoluminescence upon photon absorption. This is because for metallic SWNTs the density of states is not zero at the Fermi level, so that the excited state after absorbing a photon can relax non-radiatively to the valence band (Fig. 1.6a). From the color coding in Fig. 1.5 it can be seen that 2/3 of all SWNT chiralities are semiconducting and thus fluorescent.

Due to the many semiconducting chiralities in the raw SWNT sample with each chirality possessing unique E11 and E22 bandgap energies (Fig. 1.6b), the photoluminescence-versus-excitation plot (PLE plot) typically features many discrete peaks, where each peak corresponds to different excitation and emission wavelengths (Fig. 1.7). SWNTs coming from different synthetic sources have different ranges of
diameter distribution, resulting in different ranges of photoluminescence wavelengths of 900-1400 nm for high-pressure CO (HiPco) decomposed SWNTs (Fig. 1.7a), 1500-1700 nm for laser ablation SWNTs (Fig. 1.7b) and 1600-1900 nm for arc discharge SWNTs (Fig. 1.7c).

The photoluminescence of semiconducting SWNTs is excitonic in nature with a bound state of an electron and an electron hole after excitation, with an exciton binding energy between 300 and 400 meV. Due to the nonradiative nature of the lowest-energy excitons, as well as many external quenchers that could increase the non-radiative recombination rate of excitons, the fluorescence quantum efficiency of SWNTs in the NIR-II window is usually very low (~0.1%), significantly limiting their applications for deep tissue biological imaging with high resolution and fast acquisition rate. To address the issue of sub-optimal fluorescence quantum efficiency of SWNTs, and to expand the limited choices of biocompatible NIR-II fluorophores, the aim of my work is to enhance the NIR-II fluorescence brightness through the interaction with plasmonic nanomaterials, and to develop other NIR-II fluorophores suitable for a variety of medical applications. First, I will describe the metal enhanced NIR-II fluorescence of SWNTs, discuss the physiochemical mechanism behind this phenomenon, and present two examples of applying this interesting phenomenon for in vitro cell imaging. Second, I will place the NIR-II fluorescence property of SWNTs in the context of two major cardiovascular diseases, lower limb ischemia and cerebral arterial occlusion, and discuss the benefits of using SWNTs for non-invasive NIR-II imaging of the hind limb and cerebral vasculature structures at unprecedented depths. I have led these highly collaborative projects and made my unique
contributions by designing and building new optical setups, preparing biocompatible SWNT fluorophores, performing imaging and post-acquisition data analysis. Third, I will describe the development of two other NIR-II fluorophores, Ag$_2$S quantum dots and conjugated copolymers, for enhanced tumor imaging and ultrafast blood flow imaging.\textsuperscript{74-76} In these projects, I have contributed uniquely by performing surface functionalization and bioconjugation to make these materials water-soluble and biocompatible, as well as conducting imaging experiments and analyzing the data. Finally, I will discuss in the Appendix the assembly and operating procedures for a few NIR-II imaging setups I have built in our lab, including an NIR-II whole body imager with tunable magnification, a visible and NIR-I whole body imager with tunable magnification, a prototype NIR-II confocal microscope and a hyperspectral microscopic imaging setup.
Figure 1.1 | Current Biomedical Imaging Modalities. This triangle diagram shows the strengths and limitations of tomographic, coherent and optical imaging techniques in measures of spatial resolution, temporal resolution and penetration.
Figure 1.2 | Wavelength Dependent Photon Scattering in Tissues. The reduced scattering coefficient, $\mu'_s$, is derived from literature$^{27,34-36}$ and plotted as a function of wavelength for four different types of tissues: skin (black), muscle (red), skull (blue) and brain tissue (green).
Figure 1.3 | Absorbance of Water. The UV-Vis-NIR absorption spectrum of water is shown in this plot for a total path length of 1 mm in a quartz cuvette.
Figure 1.4 | Absorbance of Hemoglobins. The extinction coefficients of oxygenated (red) and deoxygenated (blue) hemoglobins are derived from data by courtesy of the Oregon Medical Laser Center and replotted by the author.
Figure 1.5 | Chirality of Single-Walled Carbon Nanotubes. This diagram shows conceptually how a single-walled carbon nanotube is ‘rolled up’ from a single graphene sheet through the roll-up vector (red arrow), which is a linear combination of the two base vectors ($\vec{a}_1$ and $\vec{a}_2$, black arrows) and its two scalar coefficients determine the chirality $(n, m)$ of the nanotube. Different chiralities are shown in the indexed hexagonal lattice with blue hexagons denoting semiconducting chiralities and red hexagons denoting metallic chiralities. The blank lattice has the same chiralities forming a mirror image of the labeled lattice about the armchair line.
Figure 1.6 | Band Diagrams of SWNTs. (a) A band diagram of a metallic SWNT, showing no fluorescence emission after the E22 absorption due to the lack of a band gap. (b) A band diagram of a semiconducting SWNT, showing fluorescence emission across the band gap of E11 after the E22 absorption.
Figure 1.7 | Photoluminescence versus Excitation (PLE) Maps of SWNTs. (a) PLE map of HiPco SWNTs with fluorescence emission in the 1000-1400 nm window. (b) PLE map of the laser ablation SWNTs with fluorescence emission in the 1500-1700 nm window. (c) PLE map of the arc discharge SWNTs with fluorescence emission in the 1500-1700 nm window.
nm window. (c) PLE map of the arc discharge SWNTs with fluorescence emission in the 1600-1900 nm window.
1.6 References


* The work in this chapter was published in:


2.1 Introduction

As discussed in Chapter 1, semiconducting single-walled carbon nanotubes (SWNTs) are promising fluorophores with characteristic bandgap photoluminescence in the second near-infrared window (NIR-II window, 1000-1700 nm) when being excited in the visible (400-750 nm) and traditional near-infrared window (NIR-I window, 750-900 nm). The intrinsic NIR-II fluorescence makes SWNTs good candidates for biomedical imaging in both *in vitro* and *in vivo* settings.\(^1\)\(^-\)\(^3\) However, compared to visible and NIR-I fluorophores such as organic synthetic dyes, fluorescent proteins (FPs) and quantum dots (QDs), the fluorescence quantum efficiency of SWNTs is usually very low, on the order of \(10^{-3}\).\(^4\) The low fluorescence quantum yield is a result of endogenous factors including the lowest-energy excitons that are optically forbidden,\(^5\) as well as a handful of exogenous factors such as metallic nanotube species forming bundles with semiconducting ones,\(^6\) conductive and semiconductive substrates in contact with nanotubes,\(^7\) external electric field,\(^8\) and single-molecule reactive species such as acid, base, oxygen, and diazonium compounds.\(^9\)-\(^11\) Therefore there is an urgent need to increase the NIR-II fluorescence emission, and previous efforts have been made to improve the fluorescence quantum yield through various chemical and physical means.\(^3\),\(^10\),\(^12\),\(^13\)
Although metal is usually considered as a quencher of the fluorescence of SWNTs, many organic fluorophores and QDs have been reported to exhibit metal-enhanced fluorescence (MEF) when they are placed in proximity to the surface of gold and silver nanoparticles and colloidal films.\textsuperscript{14-16} However, MEF of SWNTs has never been observed or reported till the publication of this work. It has also been reported that the distance between the fluorophores and the metal surface plays an important role in quenching or enhancing the fluorescence of organic dyes and quantum dots.\textsuperscript{17-19} Therefore, by carefully engineering the nanostructure of metal colloidal film and tuning the distance between the SWNTs and the metal coated film, it is possible to observe the MEF of the NIR-II fluorescence of SWNTs and use it for biological imaging and sensing applications.

\subsection*{2.2 Materials and Methods}

\textbf{Solution-phase synthesis of gold-on-gold (Au/Au) films on quartz.} Quartz slides were first submerged in a 3 mM chloroauric acid solution, and then under vigorous agitation, a concentrated solution of ammonium hydroxide was added to the chloroauric acid solution to reach 0.6 wt\%. With the quartz slides still immersed in such seeding solution, the container was gently shaken for 1 min, followed by a thorough wash of the quartz slides with deionized ultra-filtered (DIUF) water. Then the quartz slides were immersed into a 1 mM sodium borohydride solution with the container placed on an orbital shaker for 5 min at 100 rpm to form Au seeds onto the substrates. After a second wash step for the slides, the seeded substrates were
submerged in growth solutions consisting of 1:1 chloroauric acid and hydroxylamine at four different molar concentrations (250 μM for Au/Au-I, 500 μM for Au/Au-II, 1250 μM for Au/Au-III and 3000 μM for Au/Au-IV) under vigorous shaking. Then the growth solution containing the substrate was transferred to an orbital shaker at 100 rpm and allowed to stay on the shaker for 15 min. The Au/Au substrates were finalized by rinsing with DIUF water, and blow-dried with house air.

**UV-Vis-NIR absorbance measurement of Au/Au films.** UV-Vis-NIR absorbance spectra of the Au/Au films on quartz substrates were measured in the range of 350-1500 nm by a Cary 6000i UV-Vis-NIR spectrophotometer, background-subtracted for any absorbance contribution from the quartz substrate.

**Scanning electron microscopy (SEM) imaging of Au/Au films.** SEM images of the Au/Au films on quartz substrates were acquired on an FEI XL30 Sirion SEM with FEG source at 5 kV acceleration voltage. False colors were added to the original images to enhance visual appeal using the MATLAB software.

**Dielectric coating on the Au/Au films.** We coated the surface of the Au/Au films with two different types of dielectric layers, alkanethiol self-assembled monolayers (SAMs) and aluminum oxide (Al₂O₃). Three different alkanethiol SAMs were coated on the Au/Au films using the cysteamine, 1-propanethiol and 1-octadecanethiol molecules, respectively. The quartz substrates coated with Au/Au films were soaked in an ethanol solution of cysteamine, 1-propanethiol or 1-octadecanethiol, all at a molar concentration of 1 mM, on an orbital shaker for 1 h, and then rinsed thoroughly with ethanol and blow-dried with house air.
Low temperature atomic layer deposition (ALD) was used to coat a thin layer of dielectric material, Al$_2$O$_3$, with desired thickness on the Au/Au films. To render the Au/Au film amenable to further coating of Al$_2$O$_3$, an SAM of cysteamine was formed on the surface first according to the aforementioned procedure. The deposition of Al$_2$O$_3$ was carried out using trimethylaluminum (TMA) and water vapor as precursors and pure nitrogen as the carrier gas at a pressure of ~300 mTorr and a temperature of 100 °C. Each cycle of ALD was divided as follows: the water vapor pulse lasted 0.5 s in duration, followed by a purge time of 40 s; and then the TMA pulse lasted 0.5 s, followed by a purge time of 15 s. Each cycle coated a thin layer of Al$_2$O$_3$ with a thickness of 0.1 nm, and the number of total cycles was determined by the desired thickness of the Al$_2$O$_3$ dielectric layer on the Au/Au film.

**Preparation of water soluble SWNT fluorophores.** Raw HiPco SWNTs (Unidym) were bath sonicated in an aqueous solution containing 1 wt% sodium cholate for 1 h, and ultracentrifuged at 300,000 g for 1 h to remove bundles and other large aggregates of SWNTs in the suspension. After ultracentrifugation, only the supernatant was retained, concentrated through a 30k NMWL centrifugal filter unit (Fisher), layered to the top of a 5%/10%/15%/20%/60% iodixanol step gradient, and ultracentrifuged at 300,000 g for 1 h. Only the top 1 mL of the gradient was collected by careful fractionation and the PEGylated surfactant DSPE-mPEG(5000) (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)5000], Laysan Bio) was added to the solution to reach a surfactant concentration of 1 mg/mL. Then the suspension was bath sonicated for 5 min, dialyzed against DIUF water in a 3500MCWO dialysis membrane (Fisher) to remove all small molecules such as...
sodium cholate and iodixanol, and ultracentrifuged again at 300,000 g for 1 h to remove bundles and other aggregates formed during dialysis. The resulting suspension of surfactant-coated SWNTs can be concentrated further through the centrifugal filter unit without causing instability of the solution.

**Deposition of SWNTs on the substrates from aqueous suspension.** To image single nanotubes with NIR-II fluorescence in a widefield microscope setup, the surfactant-coated SWNT suspension as described in the preceding paragraph was mixed with polyvinylpyrrolidone (PVP) and spin-coated on different substrates using a KW-4A Chemat Technology spin-coater at 1,500 rpm. To study the distance dependent MEF of SWNTs, the surfactant-coated SWNT suspension was mixed with Triton X-100 (TX-100) to reach 0.5 wt% to improve the wetting of the solution on the substrates and 0.1 μL of this solution was drop-dried on the substrates to form a spot with the diameter of 1.5-2 mm. For photoluminescence versus excitation (PLE) measurements, 1 μL of the SWNT solution containing 0.5 wt% TX-100 was drop-dried onto the substrates to increase the signal-to-noise ratio (SNR).

**Low magnification NIR-II fluorescence microscopy imaging.** The drop-dried spots of SWNTs on Au/Au films with different coating layers were imaged under a low magnification (10×) in the epifluorescence widefield mode using a 658-nm laser diode (100 mW, Thorlabs) as the excitation source. The 658-nm laser was cleaned using a 750 nm short-pass filter (Omega) and focused to a 750 μm diameter spot on the sample through a 10× objective lens (Bausch & Lomb). The NIR-II fluorescence from the sample was allowed to pass through an 1100 nm long-pass filter and focused onto a two-dimensional InGaAs camera (Princeton Instruments 2D OMA-V) through a
200-mm tube lens. The InGaAs camera has sufficient sensitivity in the range of 800-1700 nm, beyond which the responsivity profile drops dramatically. Since the field of view under the 10× objective was ~800 µm, significantly smaller than the size of each drop-dried spot on the substrate, widefield NIR-II fluorescence snapshots were captured at different places to cover the entire spot, flatfield corrected, and then stitched manually to reconstruct the whole spot.

**High-magnification NIR-II fluorescence microscopy imaging and spectroscopy.** Single nanotube imaging was done in a high magnification, widefield microscope setup using the same 658-nm laser diode (100 mW, Thorlabs) as the excitation source. The 658-nm laser was cleaned through a 750 nm short-pass filter and focused to a 75 µm diameter spot on the sample by a 100× objective lens (Nikon). The emitted NIR-II photoluminescence from the sample was allowed to pass through a 910 nm long-pass filter and focused onto the two-dimensional InGaAs camera, with an exposure time of 300 ms.

For *in situ* fluorescence spectroscopy of single nanotubes, the same setup was used except that the 658-nm laser was focused to a much smaller, 5 µm diameter spot. Then the photoluminescence from this much smaller area of illumination was allowed to pass through a 910-nm long-pass filter and directed into a spectrometer (Acton SP2300i) equipped with a one-dimensional InGaAs linear array detector (Princeton OMA-V). The emission spectra were corrected post-collection to account for the extinction profile of the filter as well as the responsivity difference of the detector at different wavelengths.
**PLE spectroscopy of drop-dried SWNTs on different substrates.** The PLE spectra of drop-dried SWNTs on different substrates were taken on a home-built NIR-II spectroscopy setup. The excitation was provided by a white-light source of an ozone-free Xenon lamp (Oriel) with a total power of 150 W, which was filtered by a UV filter to remove the ultraviolet light, a water filter to remove the short-wavelength infrared (SWIR) light (>1400 nm), and an 850 nm short-pass filter to remove the NIR light. The excitation light cleaned by these filters was then dispersed by a monochromator (Oriel) to generate single-wavelength excitation lines with a bandwidth of 15 nm, focused onto the reflective surface of Au/Au film or bare quartz slide with surfactant-coated SWNTs drop-dried on it. The emitted fluorescence of SWNTs was collected at 90° to the incident excitation light. The reflected excitation light from the sample was rejected using a 910-nm long-pass filter and collected by the spectrometer equipped with a one-dimensional InGaAs linear array detector. The raw PLE spectra were corrected post-collection to account for the excitation power difference at different wavelengths, the extinction profile of the emission filter, and the sensitivity of the detector using the MATLAB software.

**2.3 Results and Discussion**

SWNTs with an average length of ~400 nm were solubilized by DSPE-mPEG(5000) in a stable aqueous suspension by exchanging the micelle coating of sodium cholate into the PEGylated surfactant, DSPE-mPEG coating.\textsuperscript{3,21-23} PVP was added to the SWNT suspension to yield a moderately viscous solution, which was then
spin-coated onto a bare quartz substrate (Fig. 2.1a) and an Au/Au-film-coated quartz substrate (Fig. 2.1b) made by solution-phase synthesis (see section 2.2 for more details). SEM image of the Au/Au film reveals its microscopic morphology as tortuous gold islands with abundant small gaps in between the islands (Fig. 2.1b inset). Using a 658 nm laser as the excitation source and a two-dimensional InGaAs camera for detection, we imaged SWNT ensembles based on their NIR-II fluorescence in the 900-1700 nm region, where the lower bound of detectable wavelength was set by the emission filter while the higher bound set by the cutoff of the detector sensitivity profile. The NIR-II fluorescence images taken on the two substrates with the same density of SWNTs deposited on both substrates clearly revealed much brighter fluorescence signals from SWNTs on the Au/Au substrate (Fig. 2.1d) than on the bare quartz (Fig. 2.1c).

In good agreement to widefield microscopic imaging, the spectroscopic measurement and analysis also revealed significantly enhanced NIR-II fluorescence emission from SWNTs on the Au/Au film compared to those on the bare quartz substrate. The emission spectra for ensembles of SWNTs within the same area of field of view on both substrates showed a ~8-fold enhancement of the overall NIR-II fluorescence intensity (via integration of the spectrum in the 0.9-1.7 μm range) of SWNTs on the Au/Au film (Fig. 2.1e) compared to that on bare quartz (Fig. 2.1f). We have also performed single carbon nanotube spectroscopy by focusing the excitation beam to a much smaller area, with distinct, sharp emission peaks assigned with single (n,m) chiralities (Fig. 2.1g&h) and polarization angle dependent fluorescence intensity proving single SWNTs (Fig. 2.1i&j). The single nanotube fluorescence spectroscopy
exhibited much smoother spectral profile with less noisy baseline and higher SNR for SWNTs on the Au/Au film than on the bare quartz substrate, suggesting the advantages of using Au/Au films for detecting and characterizing the otherwise weak fluorescence signals in the NIR-II spectral region.

We elucidated the mechanism of the observed MEF phenomenon of SWNTs by studying how the distance between the SWNT fluorophores and the Au/Au metal surface affected the fluorescence enhancement. To study the distance dependent MEF, we coated dielectric spacers on the Au/Au surface with different thicknesses, including alkanethiol SAMs of 1-propanethiol (~0.5 nm thick) and 1-octadecanethiol (~2 nm thick), and Al₂O₃ layers with thicknesses of 5 nm and 10 nm made by ALD on the cysteamine-coated Au/Au film (Fig. 2.2a). The same amount of SWNTs were then deposited onto bare quartz and these Au/Au films with different coatings, and imaged under a low-magnification microscopic objective to reveal a monotonically decreasing trend of fluorescence enhancement with increasing thickness of the dielectric layer (Fig. 2.2b). This finding suggested that the Au/Au nanoparticles had to be close enough to the SWNTs to afford sufficient enhancement, while the enhancement reduced as the SWNT fluorophores were spaced away from the surface of the Au/Au film.

Based on the experimental observations of the distance dependent MEF of SWNTs, we attribute the fluorescence enhancement of SWNTs on the Au/Au film to the resonant coupling of the excitonic fluorescence emission of SWNTs with surface plasmons of the colloidal Au nanoparticles in the Au/Au film. It is well known that the coupling between a fluorescent molecule and a metal particle leads to shortening of
radiative lifetime \( \frac{1}{\tau_r} \) and thus higher quantum yield \( \eta \) of the fluorescence emission, which are related by:

\[
\eta = \frac{\Gamma_r}{\Gamma_r + \Gamma_{nr}}
\]

where \( \Gamma_r \) is the radiative decay rate, and \( \Gamma_{nr} \) is the non-radiative decay rate; the radiative lifetime and non-radiative lifetime can be readily derived as the reciprocal of the decay rates due to the exponential decay nature of both the radiative and non-radiative processes.\(^{25-27}\) For fluorophores such as SWNTs with very low quantum efficiency (~0.1%),\(^4\) we can make the following assumption:

\[
\Gamma_r \ll \Gamma_{nr}
\]

thus one has

\[
\eta \approx \frac{\Gamma_r}{\Gamma_{nr}}
\]

The coupling effect between a dipole emitter and a plasmonic colloid is known to reduce rapidly as the dipole emitter are placed away from the surface of the plasmonic colloid by dielectric spacer layers, which agrees with our observation that the fluorescence enhancement decays monotonically with increasing distance.\(^{26,27}\) Therefore, as the SWNTs approach the metal surface, if the increase of the radiative decay rate (i.e., the shortening of the radiative decay time) exceeds that of the non-radiative decay rate, an increase of the quantum yield will be observed.

On the other hand, it has been reported that the high-order dark plasmon modes in the near field of the plasmonic material contribute to a dramatic increase of the non-
radiative decay rate due to energy transfer, which leads to fluorescence quenching of the fluorescence emitters when they are placed in very close proximity to the surface of the metal (i.e., placed in the near field of the plasmon modes, usually on the order of a few nanometers).\textsuperscript{27,28} In contrast, no fluorescence quenching was observed in our experiments even when the SWNTs were placed on the Au/Au film without any spacer in between. We attribute this ‘immunity to quenching’ to the surfactant layer of DSPE-mPEG(5000) coating on the surface of the SWNTs, which has a radius of gyration of \textasciitilde 3 nm and prevents the SWNT backbone from approaching too close to the Au surface.\textsuperscript{29} Therefore, due to the existence of this protective layer of surfactants, it is very unlikely for the SWNTs to get within the small quenching distance of the Au/Au film (which is likely to be < 3 nm) and become quenched.

It is well known that the fluorescence enhancement due to resonant coupling to surface plasmons is strongly dependent on the size of the metal colloids and the peak location of the surface plasmon resonance (SPR).\textsuperscript{27,30} To find out the dependence of fluorescence enhancement of SWNTs on different Au/Au films, we studied the MEF of SWNTs on a series of four Au/Au substrates with increasing gold colloidal coverage and thus red-shifted SPR features (Fig. 2.3a, where the four Au/Au films are named Au/Au-I to -IV). The PLE spectra were taken on samples with the same amount of surfactant-coated SWNTs deposited on bare quartz and the four Au/Au substrates (Fig. 2.3b-f), revealing a monotonically increasing fluorescence enhancement of the Au/Au films with increasing thickness and progressively red-shifted SPR extinction. This trend agrees well with the fluorescence enhancement of
NIR fluorophores such as indocyanine green (ICG) on silver films or gold nanospheres with progressively red-shifted SPR extinction.\textsuperscript{30,31}

To further elucidate the coupling mechanism between the SWNT fluorescence emitters and the surface plasmons of the Au/Au film, we analyzed the enhancement factors (EFs) for a group of three different chiralities, (7,5), (7,6) and (10,3), with similar excitation wavelength (~650 nm) but progressively increasing emission wavelengths (see Fig. 2.3e for the locations of these three chiralities in the PLE map). The EFs of these three chiralities were plotted against the emission wavelength and correlated with the SPR absorbance of the corresponding Au/Au film (Fig. 2.4). A good agreement was found for Au/Au-I, Au/Au-II and Au/Au-IV films, where the EFs of the three chiralities showed similar trend as the SPR absorbance profiles, suggesting higher SPR absorbance resulted in greater fluorescence enhancement, possibly due to a stronger resonant coupling between the SWNT emission and the Au/Au surface plasmons. This hypothesis of resonant coupling was further confirmed by the observation that the (10,3) chirality showed almost no fluorescence enhancement with an EF of \(~1\) (Fig. 2.4a) on Au/Au-I, which exhibited SPR absorbance of almost zero at the emission wavelength of the (10,3) chirality (~1300 nm).

In contrast to the consistent trends observed for the other three Au/Au films, the Au/Au-III film appeared to have the trend of EF slightly against the trend of the SPR extinction curve (Fig. 2.4c). We attributed this opposite trend to the very little variation in the plateau region of the extinction curve in the 1000-1400 nm range of the Au/Au-III film, which resulted in very similar strength of the surface plasmon from \(~1050\) nm [the emission wavelength of (7,5)] to \(~1300\) nm [the emission
wavelength of (10,3)]. Therefore in the case of the Au/Au-III film, the intrinsic
difference of fluorescence intensity of these three chiralities could be playing a more
important role.

2.4 Conclusion

In this work, we observed the first evidence of metal enhanced NIR-II
fluorescence of SWNTs on plasmonic Au/Au films. The MEF of SWNTs resulted
from the increase of radiative decay rate of the SWNT emitters and the shortening of
radiative lifetime, through resonant coupling of the SWNT emission to surface
plasmon modes in the Au/Au film. The fluorescence enhancement of surfactant-coated
SWNTs decreased monotonically as the nanotube fluorophores were separated from
the surface of the Au/Au film by a dielectric layer, while the surfactant-coated SWNTs
in direct contact with the metal exhibited the strongest enhancement. This quenching-
free behavior of the MEF of SWNTs suggested a small quenching distance shorter
than the thickness of the surfactant layer wrapping on the SWNT backbone. The
SWNT fluorescence enhancement was found to increase monotonically with
increasing Au colloidal coverage and progressively red-shifted SPR peak, presumably
due to the increased scattering of Au colloids in the film and re-radiation of stronger
surface plasmon modes resonantly coupled to SWNT emission. These new findings of
SWNT fluorescence enhancement by > 10 times bode well for the solution phase
synthesized Au/Au films as novel plasmonic materials in fundamental and practical
applications including detection, sensing and imaging using SWNTs as fluorescent labels and reporters in the biologically favorable NIR-II window.
2.5 Figures

Figure 2.1 | Microscope Imaging and Spectroscopy Showing MEF of SWNTs. (a&b) Schematic drawing of SWNTs embedded in a PVP matrix on the bare quartz substrate (a) and the Au/Au film (b, inset showing the SEM image of the Au/Au film).
(c&d) Widefield NIR-II fluorescence images of SWNTs on bare quartz (c, inset showing the same image rescaled by 4×) and the Au/Au film (d). (e&f) NIR-II fluorescence emission spectra of SWNT ensembles on bare quartz (e) and the Au/Au film (f). (g&h) NIR-II fluorescence emission spectra of single carbon nanotubes (circled in c&d, respectively) on bare quartz (g) and the Au/Au film (h). (i&j) Curves showing the polarization dependent NIR-II fluorescence intensity versus polarization angle for single carbon nanotubes (circled in c&d, respectively) on bare quartz (i) and the Au/Au film (j).
Figure 2.2 | Distance Dependent MEF of SWNTs. (a) A schematic drawing showing the surfactant-coated SWNTs deposited on substrates coated with different thicknesses of the dielectric layers. The NIR-II fluorescence images underneath the schematic correspond to the samples in the schematic. (b) A bar chart summarizing the average NIR-II fluorescence intensity of each sample, suggesting a monotonically decreasing trend of fluorescence enhancement versus the thickness of the dielectric spacer, with each one corresponding to an NIR-II fluorescence image in a.
Figure 2.3 | SPR Dependent MEF of SWNTs. (a) The UV-Vis-NIR extinction spectra of a series of Au/Au films (Au/Au-I to -IV) made with increasing gold colloidal coverage, increasing film thickness and thus progressively red-shifted SPR extinction features. (b-f) PLE spectra of surfactant-coated SWNTs deposited on bare quartz (b) and the four Au/Au films (c-f) shown in a.
Figure 2.4 | Resonant Coupling Between SWNT Emission and the SPR of Au/Au film. The UV-Vis-NIR extinction spectrum (black curve) and the enhancement factors for three chiralities with the same excitation wavelength of ~650 nm (red curve) are plotted for each Au/Au film with the corresponding SEM image shown in the inset.
2.6 References


Chapter 3. Metal Enhanced Molecular Imaging of Live Cells in the Entire Near-Infrared Window

* The work in this chapter was published in:


3.1 Introduction

The many benefits of fluorescence imaging in the second near-infrared window (NIR-II window, 1000-1700 nm), as described in detail in Section 1.4, well justify their applications for *in vitro* and *in vivo* biological imaging with reduced scattering, high transparency and minimum autofluorescence of cells and tissues. However, one common caveat of fluorophores in the NIR-II window and even the traditional first near-infrared window (NIR-I window, 750-900 nm) is the relatively low fluorescence quantum yields (QYs), in comparison with their counterparts in the visible window (400-750 nm). For instance, single-walled carbon nanotubes (SWNTs) with fluorescence in the 1000-1400 nm NIR-II window exhibit quantum yields in the range of 0.1% to 3%,\(^1\)-\(^3\) the commercial available and widely used NIR-I fluorophore, IRDye-800, emits fluorescence at ~800 nm upon excitation at ~785 nm with a low QY of merely ~10%,\(^4\) and another NIR-I fluorophore with similar emission at 805 nm, indocyanine green (ICG), exhibits a QY of only ~4.3%.\(^5\) In strong contrast, fluorescent molecules emitting at shorter wavelengths in the visible window typically exhibit much higher QYs, such as IRDye-700 with a QY of ~24% at an emission wavelength of 700 nm emission,\(^4\) cyanine-5 with a QY of ~30% at 660 nm emission,\(^6\) and fluorescein with a very high QY of ~91% at the emission of 521 nm.\(^7\) The
relatively low fluorescence quantum yields of NIR fluorophores in general limit their imaging capabilities as to the necessary concentration or dose needed to reach similar signal-to-noise ratio (SNR) as in the visible window.

Therefore, in order to fully exploit the spectral advantages of the NIR fluorescence in biological imaging, it is desirable to develop a facile approach of enhancing the fluorescence emission of various NIR fluorophores such as SWNTs and IRDye-800. In Chapter 2, the metal enhanced fluorescence (MEF) of SWNTs was realized on a planar gold-on-gold film (called ‘Au/Au film’) to overcome the sub-optimal fluorescence quantum efficiency of SWNTs due to the optically forbidden, intrinsic low-energy excitons, as well as the exogenous quenchers such as metallic nanotubes in a bundle, and single-molecule reactive species such as acid, base, oxygen, and diazonium compounds. In this chapter, we would like to move one step further and apply the same Au/Au plasmonic enhancing substrate for NIR fluorescence enhanced (NIR-FE) molecular imaging using SWNTs in the NIR-II window and IRDye-800 in the NIR-I window.

3.2 Materials and Methods

Solution-phase synthesis of gold-on-gold (Au/Au) films on quartz. Quartz slides were first submerged in a 3 mM chloroauric acid solution, and then under vigorous agitation, a concentrated solution of ammonium hydroxide was added to the chloroauric acid solution to reach 0.6 wt%. With the quartz slides still immersed in such seeding solution, the container was gently shaken for 1 min, followed by a
thorough wash of the quartz slides with deionized ultra-filtered (DIUF) water. Then the quartz slides were immersed into a 1 mM sodium borohydride solution with the container placed on an orbital shaker for 5 min at 100 rpm to form Au seeds onto the substrates. After a second wash step for the slides, the seeded substrates were submerged in growth solutions consisting of 1:1 chloroauric acid and hydroxylamine at four different molar concentrations: 250 μM, 500 μM, 1000 μM and 3000 μM under vigorous shaking. Then the growth solution containing the substrate was transferred to an orbital shaker at 100 rpm and allowed to stay on the shaker for 15 min. The Au/Au substrates were finalized by rinsing with DIUF water, and blow-dried with house air. The Au/Au substrate grown in the solution containing 3000 μM chloroauric acid and 3000 μM hydroxylamine gave the optimized enhancement for cell molecular imaging in the NIR window.

**UV-Vis-NIR absorbance measurement of Au/Au films.** UV-Vis-NIR absorbance spectra of the Au/Au films on quartz substrates were measured in the range of 400-1500 nm by a Cary 6000i UV-Vis-NIR spectrophotometer, background-subtracted for any absorbance contribution from the quartz substrate.

**Scanning electron microscopy (SEM) imaging of Au/Au films.** SEM images of the Au/Au films on quartz substrates were acquired on an FEI XL30 Sirion SEM with FEG source at 5 kV acceleration voltage. False colors were added to the original images to enhance visual appeal using the MATLAB software.

**Preparation of water soluble SWNT-IRDye-800-RGD bi-color conjugate.** Raw HiPco SWNTs (Unidym) were bath sonicated in an aqueous solution containing 1 wt%
sodium deoxycholate for 1 h, and ultracentrifuged at 300,000 g for 1 h to remove bundles and other large aggregates of SWNTs in the suspension. After ultracentrifugation, only the supernatant was retained, concentrated through a 30k NMWL centrifugal filter unit (Fisher), layered to the top of a 10%/20%/30%/40% sucrose step gradient, and ultracentrifuged at 300,000 g for 1 h. Only the top 1 mL of the gradient was collected by careful fractionation and a mixture of the PEGylated surfactant C18-PMH-mPEG(90k) (poly(maleic anhydride-alt-1-octadecene)-methoxy(polyethylene glycol)90,000), synthesized by our group along with DSPE-PEG(5k)-NH$_2$ (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)5,000], Laysan Bio) was added to the solution to reach a final C18-PMH-mPEG(90k) concentration of 0.75 mg/mL and a final DSPE-PEG(5k)-NH$_2$ concentration of 0.25 mg/mL. Then the suspension was bath sonicated for 5 min, dialyzed against 1x PBS in a 3500MCWO dialysis membrane (Fisher) to remove all small molecules such as sodium deoxycholate and sucrose, and ultracentrifuged again at 300,000 g for 1 h to remove bundles and other aggregates formed during dialysis. The amine-functionalized SWNTs were conjugated further with the IRDye-800 fluorophore and RGD peptide according to a previously published protocol. In brief, the SWNT solution after dialysis and ultracentrifugation was washed with DIUF water in a 30k NMWL centrifugal filter unit to remove excess surfactant and concentrated to ~300 nM. 10x PBS was added to this solution to reach a 1x PBS solution with pH 7.4, and then the solution was mixed with a DMSO solution containing 0.1 mM IRDye-800 and 1 mM sulfo-SMCC. The mixture was allowed to sit for 2 h at room temperature. After removing excess sulfo-SMCC and unbound IRDye-800 by
filtration through a 100k NMWL centrifugal filter and concentrating the solution down to as small volume as possible, RGD-SH (cyclo-RGDFC, Peptides International) was added to the solution along with tris(2-carboxyethyl)phosphine (TCEP, Sigma) at pH 7.4. The final concentrations of SWNTs, RGD-SH and TCEP should be adjusted to 300 nM, 0.1 mM and 1 mM, respectively. The conjugation reaction was allowed to proceed at 4 °C for 2 days, before centrifugal filtration to remove excess RGD-SH and TCEP through a 100k NMWL filter.

**Atomic force microscopy (AFM) imaging of SWNTs.** AFM imaging of the as-made SWNT-IRDye-800-RGD bioconjugate was acquired with a Nanoscope IIIa multimode AFM using the tapping mode. The sample for AFM imaging was prepared by immersing the SiO₂ coated Si wafer in the SWNT-IRDye-800-RGD bioconjugate suspension for 30 seconds, followed by rinsing the Si wafer with DIUF water and isopropanol, blow-drying with house air and calcination.

**Photoluminescence versus excitation (PLE) spectroscopy of drop-dried SWNTs on different substrates.** The PLE spectra of drop-dried SWNT-IRDye-800-RGD bioconjugate on different substrates were taken on a home-built NIR-II spectroscopy setup. The excitation was provided by a white-light source of an ozone-free Xenon lamp (Oriel) with a total power of 150 W, which was filtered by a UV filter to remove the ultraviolet light, a water filter to remove the short-wavelength infrared (SWIR) light (>1400 nm), and an 850 nm short-pass filter to remove the NIR light. The excitation light cleaned by these filters was then dispersed by a monochromator (Oriel) to generate single-wavelength excitation lines with a bandwidth of 15 nm, focused onto the drop-dried spot of SWNT-IRDye-800-RGD bioconjugate on the Au/Au film
or on the bare quartz substrate. The emitted fluorescence of SWNTs was collected at 90° to the incident excitation light. The reflected excitation light from the sample was rejected using a 910-nm long-pass filter and collected by the spectrometer equipped with a one-dimensional InGaAs linear array detector. The raw PLE spectra were corrected post-collection to account for the excitation power difference at different wavelengths, the extinction profile of the emission filter, and the sensitivity of the detector using the MATLAB software.

**Cell culture and staining.** All cell culture media used in this work were supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin and L-glutamine. The U87-MG brain glioblastoma cells were cultured in Low Glucose Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 1 g/L D-glucose and 110 mg/L sodium pyruvate. The MCF-7 breast cancer cells were grown in High Glucose DMEM, supplemented with 4.5 g/L D-glucose and 110 mg/L sodium pyruvate. Cells were maintained in cell culture flasks (Corning) and placed in a 37 °C humidified incubator with 5% CO₂.

For cell staining, both cell lines growing in the flasks were trypsinized at 37 °C for 5-10 min, before the SWNT-IRDye-800-RGD bioconjugate was added to the suspensions of U87-MG (α₃β₃- positive) and MCF-7 (α₃β₃- negative) cells. The final staining concentration of the SWNT-IRDye-800-RGD bioconjugate in the cell suspension ranged from 30 nM to 48 pM. The staining was carried out at 4 °C for 1 h unless otherwise noted, followed by washing the cells thoroughly with cold 1× PBS to
remove the free, unbound conjugates in the suspension. The cells were always kept at 4°C before imaging to prevent endocytosis at elevated temperatures.

**High-magnification NIR fluorescence microscopy imaging.** Cell imaging in the NIR-I window was performed on a Horiba Labram HR800 system to look at the fluorescence of IRDye-800. The Horiba Labram HR800 system was equipped with a grating of 300 line/mm with pixel binning of 3, and a 785 nm excitation laser with the power of 0.8 mW (1% power through an OD2 neutral density filter), through a 50× objective lens with long working distance. The emission from the cells was filtered through a band-rejection 785 nm filter to clean out the laser line and collected in the range of 790-820 nm by integrating the fluorescence emission in the spectra. Exposure time was set to 0.05 s with no iteration, and cell fluorescence images were acquired by stepwise mapping with a step size of 2 μm.

Cell imaging in the NIR-II window was performed on a homemade widefield microscope setup coupled to a two-dimensional InGaAs camera (Princeton 2D OMA-V) to look at the fluorescence of SWNTs. A 658-nm laser diode (100 mW, Thorlabs) cleaned through a 750 nm short-pass filter (Omega) was used as the excitation source. The 658-nm laser was focused to a 150 μm diameter spot on the sample by a 50× objective lens (Olympus), and the emitted NIR-II photoluminescence from the SWNTs was allowed to pass through an 1100 nm long-pass filter (Thorlabs) and focused onto the two-dimensional InGaAs camera, with an exposure time ranging from 300 ms (on Au/Au) to 3 s (on quartz or with low staining concentration).
For both NIR-I and NIR-II cell fluorescence images, the average fluorescence intensity and the standard deviation in each imaged cell were analyzed using the *roipolyarray* function in Matlab.

### 3.3 Results and Discussion

We made the bicolor fluorescent reporter, SWNT-IRDye-800-RGD, to afford NIR fluorescence imaging in both the NIR-I (IRDye-800 fluorescence) and NIR-II (SWNT fluorescence) windows. The SWNT backbone emitted fluorescence in the 1000-1400 nm NIR-II window upon excitation at 658 nm, while the IRDye-800 side group emitted fluorescence in the 800-1000 nm NIR-I window upon excitation at 785 nm ([Fig. 3.1a](#)). The RGD peptide was linked to the bicolor fluorescent reporter as the targeting ligand to afford selective binding to the αvβ3-integrin positive U87-MG cells over the αvβ3-integrin negative MCF-7 cells. AFM imaging ([Fig. 3.1b](#)) revealed the length distribution of the SWNT conjugates in the range from 100 nm to 3 μm, with an average length of ~1 μm.

Via solution phase synthesis, we made plasmonic enhancing substrates by coating Au/Au films on quartz ([Fig. 3.1c](#)), with the strongest surface plasmon resonance (SPR) in the >800 nm NIR window ([Fig. 3.1d](#)) allowing for the optimum MEF of both SWNTs and IRDye-800. The PLE spectra of the drop-dried samples of SWNT-IRDye-800-RGD conjugate on bare quartz and the Au/Au substrate clearly revealed fluorescence enhancement of both IRDye-800 in the NIR-I region (the emission tail in the 900-950 nm range) and SWNTs in the NIR-II region (all the other
peaks in the 1000-1400 nm range) on the Au/Au film (Fig. 3.1e&f). The average enhancement factor (EF) of SWNT photoluminescence in the NIR-II window was approximately 10 times, and the EF was about 5 times for the IRDye-800 attached to the backbone of SWNTs. This result clearly exhibited the excellent fluorescence enhancing capability of the Au/Au film for fluorophores emitting in both NIR-I and NIR-II windows including IRDye-800 and SWNTs.

To perform targeted cell staining and molecular imaging, U87-MG brain glioblastoma cells and MCF-7 breast cancer cells were trypsinized and stained with the SWNT-IRDye-800-RGD conjugate at a nanotube concentration of ~30 nM at a low temperature of 4 ºC to prevent endocytosis during staining for 1 h. After staining, the cells were thoroughly washed to remove the unbound SWNT conjugate, divided into two identical groups for each cell line, placed onto a bare quartz substrate and an Au/Au film coated substrate respectively, and imaged in a homemade widefield NIR-II fluorescence microscope immediately (see Section 3.2 for experimental details). The NIR-II fluorescence images of the αvβ3-integrin positive U87-MG cells stained with the SWNT-IRDye-800-RGD conjugate exhibited a ~9-fold higher NIR-II fluorescence signal on average (false-color coded in green) on the Au/Au film (Fig. 3.2a) than on bare quartz (Fig. 3.2b). Owing to the significantly enhanced NIR-II fluorescence of SWNTs, a much reduced exposure time of merely 300 ms was sufficient for the stained U87-MG cells on Au/Au film, in strong contrast to the much longer exposure times (1~3 s) needed for obtaining high quality cell images on bare substrate without Au.18,19 We also optimized the SPR properties of the Au/Au films and found the Au/Au substrate with the highest concentration of chloroauric acid in
the growth solution (3 mM) and thus strongest SPR extinction in the >800 nm NIR window allowed for the highest EF of SWNT-labeled NIR-II fluorescence imaging, in good agreement with our finding in Chapter 2 (Fig. 3.3).  

On the other hand, the $\alpha_v\beta_3$-integrin negative MCF-7 cells after incubation with the SWNT-IRDye-800-RGD conjugate showed very little NIR-II fluorescence signal from the SWNTs, on both Au/Au film (Fig 3.2c) and bare quartz (Fig. 3.2d). Based on the average NIR-II fluorescence intensity, we analyzed the selectivity of cell targeting using SWNT-IRDye-800-RGD, which was defined as the ratio of the NIR-II fluorescence intensity found on the $\alpha_v\beta_3$-integrin positive U87-MG cells to that on the $\alpha_v\beta_3$-integrin negative MCF-7 cells. We found the selectivity was ~17 for cells on the optimized Au/Au film (Fig. 3.2g), significantly higher than the selectivity of ~7 on bare quartz. This higher selectivity on the Au/Au film suggested the plasmonic enhancing substrate selectively enhanced the signal while not enhancing the background to a nearly similar level, resulting in a higher positive to negative ratio.  

It is noteworthy that although the cells in the images appeared round-shaped, they were still alive and assumed the round shape only temporarily, since they were imaged immediately after staining and washing in a cell suspension and had not had enough time to adhere to the substrate yet. To demonstrate that these stained cells in the NIR-II fluorescence images were alive, we monitored the same U87-MG cells in situ over a period of 6 h after the first imaging (Fig. 3.4a). The cells were maintained in the cell medium with the temperature increased to 37 °C in a temperature controlled microscopic imaging chamber with a continuous CO$_2$ gas flow of 1 L/min. Both the bright field optical image and NIR-II fluorescence image for the same cells after 6 h of
incubation at 37 °C clearly showed cell adhesion to the Au/Au surface (Fig. 3.4b), suggesting the evidence of live cells.

To study the mechanism of metal enhanced NIR-II fluorescence imaging of cells, we trypsinized and stained the same U87-MG and MCF-7 cells with the SWNT-IRDye-800-RGD conjugate at 37 °C for 1 h, and compared the images with those stained at 4 °C. The staining temperature of 37 °C was known to lead to endocytosis of SWNTs inside live cells. Both cell lines stained at 37 °C were placed onto bare quartz and the Au/Au substrate for NIR-II fluorescence imaging, where an EF of merely ~2 times was found for the positive U87-MG cells plated on the Au/Au film (Fig. 3.2h) compared to those on bare quartz (Fig. 3.2i), in strong contrast to the EF of ~9 times observed for U87-MG cells stained at 4 °C. It was also noticeable that the negative MCF-7 cells had very higher false-positive signals (Fig. 3.2j&k), owing to the anticipated increase of non-specific cellular uptake of the nanotube conjugates at an elevated temperature of 37 °C than at 4 °C.

According to the discussion in Chapter 2, the enhanced NIR-II fluorescence of SWNT in the molecular targeting conjugate, SWNT-IRDye-800-RGD, was attributed to the strong coupling of the nanotube fluorescence emission to the surface plasmon modes in the Au/Au film. As a result, the fluorescence quantum efficiency of SWNTs was increased through a combined effect of surface plasmon re-radiation of the Au nano-colloids and the radiative lifetime shortening of nanotube excitons. In the aforementioned work, we observed higher fluorescence enhancement when the surfactant-wrapped SWNTs were placed closer to the Au/Au surface rather than further, and found a distance-dependent relationship for EF versus the nanotube-gold
distance with a half-decay distance of approximately 5 nm, on the same order of length as the cell membrane thickness. When the U87-MG cells were stained at 4 °C, most of the nanotube conjugates were blocked from active internalization and cross-membrane transport, and the SWNTs on the outside of the cell membrane interfaced with the plasmonic Au/Au surface and thus coupled strongly to the SPR modes in the Au/Au film. This strong coupling was responsible for the large fluorescence enhancement of ~9 times on gold compared to on bare quartz (Fig. 3.2e&f). Conversely, when stained at 37 °C, the nanotube conjugates were internalized into the cell cytoplasm through endocytosis and thus became spatially separated by the lipid bilayer of the cell membrane from the gold surface, leading to a much reduced EF of ~2 times (Fig. 3.2l&m). It was also worth noting that the NIR-II fluorescence intensity decreased by ~6 times for U87-MG cells stained with the SWNT conjugates at 4 °C from immediately after staining to after 37 °C incubation in cell medium for 6 h, during which time the cells actively transported the membrane-bound SWNTs to the cytoplasm via the endocytotic process, thus spatially separated the SWNTs away from the Au/Au surface and reduced effective coupling to the surface plasmons (Fig. 3.4).

The distance-dependent NIR-II fluorescence enhancement of SWNTs on the Au/Au film could also help explain the increased selectivity of targeted cell imaging experiments (~17 on Au/Au versus ~7 on bare quartz, Fig. 3.2g). For the αvβ3-negative MCF-7 cells, the detected non-zero fluorescence signal was a combination of the cellular autofluorescence and the fluorescence of SWNTs non-specifically taken up by the cells. In both cases, the fluorescence was distributed throughout the entire cell body in three dimensions rather than confined on the outside of the cell.
membranes, and thus was barely enhanced by the underlying Au/Au film. On the other hand, for the α,β₃-positive U87-MG cells, most of the detected signal came from the specifically targeted receptor sites on the cell membrane, which were in the proximity of the Au/Au surface and thus could be significantly enhanced due to strong, near-field coupling with the SPR modes. As a result, the Au/Au substrate preferentially enhanced the specific fluorescence signal located on the cell membrane while keeping the non-specific signal less enhanced, leading to a higher positive-to-negative ratio and an improved selectivity of staining.

Another explanation on the increased targeting selectivity on the Au/Au film attributed this observation to the non-linear nature of the enhancement effect. It has been reported that the surface enhanced Raman scattering (SERS) followed a non-linear relationship to the Raman probe concentration, where analytes with higher concentrations were usually enhanced to a higher degree owing to a better chance of occupying the ‘hot spots’ with greater enhancement effect.²⁸⁻²⁹ In our experiments of fluorescence enhancement, the α,β₃-negative MCF-7 cells did not get enhanced nearly as much as the the α,β₃-positive U87-MG cells, possibly due to much fewer nanotube fluorophores on the membranes than the positive cells. This non-linear MEF effect led to a ‘magnified’ targeting selectivity as measured by the NIR-II fluorescence imaging.

Besides enhancing the NIR-II fluorescence of SWNTs, we have shown that the Au/Au film was capable of enhancing the NIR-I fluorescence of a representative dye widely used for biological imaging, IRDye-800.³⁰⁻³¹ We found a ~5-fold fluorescence enhancement of IRDye-800 by comparing the two PLE maps of the SWNT-IRDye-800-RGD conjugate directly deposited on bare quartz and the Au/Au film (Fig.
We then stained the αvβ3-integrin positive U87-MG cells with the SWNT-IRDye-800-RGD bioconjugate and carried out NIR-II fluorescence imaging of cells placed on bare quartz and the Au/Au film in the IRDye-800 fluorescence channel (790-820 nm in the NIR-I window). The images shown in Fig. 3.5a&b clearly revealed a prominent fluorescence enhancement of the NIR-I fluorescence of IRDye-800 on the Au/Au film versus on bare quartz with an EF of ~6 times. Similar to the NIR-II fluorescence images revealing the distribution of SWNTs on cells, the αvβ3-integrin negative MCF-7 cells showed negligible fluorescence signals of IRDye-800 under the same conditions of staining and imaging (Fig. 3.5c&d), confirming the covalent conjugation of SWNT and IRDye-800. The MEF of IRDye-800 in the context of cell targeted imaging led to a positive/negative selectivity ratio of ~16 on the Au/Au film versus ~4 on bare quartz (Fig. 3.5e). We also confirmed that the optimized Au/Au film with the strongest SPR extinction in the >800 nm NIR window to afford the highest fluorescence enhancement of SWNTs also provided the highest EF of IRDye-800 in the setting of targeted cellular imaging (Fig. 3.6). All the aforementioned results suggested the generality of fluorescence enhanced cell imaging using the plasmonic Au/Au film in the broadly defined, entire NIR window.

One of the long-existing problems of using SWNTs as fluorescent tags for selective cell imaging remained the deficient quantum yield (0.1-3.0%),1-3 which was even lower than most of the organic NIR dyes (4-10%).4,5 As a result, in order to perform fluorescent cell imaging in the NIR-II window with sufficient SNR, a relatively high concentration (~60 nM) of SWNTs was usually needed for cell staining, which almost saturated all ligand binding sites on the cell membranes.32 Therefore it
remained desirable to develop molecular cell imaging in the NIR-II window with higher sensitivity while not losing the image quality. To this end, we stained the $\alpha_v\beta_3$-integrin positive U87-MG cells at a series of decreasing concentrations of the SWNT-IRDye-800-RGD conjugate ranging from 30 nM to 48 pM, and performed NIR-II microscopic imaging of the stained cells placed on the Au/Au films and the bare quartz slides. The NIR-II fluorescence images showed discernible signals from the U87-MG cells down to a staining concentration as low as 48 pM on the Au/Au film (Fig. 3.7a-e), in strong contrast to the detection limit of 1.2 nM on bare quartz (Fig. 3.7f-j), below which no detectable signal was observed due to lack of SNR. Therefore, the sensitivity of NIR-II fluorescence based molecular imaging was significantly improved with a ~25x lower detection limit by the plasmonic enhancing Au/Au substrates (Fig. 3.7k).

We also estimated the number of SWNTs (i.e., the number of occupied $\alpha_v\beta_3$-integrin receptors) on the U87-MG cell membranes at the lowest staining concentration of 48 pM. First we have the equilibrium of dissociation of the $\alpha_v\beta_3$-RGD complex as follows,

$$\alpha_v\beta_3 - \text{RGD} \leftrightarrow \alpha_v\beta_3 + \text{RGD}$$

which has a dissociation constant $K_d$ of 41.70 nmol/L as reported in previous literature.\textsuperscript{33} Then one immediately has

$$\frac{[\alpha_v\beta_3][\text{RGD}]}{[\alpha_v\beta_3 - \text{RGD}]} = 4.17 \times 10^8 \text{ M}$$
At a staining concentration of 48 pM for the SWNT-IRDye-800-RGD conjugate, since there were on average ~6 RGD ligands per conjugate molecule and the conjugate was always in large excess in the staining solution, we can derive the concentration of unbound RGD as follows,

\[
[\text{RGD}] = 48 \text{ pM} \times 6 = 2.88 \times 10^{-10} \text{M}
\]

Thus

\[
\frac{[\alpha_v \beta_3]}{[\alpha_v \beta_3 - \text{RGD}]} = 145
\]

which suggests that the \( \alpha_v \beta_3 \)-integrin receptors on the cell membranes of U87-MG cells were highly unsaturated, with only \( \frac{1}{145+1} \approx 0.68\% \) of all receptors being occupied by SWNT-RGD. We can also estimate the percentage of receptor occupancy at a typical staining concentration of 60 nM without using the enhancing Au/Au substrate\(^3\) as follows,

\[
\frac{[\alpha_v \beta_3]}{[\alpha_v \beta_3 - \text{RGD}]} = \frac{K_d}{[\text{RGD}]} = \frac{4.17 \times 10^{-8} \text{M}}{60 \text{ nM} \times 6} = 0.116
\]

meaning only \( \frac{0.116}{0.116+1} \approx 10\% \) of the \( \alpha_v \beta_3 \)-integrin receptors were unoccupied with a high occupancy percentage of ~90%.

For \( \alpha_v \beta_3 \)-integrin positive cells such as U87-MG cells, the expression level of the \( \alpha_v \beta_3 \)-integrin is usually on the order of \( 10^5 \) integrins/cell.\(^3\) Therefore at the lowest staining concentration of 48 pM, given a percentage of occupancy of ~0.68% derived in the preceding paragraphs, on average there should be ~680 \( \alpha_v \beta_3 \)-integrin forming the bound complex with the SWNT-IRDye-800-RGD conjugate. This sets the
detection limit of membrane integrins using SWNTs as NIR-II fluorescent tags. Since there were ~6 RGD residues per nanotube molecule, we estimate an average number of approximately 113 SWNTs per cell, corresponding to ~76 semiconducting SWNTs with NIR-II fluorescence. However, since only the SWNTs located at the interface between the bottom cell membrane and the Au/Au surface could be efficiently enhanced for imaging, and only a handful of chiralities could be resonantly excited by the 658-nm laser used for imaging, we estimate a total of ~10 SWNTs contributing to the NIR-II fluorescence signals detected for cell imaging at the lowest staining concentration of 48 pM.

3.4 Conclusion

In this work, the plasmonic Au/Au substrates were employed for the first time for the NIR fluorescence enhanced molecular imaging of live cells in the entire NIR window by detecting the NIR-II fluorescence of SWNTs and the NIR-I fluorescence of IRDye-800. The optimized plasmonic Au/Au film was capable of enhancing the NIR-II fluorescence of SWNTs by ~9 times and the NIR-I fluorescence of IRDye-800 by ~6 times with significantly improved selectivity (up to a positive/negative ratio of ~17) and sensitivity (down to ~680 integrins per cell at a staining concentration of 48 pM) of molecular imaging. We performed cell staining at different temperatures and further confirmed the distance-dependent MEF of SWNTs and the location of the fluorescent tags in the cell after staining. We envisage the NIR fluorescence enhanced cell imaging as a useful tool allowing us to image and track the transmembrane
behavior of single carbon nanotube molecules with nanometer accuracy and to image the distribution of cell membrane receptors with low abundance.
3.5 Figures

Figure 3.1 | MEF of SWNT-IRDye-800-RGD Conjugate. (a) Schematic drawing of the SWNT-IRDye-800-RGD conjugate. (b) An AFM image of the SWNT-IRDye-800-RGD conjugate deposited on Si wafer. (c) A digital photograph of the Au/Au film coated substrate. The inset shows a typical SEM image of the Au/Au film. (d) A UV-Vis-NIR extinction spectrum in the range of 400-1500 nm of the Au/Au film shown in c. (e&f) The PLE spectra of the SWNT-IRDye-800-RGD conjugate deposited on quartz (e) and the Au/Au film (f).
Figure 3.2 | Fluorescence Enhanced Molecular Imaging of Cells in the NIR-II Window. (a-d) NIR-II fluorescence images of the positive U87-MG cells stained with SWNT-IRDye-800-RGD at 4°C on the Au/Au film (a) and on bare quartz (b), and the images of negative MCF-7 cells treated in the same way on the Au/Au film (c) and on bare quartz (d). (e&f) Schematic drawings showing the SWNTs sandwiched between the gold surface and the cell membrane were enhanced with higher NIR-II fluorescence emission (e), in comparison with the SWNT-stained cell on bare quartz.
(f) (g) A bar chart diagram showing the average NIR-II fluorescence intensities in a-d. (h-k) NIR-II fluorescence images of the positive U87-MG cells stained with SWNT-IRDye-800-RGD at 37°C on the Au/Au film (h) and on bare quartz (i), and the images of negative MCF-7 cells treated in the same way on the Au/Au film (j) and on bare quartz (k). (l&m) Schematic drawings showing the SWNTs internalized by the cell on the Au/Au film were not enhanced due to spatial separation (l), in comparison with the SWNTs internalized by the cell on bare quartz (m). (n) A bar chart diagram showing the average NIR-II fluorescence intensities in h-k.
Figure 3.3 | Optimization of the Au/Au Substrates for MEF of SWNTs on Cells.

(a-e) NIR-II fluorescence images of the αvβ3-integrin positive U87-MG cells stained with the SWNT-IRDye-800-RGD conjugate under the same conditions but placed on Au/Au substrates synthesized from decreasing chloroauric acid concentrations and thus with decreasing Au colloidal coverages (a-d), and on quartz (e). (f) A bar chart showing the EFs of the average cell NIR-II fluorescence from the SWNTs for different substrates shown in a-e.
Figure 3.4 | Proof of Live Cells on Au/Au Substrate. (a) An NIR-II fluorescence image of the $\alpha_v\beta_3$-integrin positive U87-MG cells on the Au/Au substrate immediately after the 1 h staining and washing. (b) An NIR-II fluorescence image of the same U87-MG cells on the Au/Au substrate after 6 h of incubation at 37 °C with continuous gas flow of CO$_2$. Note that the NIR-II fluorescence image is scaled up by 4x for visual clarity. The inset shows the same cells in the field of view under bright field, white light illumination.
Figure 3.5 | Fluorescence Enhanced Molecular Imaging of Cells in the NIR-I Window. (a-d) NIR-I fluorescence images of the positive U87-MG cells stained with SWNT-IRDye-800-RGD at 4°C on the Au/Au film (a) and on bare quartz (b), and the images of negative MCF-7 cells treated in the same way on the Au/Au film (c) and on bare quartz (d). (e) A bar chart diagram showing the average NIR-I fluorescence intensities in a-d.
Figure 3.6 | Optimization of the Au/Au Substrates for MEF of IRDye-800 on Cells. (a-e) NIR-I fluorescence images of the \( \alpha_v\beta_3 \)-integrin positive U87-MG cells stained with the SWNT-IRDye-800-RGD conjugate under the same conditions but placed on Au/Au substrates synthesized from decreasing chloroauric acid concentrations and thus with decreasing Au colloidal coverages (a-d), and on quartz (e). (f) A bar chart showing the EFs of the average cell NIR-I fluorescence from the IRDye-800 for different substrates shown in a-e.
Figure 3.7 | Detection Limit of NIR-II Fluorescence Based Cell Imaging. (a-e) NIR-II fluorescence images of αvβ3-integrin positive U87-MG cells stained at a series of decreasing concentrations of RGD-SWNT ranging from 30 nM to 48 pM and placed on the Au/Au films for imaging. (f-j) NIR-II fluorescence images of U87-MG cells stained in the same way as in a-e but placed on the bare quartz slides for imaging. The insets in i&j showed corresponding bright field images taken with white light illumination. Note some images were scaled up by a certain multiplier as denoted. (k) Average NIR-II photoluminescence (PL) intensity of cells plotted as a function of
staining concentration of RGD-SWNT for cells placed on the Au/Au film (red) and bare quartz (blue).
3.6 References


Chapter 4. Imaging and Tracking of Single Carbon Nanotube Endocytosis

Facilitated by Metal Enhanced Fluorescence

* The work in this chapter was published in:


4.1 Introduction

Nanoscopic materials with unique physical and chemical properties have received growing interests in biomedical applications, while it remains an open question as to how the exogenous nanoparticles such as quantum dots (QDs)\(^1\) and carbon nanotubes\(^2-8\) interact with live cells, including the internalization pathway and intracellular transport.\(^9\) One of the much studied and debated issues is how the cell uptake pathways of external nanoparticles depend on the size, shape and surface coating of certain nanomaterials.

Taking carbon nanotubes as an example, there have been two distinct internalization pathways suggested by different groups to describe the uptake process of nanotubes by live cells, including the energy-independent insertion/diffusion across the cell membrane,\(^2,10-12\) and the energy-dependent clathrin mediated endocytosis.\(^4-8,13,14\) However, most of the previous studies on the internalization pathway investigated carbon nanotube ensemble\(^2,4-8,10\) while only a few studies focused on the single carbon nanotube behavior when interacting with live cells.\(^3,15\) Furthermore, for fluorescence imaging on the single nanotube level, there has been no study of direct imaging and tracking of the carbon nanotubes in the axial direction (the \(z\) direction) in
addition to the in-plane directions (x and y directions), limiting direct visualization of the cellular internalization process of single carbon nanotubes in three dimensions (3D).

Tracking the behavior and motion of specific molecules and the single events those molecules undergo on and near the cell membrane in a fluorescence microscope can provide useful information and help elucidate the complex mechanisms of many biologically significant processes on a sub-cellular and molecular level. In order to image the internalization process of membrane receptor-bound single carbon nanotube molecules, it is highly desirable to track the cross-membrane motion in real time with high spatial resolution on the same order as the membrane thickness (~3 nm). Although a variety of 3D microscopic imaging techniques have been developed to locate and track single nanoparticles with high axial resolution and real-time recording capability, it still remains highly challenging to image and track the dynamic process of a single carbon nanotube molecule crossing the cell membrane with sub-10 nm axial resolution and elucidate the uptake pathway based on the kinetics of nanotubes during this process.

In previous chapters, we have discussed the metal-enhanced fluorescence (MEF) of single-walled carbon nanotubes (SWNTs) on solution-phase grown colloidal gold films (named ‘Au/Au films’) with the hypothesized mechanism and applications for NIR-II fluorescence enhanced cell molecular imaging. One interesting feature of the SWNT fluorescence enhancement is the distance-dependent degree of enhancement that the enhancement effect decreases with increased gold-nanotube separation distance due to reduced coupling between the nanotube excitons and the
gold surface plasmon modes. Since the distance dependent MEF is very sensitive to nanometer changes of the gold-nanotube distance with an exponential decay distance (i.e., the \(1/\varepsilon\) decay distance) of a mere \(~6\) nm,\(^{23,24}\) it provides us with a nanoscopic ‘ruler’ that measures sub-10 nm distance changes based upon the changes of NIR-II fluorescence. By taking advantage of this interesting phenomenon, we will demonstrate in this chapter that imaging and tracking of the dynamic process of a single carbon nanotube molecule crossing the cell membrane can be achieved with unprecedented sub-10 nm axial resolution to shed light on the uptake pathway for individual SWNTs.

4.2 Materials and Methods

**Solution-phase synthesis of gold-on-gold (Au/Au) film on glass.** A glass slide was first submerged in a 3 mM chloroauric acid solution, and then under vigorous agitation, a concentrated solution of ammonium hydroxide was added to the chloroauric acid solution to reach 0.6 wt%. With the glass slide still immersed in such seeding solution, the container was gently shaken for 1 min, followed by a thorough wash of the glass slide with deionized ultra-filtered (DIUF) water. Then the glass slide was immersed into a 1 mM sodium borohydride solution with the container placed on an orbital shaker for 5 min at 100 rpm to form Au seeds onto the substrates. After a second wash step for the slide, the seeded substrate was submerged in growth solutions consisting of 1 mM chloroauric acid and 1 mM hydroxylamine under vigorous shaking. Then the growth solution containing the substrate was transferred to an orbital shaker at 100
rpm and allowed to stay on the shaker for 15 min. The Au/Au coated substrate was finalized by rinsing with DIUF water, soaking in a 1 mM cysteamine ethanol solution for 1 h, rinsing with anhydrous ethanol, and blow-dried with house air.

**UV-Vis-NIR absorbance measurement of the Au/Au film.** UV-Vis-NIR absorbance spectra of the Au/Au film were measured in the range of 400-1200 nm by a Cary 6000i UV-Vis-NIR spectrophotometer, background-subtracted for any absorbance contribution from the glass substrate.

**Scanning electron microscopy (SEM) imaging of Au/Au films.** SEM images of the Au/Au film on glass substrate were acquired on an FEI XL30 Sirion SEM with FEG source at 5 kV acceleration voltage. Grayscale was used to plot the SEM image.

**Dielectric coating on the Au/Au films.** Low temperature atomic layer deposition (ALD) was used to coat a thin layer of dielectric material, Al₂O₃, with desired thickness on the cysteamine-coated Au/Au films. The deposition of Al₂O₃ was carried out using trimethylaluminum (TMA) and water vapor as precursors and pure nitrogen as the carrier gas at a pressure of ~300 mTorr and a temperature of 100 °C. Each cycle of ALD was divided as follows: the water vapor pulse lasted 0.5 s in duration, followed by a purge time of 40 s; and then the TMA pulse lasted 0.5 s, followed by a purge time of 30 s. Each cycle coated a thin layer of Al₂O₃ with a thickness of 0.1 nm, and the number of total cycles was determined by the desired thickness of the Al₂O₃ dielectric layer on the Au/Au film.

**Preparation of water soluble SWNT-RGD conjugate.** Raw high-pressure CO conversion (HiPco) SWNTs (Unidym) were bath sonicated in an aqueous solution
containing 1 wt% sodium deoxycholate for 1 h, and ultracentrifuged at 300,000 g for 1 h to remove bundles and other large aggregates of SWNTs in the suspension. After ultracentrifugation, only the supernatant was retained, concentrated through a 30k NMWL centrifugal filter unit (Fisher), layered to the top of a 10%/20%/30%/40% sucrose step gradient, and ultracentrifuged at 300,000 g for 1 h. Only the top 1 mL of the gradient was collected by careful fractionation and a mixture of the PEGylated surfactant C18-PMH-mPEG(90k) (poly(maleic anhydride-alt-1-octadecene)-methoxy(polyethylene glycol)90,000), synthesized by our group along with DSPE-PEG(5k)-NH2 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)5,000], Laysan Bio) was added to the solution to reach a final C18-PMH-mPEG(90k) concentration of 0.75 mg/mL and a final DSPE-PEG(5k)-NH2 concentration of 0.25 mg/mL. Then the suspension was bath sonicated for 5 min, dialyzed against 1x PBS in a 3500MCWO dialysis membrane (Fisher) to remove all small molecules such as sodium deoxycholate and sucrose, and ultracentrifuged again at 300,000 g for 1 h to remove bundles and other aggregates formed during dialysis. The amine-functionalized SWNTs were conjugated further with RGD peptide according to a previous protocol. In brief, the SWNT solution after dialysis and ultracentrifugation was washed with DIUF water in a 30k NMWL centrifugal filter unit to remove excess surfactant and concentrated to ~300 nM. 10x PBS was added to this solution to reach a PBS concentration of 1x with pH of 7.4, and then the solution was mixed with a DMSO solution containing 1 mM sulfo-SMCC. The mixture was allowed to sit for 2 h at room temperature. After removing excess sulfo-SMCC by filtration through a 100k NMWL centrifugal filter and concentrating the solution down
to as small volume as possible, RGD-SH (cyclo-RGDFC, Peptides International) was added to the solution along with tris(2-carboxyethyl)phosphine (TCEP, Sigma) at pH 7.4. The final concentrations of SWNTs, RGD-SH and TCEP should be adjusted to 300 nM, 0.1 mM and 1 mM, respectively. The conjugation reaction was allowed to proceed at 4 °C for 2 days, before centrifugal filtration to remove excess RGD-SH and TCEP through a 100k NMWL filter.

Atomic force microscopy (AFM) imaging of SWNTs. AFM imaging of the as-made SWNT-RGD conjugate was acquired with a Nanoscope IIIa multimode AFM performing in the tapping mode. The sample for AFM imaging was prepared by drop-drying 0.5 μL of a very diluted solution (0.45 nM SWNTs and 0.05 wt% Triton X-100) on glass in the same way as preparing samples for the measurement of the distance-dependent fluorescence enhancement of SWNTs on Au/Au film. Then the glass substrate was calcined at a temperature of 350 °C for 15 min.

Distance dependent MEF measurement. The surfactant-coated SWNT-RGD suspension was diluted to a very low concentration of 0.45 nM and mixed with Triton X-100 to reach 0.05 wt%. Then 0.5 μL of this solution was drop-dried on the glass substrate and Au/Au substrates with different thicknesses of Al₂O₃ coating to form a uniform spot with the diameter of ~2 mm. All drop-dried spots were imaged under a low magnification (10×) in the epifluorescence widefield mode using a 658-nm laser diode (100 mW, Thorlabs) as the excitation source. The 658-nm laser was cleaned using a 750 nm short-pass filter (Omega) and focused to a 750 μm diameter spot on the sample through a 10× objective lens (Bausch & Lomb). The NIR-II fluorescence
from the sample was allowed to pass through an 1100 nm long-pass filter and focused onto a two-dimensional (2D) InGaAs camera (Princeton Instruments 2D OMA-V) through a 200-mm tube lens. The InGaAs camera has sufficient sensitivity in the range of 800-1700 nm, beyond which the responsivity profile drops dramatically. Since the field of view under the 10× objective Images was \(~800\ \mu\text{m}\), significantly smaller than the size of each drop-dried spot on the substrate, widefield NIR-II fluorescence snapshots were captured in the area scanning mode to cover the entire spot, flatfield corrected, and then stitched automatically to reconstruct the whole spot. For the stitched NIR-II images of the spots, the average fluorescence intensity and the standard deviation of each spot were analyzed using the `roipolyarray` function in Matlab.

**Cell culture and staining.** The U87-MG brain glioblastoma cells were cultured in Low Glucose Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 1 g/L D-glucose, 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin and L-glutamine. Cells were maintained in cell culture flasks (Corning) and placed in a 37 °C humidified incubator with 5% CO₂.

For cell staining, the αvβ3- positive U87-MG cells growing in the flasks were trypsinized at 37 °C for 5-10 min, before the SWNT-RGD bioconjugate was added to the suspension of U87-MG cells to reach a staining concentration of 1 nM for imaging on the glass substrate and a concentration of 20 pM for imaging on the Au/Au film. The staining was carried out at 4 °C for 1 h unless otherwise noted, followed by washing the cells thoroughly with cold 1× PBS to remove the free, unbound
conjugates in the suspension. The cells were always kept at 4 °C before imaging to prevent endocytosis at elevated temperatures. To carry out the hypertonic treatment, when the U87-MG cells were still growing in the incubator, the original growth medium was replaced with 1x PBS solution supplemented with sucrose at a high concentration of 0.45 M. To carry out the K⁺-depletion treatment, when the U87-MG cells were still growing in the incubator, the original growth medium was replaced with K⁺-free buffer containing 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1.4 M NaCl and 25 mM CaCl₂. In both treatments, the cells were allowed to stay with the corresponding medium in the incubator at 37 °C for 30 min, before they were trypsinized and stained in the same way as aforementioned.

**High-magnification NIR-II fluorescence microscopy imaging and tracking of single nanotubes.** After the U87-MG cells were stained and the excess, unbound SWNT-RGD was washed away, 5 µL of the cell suspension was mixed with 200 µL of 1x PBS or K⁺-free buffer, and 20 µL of the diluted cell suspension was transferred into an 8-well chambered coverglass (Lab-Tek™). Then an Au/Au substrate or a bare glass substrate was placed on top of the 20 µL cell suspension. Due to the capillary force, a very thin layer of cell suspension should be formed between the substrate (top) and coverglass (bottom), and the capillary force ensured that only a monolayer of U87-MG cells residing in between and that the cell membrane was drawn to the proximity of the Au/Au surface due to the ‘sucking’ force of surface tension. The chambered coverglass was kept in a temperature controlled microscopic imaging chamber (BC-260W, 20/20 Technology, Inc.) at 4 °C during sample transfer and
before imaging started. A heat exchanger (HEC-400, 20/20 Technology, Inc.) was used to control the temperature of the chambered coverglass and the cells inside the chambers. To initiate the endocytotic process of single carbon nanotubes into cells, the chamber temperature was increased from 4 ºC and allowed to reach and stabilize at the desired temperature, which usually took ~2 min. A gas purging system (GP-502, 20/20 Technology, Inc.) was used to supply CO₂ gas flow at 1 L/min during imaging.

Single nanotube NIR-II fluorescence imaging on live cells was performed in a homemade widefield microscope setup coupled to a 2D InGaAs camera (Princeton 2D OMA-V) to look at the fluorescence of each individual SWNT. A 658-nm laser diode (100 mW, Thorlabs) cleaned through a 750 nm short-pass filter (Omega) was used as the excitation source. The 658-nm laser was focused to an ~80 µm diameter spot on the sample by a 100× objective lens (Nikon), and the emitted NIR-II photoluminescence from the SWNTs was allowed to pass through an 1100 nm long-pass filter (Thorlabs) and focused onto the 2D InGaAs camera, with an exposure time ranging from 3 s due to the ultra-low staining concentration and the weak fluorescence emission of single carbon nanotubes. Time course NIR-II fluorescence images were recorded continuously with the InGaAs camera for the entire internalization process of single carbon nanotubes. For the acquired NIR-II cell fluorescence images, Matlab was used for flat-field correction, extraction of single nanotube trajectories and analysis of dynamic fluorescence changes in the time course.
4.3 Results and Discussion

The HiPco SWNTs used in this study had a diameter distribution ranging from ~0.7 nm to ~1 nm, but after coating with the mixed surfactants of C18-PMH-mPEG(5kD for each PEG chain and 90kD in total) and DSPE-PEG(5kD)-NH₂, the nanotube-surfactant complex formed a cylinder with an overall diameter of ~15 nm, consisting of an inner core of ~1 nm nanotube backbone, and a shell of PEGylated surfactant with ~7 nm hydrodynamic diameter on both sides of the nanotube backbone (Fig. 4.1a). The length distribution of the HiPco SWNTs used in this study ranged from 100 nm to 3 μm with an average length of ~1 μm, as revealed by the AFM image in Fig. 4.1b.

The plasmonic Au/Au film used in this study was comprised of nano-islands of gold colloids with a considerable number of nanometer-sized gaps in between acting as ‘hot spots’, as revealed by the SEM image in Fig. 4.1c. This Au/Au film exhibited a surface plasmon resonance (SPR) peak at ~800 nm extending all the way to >1200 nm in the UV-Vis-NIR extinction spectrum (Fig. 4.1c inset), facilitating the MEF of SWNTs with photoluminescence in the NIR-II region. To calibrate the ‘nanoscopic ruler’ based on the distance dependent fluorescence enhancement, the as-synthesized Au/Au films were coated with dielectric Al₂O₃ layers with increasing thicknesses from 0 nm to 20 nm, and the surfactant-coated SWNTs were drop-cast onto these films from an aqueous solution (see Section 4.2 for more details). To ensure there was no additional separating layers between the SWNTs and the Au/Au surface due to multiple layers of drop-dried SWNTs, AFM image was taken for SWNTs drop-cast on
the bare glass substrate with same concentration and revealed a low surface density of SWNTs in the spot forming only a monolayer or sub-monolayer of SWNTs (Fig. 4.1b). By taking NIR-II fluorescence images of drop-dried SWNTs on the bare glass and the Au/Au substrates coated with increasing spacer thicknesses, a monotonically decreasing relationship was found for the enhancement factor (EF) versus the Au-SWNT separation distance, from the maximum EF of ~8 to the minimum EF of ~1, with an exponential decay distance (i.e., the 1/e decay distance) of a mere ~6 nm (Fig. 4.1d). It is noteworthy that the Au-SWNT separation distance in Fig. 4.1d corresponded to the minimum distance between any part of a carbon nanotube and the Au surface, since the SWNTs were long (average length ~1 μm) and it was very unlikely for the entire length of an SWNT to rest perfectly flat on the Au/Au film.

We then probed the cross-membrane motion of single carbon nanotube fluorophores in the normal direction of the Au/Au surface by taking advantages of the ultra-sensitive MEF of SWNTs to the Au-SWNT distance. We stained the α,β3-positive U87-MG cells with the SWNT-RGD conjugate at a very dilute concentration of ~20 pM to increase the chance of finding single nanotubes rather than aggregates on the cell membrane. The cells were stained and transferred to an Au/Au substrate at 4 °C to prevent any unwanted internalization before imaging started. The inset of Fig. 4.2a clearly showed a bright spot with NIR-II fluorescence under the 658-nm excitation, which corresponded to a single emission peak at ~1150 nm in the fluorescence spectrum and was assigned to the (7,6) chirality (Fig. 4.2a). The NIR-II emission fluorescence of this bright spot was found to depend on the polarization
angle of the laser excitation with sinusoidal fluctuation (Fig. 4.2b), further confirming the evidence of a single nanotube observed for this spot.

Once an individual SWNT was identified and proved as a single nanotube, the temperature of the imaging chamber, in which the cells and the Au/Au substrate were placed, was increased from 4 °C to 37 °C within ~2 min *in situ*. After the temperature reached 37 °C and stabilized, the camera started recording NIR-II fluorescence images continuously (Fig. 4.2c-e), where one could clearly observe a monotonic decrease of the NIR-II fluorescence intensity over a period of ~5 min, with in-plane motion recorded as the trajectory shown in Fig. 4.2i. The NIR-II fluorescence intensity of this specific SWNT plotted as a function of time revealed some intensity fluctuations at the beginning, followed by a monotonic decrease in intensity by ~7 times, which was fitted into a first-order exponential decay (*1/e* decay time = 121 s, Fig. 4.2j). We also repeated the single nanotube tracking on Au/Au films with two other independent experiments performed at 37 °C and found similar fluorescence intensity decays with fitted *1/e* decay times of 103 s and 111 s, respectively (Fig. 4.7i-l).

The observed fluorescence decay on cells could have been due to many factors other than the motion of the single nanotube in the direction perpendicular to the Au/Au surface. First, the change of pH from the PBS-dominant extracellular environment (pH = 7.4) to the more acidic environment of endosomes and lysosomes (pH ~ 5)\(^29\) could cause the decrease of the NIR-II fluorescence intensity of SWNTs. To rule out this possibility, we anchored the same SWNTs with activatable SH terminus on the Au/Au surface without involving any cells, soaked the Au/Au film in the 1x PBS solution, and then adjusted the pH of the immersion medium in the range
of 5 ~ 9. The lowest pH of 5 was intended to mimic the pH inside endosomes and lysosomes. Although some decrease of NIR-II fluorescence was observed for the SWNTs at both lower and higher pH values, this only comprised a reduction of 15% in fluorescence intensity at the maximum (Fig. 4.3), and thus had negligible contribution to the ~7-fold decay observed in Fig. 4.2j. Second, since the SWNT was most efficiently excited by polarized light with the angle of polarization parallel to the nanotube axis, the decay of nanotube fluorescence could be due to the rotation of the nanotube axis in the \( x-y \) plane and thus less efficient excitation of the individual SWNT. We performed a control experiment to turn the previously linearly-polarized excitation light into circularly polarized light (Fig. 4.4a), which exhibited equal component of the electric field strength along any given direction in the \( x-y \) plane (Fig. 4.4b&c). With this ‘depolarized’ excitation, we carried out the same single nanotube tracking experiment as with the linearly-polarized light (Fig. 4.4d), where very similar behavior of the fluorescence decay was found at 37 °C (1/e decay time = 114 s, Fig. 4.4e). This finding suggested minimum in-plane rotation of a single nanotube during the internalization process, which would otherwise cause huge fluctuation of the detected NIR-II fluorescence intensity as reported for freely-rotating SWNTs in water. This limited degree of rotation could be attributed to the local confinement of membrane-bound SWNTs through strong interactions between the SWNT-RGD conjugate and the integrin receptors on the cell membrane. Third, it remained possible for the membrane-bound individual SWNTs to change orientation from lying flat in the \( x-y \) plane to pointing to the \( z \)-direction during the uptake process, as observed for large-diameter, multi-walled carbon nanotubes (MWNTs), where the nanotube axis
turned perpendicular to the electric field vector of the excitation and thus became not excitable by the laser. However, we believe this is also unlikely for SWNTs with much smaller diameter, which could cause significantly elevated elastic energy,\textsuperscript{32} and for membrane-bound SWNTs with estimated rotation time of a few seconds, which disagreed with the time scale of our observed fluorescence decay (>100 s).

With these three possibilities causing the decrease of NIR-II fluorescence ruled out in the preceding discussions, the observed fluorescence decay shown in Fig. 4.2j could only be attributed to the axial motion of single carbon nanotubes away from the Au surface during the uptake process. Based on the experimental data and the ultra-sensitive dependence of fluorescence enhancement on nanometer distance changes between Au and SWNT, we proposed the following internalization pathway of SWNTs by live cells: when the $\alpha_v\beta_3$-positive U87-MG cells were incubated with SWNT-RGD conjugate at 4 °C, SWNT-RGD selectively bound to the $\alpha_v\beta_3$-integrin receptors on the cell membrane without crossing the membrane and entering the cytoplasm due to the low temperature of 4 °C that blocked the energy-dependent endocytosis process. When the temperature was elevated from 4 °C to 37 °C, the active uptake process of SWNTs was activated by the cell. Within the first 20 s, the membrane-bound single carbon nanotube was still sandwiched between the cell membrane and the Au/Au film (Fig. 4.2f), and this close proximity of the single nanotube to the Au surface led to the highest NIR-II fluorescence due to the maximum enhancement (Fig. 4.2c). It has been reported that the distance from the cell plasma membrane to the surface of a hydrophilic substrate could reach 4~8 nm in less than 5 min after sedimentation according to the Derjaguin–Landau–Verwey–Overbeek
Then the cells started the active uptake process by recruiting clathrin molecules and assembling them on the inner surface of the plasma membrane to form a clathrin-coated pit (Fig. 4.2g). The clathrin-coated pit caused the cell membrane to invaginate and drew the membrane-bound SWNT away from the Au surface, reducing the fluorescence signal of this specific single nanotube due to the less efficient coupling between Au and SWNT (Fig. 4.2d). Over time this clathrin-coated pit continued to grow and wrap around the single nanotube, before it finally pinched off and resulted in a complete vesicle enclosing the SWNT (Fig. 4.2h). Due to the formation of the vesicle inside the cytoplasm, at this point there were two lipid bilayers between the SWNT and the Au surface, with a total distance of >20 nm separating the SWNT from the Au surface. Owing to the ultra-sensitive ‘nanoscopic ruler’ of fluorescence enhancement, this large distance of separation led to no enhancement by the Au/Au substrate and the NIR-II fluorescence of this single nanotube almost vanished (Fig. 4.2e). Ehrlich et al. reported that this entire endocytosis procedure including an ordered sequence of highly regulated events usually took tens of seconds to a few minutes to complete, where the total time of endocytosis depended on the size of the cargo molecule, in good agreement with our observation (a total time of ~250 s for the NIR-II fluorescence decay to complete).

To further prove our observed NIR-II fluorescence decay was associated with the energy-dependent endocytosis process, we performed single nanotube tracking experiments on live cells at three other temperatures including 4 °C, 25 °C and 42 °C. It has been reported that active uptake is impaired at temperatures lower than 37 °C.
and is completely blocked at 4 °C, while cell functions become more active at higher temperatures until damages to the cells are induced by excessive heating.

In a typical single nanotube tracking experiment performed at 4 °C, a single carbon nanotube proved by polarization dependence (Fig. 4.5a) was tracked for >900 s as it moved on the cell membrane with an in-plane trajectory shown in Fig. 4.5b. In obvious contrast to the fluorescence decay curve in Fig. 4.2j, no significant decrease of the NIR-II fluorescence intensity associated with this specific nanotube was found except for some fluctuations in the fluorescence intensity (Fig. 4.5c). This finding supported our hypothesis that endocytosis should be completely blocked at 4 °C, while the random fluctuations in the fluorescence intensity was attributed to the transient changes of the electrostatic environment surrounding this specific single nanotube. NIR-II fluorescence fluctuation with similar amplitude was found for a single carbon nanotube anchored on the Au/Au substrate in the absence of any cells (Fig. 4.6), suggesting the biological environment near the cell membrane was not unique to cause the intensity fluctuation. The single nanotube tracking at 4 °C on the Au/Au film was repeated with two other independent experiments and similar fluorescence intensity plot versus time was found with no decay of signal (Fig. 4.7a-d).

When single nanotube tracking was performed at 25 °C, a much longer 1/e decay time of 327 s was found than that at 37 °C (100~120 s), further confirming the transmembrane motion of individual SWNTs was energy-dependent (Fig. 4.5d-f). At an elevated temperature of 42 °C, we saw a precipitous decrease of the nanotube fluorescence intensity within only ~50 s (Fig. 4.5g-i), consistent with the previous finding that the endocytosis of mammalian cells was enhanced at 42 °C without
significant damages.\textsuperscript{39} The single nanotube tracking at 25 °C and 42 °C on the Au/Au film was repeated with two more independent experiments for each temperature (\textbf{Fig. 4.7e-h} & \textbf{Fig. 4.7m-p}). To show the disappeared SWNT still existed inside the cell rather than having drifted away from the field of view, we imaged a cell by using a 20-fold higher excitation power density to recover the signal of a ‘disappeared’ single nanotube after its signal vanished after endocytosis (\textbf{Fig. 4.8}).

Control experiments of single nanotube tracking were also carried out for SWNT-RGD-stained live U87-MG cells on bare glass at all temperature to match the conditions used for tracking on the Au/Au film (\textbf{Fig. 4.9}). It was noteworthy that no significant fluorescence decay of single carbon nanotubes was observed for cells placed on bare glass over much longer tracking times, suggesting the distance-dependent MEF of SWNTs on the Au/Au enhancing substrate was the only cause of the drastic fluorescence decrease during the transmembrane displacement of membrane-bound single nanotubes.

The single nanotube imaging and tracking on cells placed on the enhancing Au/Au substrates was reproduced with three independent experiments for each temperature (\textbf{Fig. 4.7}), from which average $1/e$ decay times representing the different kinetics of endocytosis at 25 °C, 37 °C and 42 °C were derived (\textbf{Fig. 4.10a}). The temperature-dependent rate of endocytosis clearly revealed an energy dependent process of single nanotube internalization, with an activation barrier of 120±37 kJ/mol for this process based on Arrhenius fitting (\textbf{Fig. 4.10b}). It was noteworthy that this extracted activation barrier for SWNTs was significantly higher than reported for
some proteins undergoing the same clathrin-mediated endocytosis \[ E_a \approx 55 \text{ kJ/mol} \] for mannosylated albumin with a molecular weight (MW) of \(~67 \text{ kDa}\), and \[ E_a \approx 40 \pm 13 \text{ kJ/mol} \] for horseradish peroxidase with an MW of \(~44 \text{ kDa}\), which could be attributed to the much larger MW of \(~1 \text{ MDa}\) for SWNTs than the proteins.

We have also analyzed the mean square displacement (MSD) of the in-plane 2D trajectories of single carbon nanotubes moving on the membrane at different temperatures to verify the energy-dependent endocytosis process. **Fig. 4.10c** shows the curves of MSD plotted as a function of lag time at different temperatures, where one can see an almost linear increase of MSD mixed with a small quadratic component in the initial phase for all temperatures, indicating dominant Brownian motion of the single nanotubes at the beginning of the tracking experiment when the vesicles formed gradually.\(^42\) We extracted the Brownian diffusivity (diffusion constant) at each temperature and found our measured diffusivity \((10^3 \text{ to } 10^4 \text{ nm}^2/\text{s})\) agreed well with that of membrane receptors \((10^4 \text{ nm}^2/\text{s})\),\(^43\) indicating the membrane-bound SWNT-RGD formed a strong complex with the membrane receptors so the motion of the single nanotube was dominated by that of the receptor. The measured diffusivity of SWNTs was also found 2-3 orders of magnitude smaller than that of free SWNTs in water \((>1 \mu\text{m}^2/\text{s})\),\(^30\) further confirming the confined motion of carbon nanotubes on the cell membrane and the hindered rotation of SWNT during endocytosis.

After the initial phase of the dominant Brownian motion of the SWNT, an abrupt turning point followed by a sharp increase was observed in the MSD plot for temperatures of \(25 \text{ °C}, 37 \text{ °C}\) and \(42 \text{ °C}\) (red, blue and pink solid curves in **Fig. 4.10c**, \(40\).
respectively). The MSD curve after the turning point fit nicely to a quadratic function with significantly increased convective diffusivity (compare the red and blue bars in Fig. 4.10d). Taken together, we rationalized the initial phase of dominant Brownian motion as the vesicle formation process, while the sharp turn indicated the complete formation of the vesicle encapsulating the SWNT, followed by convective diffusion corresponding to the active vesicular transport by motor proteins into the cytoplasm.\textsuperscript{3,44} Moreover, the MSD curve at 4 °C showed a continuous Brownian motion without obvious change of the diffusion pattern, in good agreement with our hypothesis that the endocytosis of SWNTs should be completely blocked at 4 °C.

It has been reported that the cells initiate the endocytosis process by recruiting clathrin molecules on the inner surface of the cell plasma membrane.\textsuperscript{4} Therefore to further confirm our observation of the nanotube fluorescence decay on the Au/Au substrate was due to endocytosis, we employed two methods, sucrose hypertonic treatment and potassium depletion, to prevent the formation of the clathrin lattice on the membrane and thus perturb the endocytotic pathway.\textsuperscript{45} The results shown in Fig. 4.11a-d suggested no fluorescence decrease for membrane-bound single nanotubes placed on the Au/Au film over much longer tracking times than previous experiments at the same temperature of 37 °C, indicating successful blocking of the endocytosis process with both treatments. The blocked endocytosis was attributed to abnormal polymerization of clathrin into empty microcages rather than normal clathrin-coated pits (Fig. 4.11e), induced by both the hypertonic incubation and potassium depletion treatments.\textsuperscript{45}
4.4 Conclusion

In this work, the ultra-sensitive distance dependence of NIR-II fluorescence enhancement of SWNTs on an Au/Au plasmonic substrate was exploited to probe the transmembrane motion of single carbon nanotubes with ~10 nm displacements via tracking the changes of NIR-II fluorescence intensity. The temperature dependent endocytosis rate by analyzing the fluorescence decay curve, and the temperature dependent diffusion pattern derived from the MSD analysis of the in-plane trajectory, both suggested the energy-dependent endocytosis as the cellular uptake pathway of single nanotubes. Negative control experiments exploiting sucrose hypertonic incubation and potassium depletion further confirmed the receptor-mediated endocytosis pathway involving the formation of clathrin-coated pits. We envisage this ‘nanoscopic ruler’ based on MEF of SWNTs allows for imaging many biologically significant processes in and out of the cell, including exocytosis, neuron firing and intracellular trafficking.
4.5 Figures

**Figure 4.1** | The Ruler Effect of the Fluorescence Enhancement of SWNTs. (a) A schematic drawing showing an SWNT (the red core) coated with a surfactant shell (the cyan shell) and placed on an Au/Au film with a dielectric layer of certain thickness $d$ separating the SWNT-surfactant complex from the gold surface. The green triangles denote the RGD ligands covalently attached to the PEG chains. (b) An AFM image of the SWNT-RGD conjugate deposited on a glass substrate. (c) An SEM image of the Au/Au film, with the inset showing the UV-Vis-NIR extinction spectrum of the Au/Au film. (d) The fluorescence enhancement factor of SWNTs on the Au/Au film.
plotted as a function of the Au-SWNT distance (black squares) and fitted to first-order exponential decay with a $1/e$ decay distance of ~6 nm (red curve).
Figure 4.2 | Single Nanotube Imaging and Tracking on Cells on the Au/Au film at 37 °C. (a) A fluorescence emission spectrum of a bright spot corresponding to a single SWNT on the cell shown in the inset image. (b) The photoluminescence (PL) of the single nanotube shown in a plotted as a function of polarization angle. The inset shows
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4.6 References


Chapter 5. *In Vivo* Vascular Imaging of Mouse Hind Limb Using Single-Walled Carbon Nanotubes in the Second Near-Infrared Window

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5.1 Introduction

Testing the efficacy of treatments for peripheral arterial diseases (PADs) is highly dependent on physiological imaging to visualize blood vessels and measure blood flow and tissue perfusion in a quantitative manner. However, current imaging modalities for assessing the anatomical vessel structures and the hemodynamics in small peripheral blood vessels in an *in vivo* setting with minimum invasiveness remain suboptimal.¹ For anatomical imaging of vasculature, X-ray mediated microscopic computed tomography (micro-CT) and magnetic resonance imaging (MRI) are capable of resolving features down to ~100 μm with almost unlimited penetration depth, but both imaging modalities are unable to image small vessels with <50 μm diameters and are limited by difficulties in evaluating vascular hemodynamics in real time owing to the long data acquisition time.² ³ For hemodynamic imaging and measurement, Doppler ultrasonography can measure fast blood flow with a high temporal resolution of ~1 kHz, but the spatial resolution of ultrasound remains subject to the depth of penetration and speckle artifacts.⁴ ⁵
In vivo optical imaging techniques based on fluorescence have inherent advantages over tomographic imaging methods such as CT and MRI, owing to the high spatial and temporal resolutions.6,7 As we have discussed in the preceding chapters, single-walled carbon nanotubes (SWNTs) are an optically active material with intrinsic fluorescence in the second near-infrared window (NIR-II window, 1.0–1.7 µm) when excited in the visible (400-750 nm) and traditional near-infrared window (NIR-I window, 750-900 nm) with large Stokes shifts of up to ~400 nm. The NIR-II imaging window benefits from deeper tissue penetration than the extensively explored NIR-I window,8-13 owing to the negligible tissue autofluorescence, minimum photon absorption14,15 and reduced scattering of longer emission wavelengths.16-19

In this chapter we exploit the NIR-II fluorescence of biocompatible SWNTs for in vivo hind limb vascular imaging and hemodynamic measurement in the context of a mouse PAD model, acute hind limb ischemia. By comparing the NIR-II imaging with micro-CT, ultrasound and laser Doppler, we will show that a single modality of NIR-II fluorescence imaging enables multifunctional capabilities with many strengths including high spatial resolution (~30 µm), fast acquisition rate (<200 ms per frame), good tissue penetration depth (millimeters below the skin), vessel type differentiation and blood flow quantification, allowing for unprecedented sharpness of visualizing subcutaneous vascular features with fluorescence-based imaging technique in vivo.
5.2 Materials and Methods

Preparation of water soluble and biocompatible SWNT-IRDye-800 conjugate. Raw high-pressure CO conversion (HiPco) SWNTs (Unidym) were bath sonicated in an aqueous solution containing 1 wt% sodium deoxycholate for 1 h, and ultracentrifuged at 300,000 g for 1 h to remove bundles and other large aggregates of SWNTs in the suspension. The supernatant was carefully collected and a mixture of DSPE-mPEG(5k) (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol, 5000)], Laysan Bio) along with DSPE-PEG(5k)-NH₂ (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)5,000], Laysan Bio) was added to the solution to reach a final DSPE-mPEG(5k) concentration of 0.75 mg/mL and a final DSPE-PEG(5k)-NH₂ concentration of 0.25 mg/mL. Note that to make biocompatible SWNTs without the IRDye-800 labels, 100% of the DSPE-mPEG(5k) was added to the supernatant to reach a final concentration of 1.0 mg/mL. Then the suspension was bath sonicated for 5 min, dialyzed against 1x PBS in a 3500MWCO dialysis membrane (Fisher) to remove sodium deoxycholate, and ultracentrifuged again at 300,000 g for 1 h to remove bundles and other aggregates formed during dialysis. The amine-functionalized SWNTs were conjugated further with IRDye-800 according to a previous protocol. In brief, the SWNT solution after dialysis and ultracentrifugation was washed with DIUF water in a 30k NMWL centrifugal filter unit to remove excess surfactant and concentrated to ~300 nM. 10× PBS was added to this solution to reach a PBS concentration of 1× with pH of 7.4, and then the solution was mixed with a DMSO solution of IRDye-800 NHS ester (LI-COR) at a concentration of 0.1 mM. The
mixture was allowed to sit for 1 h at room temperature before the reaction reached completion. Excess IRDye-800 was removed by filtration through a 100k NMWL centrifugal filter, and the as-prepared SWNT-IRDye-800 conjugate solution was stored at 4 °C and away from any light illumination to avoid photobleaching of IRDye-800.

**UV-Vis-NIR absorbance measurement of the SWNT-IRDye-800 solution.** UV-Vis-NIR absorbance spectrum of the SWNT-IRDye-800 solution was measured in the range of 500-820 nm by a Cary 6000i UV-Vis-NIR spectrophotometer, background-subtracted for any absorbance contribution from the solvent.

**NIR fluorescence spectroscopy of the SWNT-IRDye-800 solution.** The NIR fluorescence spectrum of the SWNT-IRDye-800 solution was taken on a home-built NIR spectroscopy setup. The excitation was provided by a white-light source of an ozone-free mercury/xenon lamp (Oriel) with a total power of 200 W, which was filtered by a UV filter to remove the ultraviolet light, a water filter to remove the short-wavelength infrared (SWIR) light (>1400 nm), and an 850 nm short-pass filter to remove the NIR light. The excitation light cleaned by these filters was then dispersed by a monochromator (Oriel) to generate a single-wavelength excitation with a center wavelength of 785 nm and a bandwidth of 15 nm, focused onto a solution containing the SWNT-IRDye-800 conjugate in a 1-mm path cuvette (Starna Cells, Inc.). The emitted fluorescence of the solution was collected in a transmission geometry at an angle of 180° to the incident excitation light. The transmitted excitation light was rejected using a 790-nm long-pass filter (Semrock) and collected by the spectrometer (Acton SP2300i) equipped with a one-dimensional InGaAs linear
array detector (Princeton OMA-V). The raw fluorescence spectrum was corrected post-collection to account for the extinction profile of the emission filter and the sensitivity of the detector using the MATLAB software.

**Determination of the cytotoxicity of SWNTs.** The *in vitro* cytotoxicity of SWNTs on human dermal microvascular endothelial cells (Lonza) was determined by an MTS assay using the CellTiter 96 kit (Promega). Approximately 5000 cells were plated into each well of the 96-well plate and incubated with 100 μl of EGM2MV growth media (Lonza). The cells were allowed to adhere to the bottom of each well before the growth medium was removed and replaced with the medium spiked with SWNTs at different concentrations (*n* = 3 for each concentration). The cells were kept in a 37 °C humidified incubator with 5% CO<sub>2</sub> in the presence of SWNTs at different concentrations for 24 h, before the SWNT-spiked medium was removed from each well and replaced with fresh cell medium. Immediately after the medium was replaced, 15 μL of CellTiter 96 was added to each well and used as the colorimetric indicator of cell viability. The cells were allowed to incubate with the CellTiter 96 for 1 h in a humidified, 5% CO<sub>2</sub> atmosphere at 37 °C, before the color change in each well was quantified with a 96-well plate reader, which measured the absorbance of the medium at 490 nm. Cell viability was plotted as a fraction of the absorbance of the control wells incubated without SWNTs.

**Laboratory mouse handling and surgery.** All experiments involving the use of laboratory animals were carried out under the approval of Stanford University’s Administrative Panel on Laboratory Animal Care. Female athymic nude mice (Charles River, 6-13 weeks old) were used in this study. Induction of unilateral hind limb
ischemia was performed according to our previously published protocols.\textsuperscript{21-23} A group of 4 control mice without receiving the surgery and a group of 3 mice with induced hind limb ischemia were used for the femoral vessel imaging and velocimetry study, while a group of 6 ischemic mice was used for both the short-term and long-term revascularization studies. For injection of the NIR-II contrast agent, a 28 gauge syringe needle was inserted into the lateral tail vein, and a bolus of 200 \( \mu \text{L} \) SWNT solution was allowed to be injected into the tail vein during the first frames of the dynamic NIR-II fluorescence imaging. All mice were anesthetized before surgery, injection and imaging in a rodent anesthesia machine supplying a mixed gas flow of 2 L/min O\textsubscript{2} and 3\% Isoflurane. To keep the mouse anesthetized during imaging, a nose cone was used to cover the face of the mouse and deliver a mixed gas flow of 1.5 L/min O\textsubscript{2} and 3\% Isoflurane.

\textit{In vivo static fluorescence imaging in the NIR windows.} For static fluorescence imaging of live animals in the NIR-I and NIR-II windows, the mouse was placed on a heated stage one at a time in the supine position at approximately 10 min post injection of the fluorophores. The excitation was provided by a 785-nm laser (Renishaw), fiber-coupled to a collimator with a focal length of 4.5 mm (Thorlabs), cleaned by a 790-nm bandpass filter with a 10-nm bandwidth (Thorlabs) to remove the long-wavelength photons, and directed to heated stage with a power density of 8 mW·cm\textsuperscript{-2}. For fluorescence imaging in the NIR-I window, the emitted photons from the animal were filtered through a 790-nm long-pass filter (Semrock) and an 850-nm short-pass filter (Thorlabs) to collect NIR-I fluorescence in the 790-850 nm region, and focused onto a silicon CCD camera with a two-dimensional (2D) array of 1344 ×
1024 pixels (Hamamatsu). For fluorescence imaging in the NIR-II window, the emitted photons from the animal were filtered through a 900-nm long-pass filter (Thorlabs) and an 1100-nm long-pass filter (Thorlabs) to collect NIR-II fluorescence in the 1100-1700 nm region (note the upper bound of detection range was determined by the sensitivity profile of the camera), and focused onto a 2D indium-gallium-arsenide (InGaAs) array of 320 × 256 pixels (Princeton Instruments). A silver mirror fixed on a flip mount was used to direct the emitted fluorescence to the Si or the InGaAs camera.

Different in vivo imaging magnifications were obtained by using different lens sets. The magnification of 1× corresponded to the field of view that covered the entire mouse body and was achieved by using two touching achromats with focal lengths of 75 mm and 200 mm (Thorlabs). The 2.5× magnification included one hind limb of the mouse in the field of view at a time, and was achieved by placing the 200 mm achromat approximately 200 mm away from the 75 mm achromat. The 7× magnification covered only part of the hind limb and was obtained by using two achromats with focal lengths of 150 mm and 200 mm and separated by ~150 mm.

For NIR-I fluorescence images captured by the Si camera, a binning of 4 was used to reduce the array size from the original 1344 × 1024 pixels to 336 × 256 pixels, matching that of images taken by the InGaAs camera, which had an array size of 320 × 256 pixel under a binning of 1.

In vivo dynamic fluorescence imaging in the NIR-II window. The video-rate dynamic fluorescence imaging was carried out on the same imaging system as in the
static fluorescence imaging, employing the InGaAs camera only in the NIR-II window. The excitation was provided by an 808-nm laser (RMPC lasers), fiber-coupled to a collimator with a focal length of 4.5 mm (Thorlabs), cleaned by an 850-nm short-pass filter and a 1000-nm short-pass filter (Thorlabs) to remove the long-wavelength photons, and directed to the mouse in the supine position and mounted on the heated stage to reach a power density of 140 mW·cm⁻². The same emission filters as used for the static NIR-II fluorescence imaging as aforementioned were employed here to filter out the excitation light. A magnification of 2.5× was used for the hind limb video-rate imaging of femoral blood flow, while a magnification of 1× was used for the dynamic tissue perfusion imaging with internal comparison between the control limb and the ischemic limb. Different magnifications were realized by choosing the right lens pair specified in the preceding paragraphs. To capture video-rate images, the 2D InGaAs detector was allowed to expose continuously with an exposure time of 100 ms immediately after a bolus injection of 200 μL of the SWNTs at 0.10 mg/mL, and the images were acquired by a user-written LabVIEW software with an additional overhead time of 87.5 ms between two consecutive frames. The exposure time and the overhead time comprised a complete cycle of 187.5 ms for each image acquisition with a frame rate of 5.3 frames/s.

The video-rate images taken in the aforementioned manner were used for dynamic contrast-enhanced visualization using principal component analysis (PCA), in a similar way to previous publications by the Hillman group²⁴ and our group.¹⁷ In a typical image processing task by the MATLAB software, the first 200 frames immediately after SWNT injection were loaded into a three-dimensional (3D) array
and reshaped into a 2D array, which was fed into the MATLAB built-in `princomp` function for PCA analysis. From the output of the `princomp` function, we took the negative fourth principal component to represent the pixels appearing early in the 200 frames and assigned them the arterial features (color-coded in red), while the negative second principal component represented the pixels appearing later in the 200 frames and was thus assigned the venous features (color-coded in blue).

Quantification of blood velocity and tissue perfusion based on dynamic NIR-II fluorescence imaging. The time-course NIR-II fluorescence images obtained from the dynamic NIR-II fluorescence imaging were first loaded into the MATLAB software. For the quantification of blood velocity in the femoral artery, the average NIR-II intensity within a certain region of interest (ROI) corresponding to the femoral artery was computed for all time-course images and plotted as a function of time post injection. The boundary of the selected ROI of the femoral artery was determined by PCA. For the quantification of tissue perfusion, the average NIR-II fluorescence intensity within symmetric ROIs in both control and ischemic hind limbs was extracted from the time-course NIR-II images. The average NIR-II intensity was normalized against the maximum intensity in the entire time course for the blood velocity quantification, and against the maximum intensity of the control hind limb for the tissue perfusion measurement when plotted against time, in order to compensate for the difference in the amount and the brightness of the injected SWNTs. The normalized NIR-II intensity was expressed in units of %. The intensity-time plot usually featured a linear rise phase, followed by a decrease of fluorescence intensity or a plateau region. We used the linear rise phase to fit into a linear function with its
slope in units of %/s, which could be translated into the corresponding blood velocity in units of cm/s by applying the intensity-to-velocity conversion coefficient in units of cm%/%. The blood flow ($F$, in ml/min) was related with the blood velocity ($v$, in cm/s) via the following equation:

$$F = \pi \left( \frac{d}{2} \right)^2 \cdot v \cdot 6 \times 10^{-7}$$

where the vessel diameter is expressed in units of μm. The relative tissue perfusion ($RTP$, in percentage) was derived from the linear-fit slopes of the ischemic limb ($slope_{\text{ischemic}}$) and the control limb ($slope_{\text{control}}$) as follows,

$$RTP = \frac{slope_{\text{ischemic}}}{slope_{\text{control}}} \times 100\%$$

On the other hand, the intensity-to-velocity conversion coefficient was validated in a simplified tubing flow experiment as follows. An aqueous solution of SWNTs with a certain concentration was propelled into a water-filled thin catheter tubing with known diameter by a syringe pump at a preset velocity. The ‘standard condition’ referred to the following parameters: SWNT concentration = 0.10 mg/ml, SWNT fluorescence quantum yield (QY) ≈ 2.5%, tubing diameter = 760 μm, tubing length within ROI (i.e., ROI length) = 2.5 cm, fluid velocity = 1.4 cm/s and tubing length before ROI (i.e., pre-ROI length) = 8.5 cm. To find out what variables among the aforementioned parameters affected the intensity-to-velocity conversion coefficient, only one of these parameters was changed at a time: SWNT concentration changed to 0.025 mg/ml, SWNT fluorescence QY changed to 5.0%, tubing diameter changed to 380 μm, ROI length changed to 1.25 cm, fluid velocity changed to 0.14
cm/s and pre-ROI length changed to 19.5 cm. Under the ‘standard condition’ and each changed condition, the average NIR-II fluorescence intensity within the ROI length was computed by MATLAB, normalized against the maximum intensity in the plateau region, and plotted as a function of time. The linear rise phase at the beginning of the curve was fit into a linear function with its slope in units of %/s, which was used to divide the preset fluid velocity in units of cm/s to derive the intensity-to-velocity conversion coefficient in units of cm/%.

The intensity-to-velocity conversion coefficient was also validated by numerical simulation, where a linear flow model with axial mixing in a tubing system was used. The distribution of NIR-II fluorescence intensity as a function of axial position at the flow front inside the tubing was simulated with a sigmoidal function of time and velocity as follows:

\[
F(x, v, t) = \frac{I \cdot \varepsilon cd \cdot QY}{1 + e^{\frac{x}{vt}}} \]

where \( I \) is the power density of the excitation light, \( \varepsilon \) is the absorption coefficient of the SWNT solution at the excitation wavelength, \( A_0 \) (~0.001 cm) is the initial degree of mixing, \( K \) (~0.5) is the mixing constant, \( c \) is the SWNT concentration, \( d \) is the diameter of the tubing, \( QY \) is the fluorescence quantum yield of SWNTs, and \( v \) is the velocity of the SWNT solution moving inside the tubing. In the simulation we varied \( c, d, QY \) and \( v \) to find which variable affected the conversion coefficient. The normalized NIR-II fluorescence intensity in a certain ROI with length of \( L_{ROI} \) was computed numerically in MATLAB as follows and plotted against the time \( t \):
\[ I_{norm}(t) = \frac{\int_{x=L_{roi-ROI}}^{x=L_{roi+ROI}} F(x, v) dx}{\int_{x=L_{roi-ROI}}^{x=L_{roi+ROI}} F(x, v, +\infty) dx} \]

Similar to the experiments, the linear rise phase at the beginning of the \( I_{norm}(t) \) curve was fit to a linear equation, with its slope extracted to derive the intensity-to-velocity conversion coefficient:

\[ \text{Coeff.} = \frac{\nu}{\hat{C}t} \]

**Micro-CT imaging of hind limb vasculature.** Micro-CT images were acquired with a micro-CT scanner (MicroCAT II, Siemens Preclinical Solutions), where the different parameters were set as follows: X-ray voltage 80 kVp, anode current 50 mA, exposure time 2 s per acquisition, and total acquisitions 576 frames through the entire 360° rotation. At 1 h before micro-CT scanning, the mouse was intravenously injected with Fenestra VC (Advanced Research Technologies), an iodine-based blood pool contrast agent at an injection dose of 0.3 ml/20 g body weight through a 28-gauge syringe needle. To prevent embolization, the Fenestra was injected slowly into the tail vein over a period of 30-60 s. The 3D images were reconstructed by COBRA 1.5 with a voxel size of 40 μm, and visualized using Amira 5.4.

**Blood velocity quantification based on ultrasound.** Ultrasound measurements of the femoral artery blood velocity were carried out using a linear real-time transducer (40 MHz) connected to a Vevo 2100 ultrasound system (VisualSonics). We identified the femoral artery by Duplex-ultrasonography (B-Mode and power Doppler) and recorded
the blood flow profiles by cw-Doppler imaging. The Vevo 2100 device software was employed to measure important parameters such as the velocity-time integrals (VTI) and cardiac cycle length (CL). Then the femoral flow ($F$) was derived as follows,

\[ F = SV \times HF = CSA \times VTI \times HF = \frac{\pi \left( \frac{d}{2} \right)^2 \cdot VTI \cdot 6 \times 10^4}{CL} \]

where $SV$ denotes the stroke volume, $HF$ denotes the heart frequency, $CSA$ denotes the arterial cross-sectional area, and $d$ is the arterial diameter and could be obtained from NIR-II fluorescence imaging. The femoral flow measurement was averaged from three cardiac cycles.

**Tissue perfusion measurement based on laser Doppler.** The tissue perfusion of mouse hind limb was measured with laser Doppler spectroscopy (PeriSCan PIM3, Permed AB). Before measurement, the mice were anesthetized in a rodent anesthesia machine under a mixed gas flow of 1 L/min O$_2$ and 3% Isoflurane. The animals were pre-warmed by a heating pad to 37.5 °C body temperature and then placed in the supine position during the laser Doppler measurement. Recovery of hind limb tissue perfusion over time was expressed as the ratio of tissue perfusion in the ischemic limb over that in the the non-ischemic, control limb.$^{21}$

**Fluorescent microbead based tissue perfusion measurement.** We used the fluorescent bead perfusion assay to validate the measured $RTP$ from NIR-II fluorescence imaging. A short-term study for up to 10 days after the induced acute hind limb ischemia was performed. On postoperative day 0, 3, 7 and 10, the mice were anesthetized and injected intracardially with the $5\times10^5$ fluorescent microbeads (blue-
green FluoSpheres 15 µm diameter), heparin (2000 U) and nitroglycerin (500 mg) sequentially. Then the mice were euthanized, and then the injected microbeads trapped in the tissue were extracted by digestion in a 0.5 M KOH solution at 52 ºC, dissolved in cellosolve acetate, and quantified using a fluorescence plate reader, according to previously established protocols.\textsuperscript{26} RTP in this method was calculated as follows,

\[ RTP = \frac{FL_{\text{ischemic}}}{FL_{\text{control}}} \times \frac{m_{\text{control}}}{m_{\text{ischemic}}} \]

where the FL denotes the fluorescence intensity, which was normalized against the total mass of the excised tissue, \( m \).

**Microvascular density quantification with immunofluorescence.** Microvascular density in the ischemic hind limb on different postoperative days was quantified with immunofluorescence staining and imaging to validate the NIR-II fluorescence based vessel number quantification. In brief, the ischemic gastrocnemius tissues were dissected, frozen, and sectioned into slices for immunofluorescence staining for the endothelial biomarker CD31. A minimum of 3 slices were stained and quantified for each mouse. The slices were imaged under a fluorescence microscope, where the blood vessels with CD31-expressing endothelial cells were quantified for microvascular density in units of vessel number/mm\(^2\).\textsuperscript{21}

### 5.3 Results and Discussion

To show the benefits of NIR-II fluorescence over NIR-I for *in vivo* animal imaging, we exploited the dual-color emission of the SWNT-IRDye-800 conjugate
(see Section 5.2 for its synthesis) with the SWNT backbone emitting in the >1000 nm NIR-II window and the IRDye-800 label fluorescing at ~800 nm in the NIR-I window, when the conjugate was excited by the same 785-nm laser (Fig. 5.1a&b). This bicolor conjugate with fluorescence emission in both NIR-I and NIR-II windows helped ensure the co-localization of SWNTs and IRDye-800 molecules, enabling us to visualize the same area of interest (e.g., same blood vessel or tissue) in the two different spectral windows and to evaluate the imaging performance of photons with distinct wavelengths in an in vivo whole-animal setting.

For in vivo live mouse imaging in the two NIR windows, a 200 μL solution of SWNT-IRDye-800 conjugates at a mass concentration of 0.10 mg/mL with bright NIR-II fluorescence shown in Fig. 5.1c was injected intravenously to reach an equivalent mass dose of 1.0 mg/kg body weight. After a roughly 10-fold dilution of the injected material by blood, the maximum SWNT concentration in the mouse circulation was estimated as ~17× lower than the half maximal inhibitory concentration (IC50) of microvascular endothelial cells (Fig. 5.2), suggesting minimum toxicity to the organism. After the injection of the SWNT-IRDye-800 conjugates, there should be a time window of a few hours within which the vascular imaging could be carried out with sufficient image quality, given the circulation half-life of DSPE-mPEG-coated SWNTs was ~5 h. The mouse injected with SWNT-IRDye-800 was illuminated by a 785-nm laser at a power density of 8 mW/cm², and the emitted fluorescence was collected in the NIR-I window with a Si camera and in the NIR-II window with an InGaAs camera (Fig. 5.1d).
The fluorescence images taken in the NIR-I window by collecting the fluorescence from the IRDye-800 labels exhibited blurry features and indistinct vascular anatomy at all three different magnifications including 1× (whole body, Fig. 5.1e top), 2.5× (entire hind limb, Fig. 5.1f top) and 7× (partial hind limb, Fig. 5.1g top). Their corresponding line intensity profiles all featured very broad peaks owing to the shorter-wavelength NIR-I photons that underwent significant scattering and absorption of the turbid tissue (Fig. 5.1e–g, bottom). In striking contrast, the same mouse imaged in the longer-wavelength, NIR-II region by collecting fluorescence from the SWNTs exhibited much crisper images of vascular structures at all magnifications (Fig. 5.1h–j, top). Furthermore, many smaller, higher-order branches of the blood vessels were clearly resolved and visualized in the NIR-II window at higher magnifications (Fig. 5.1i&j, top), owing to the substantially reduced scattering of photons.17 Cross-sectional line intensity profiles of the NIR-II vascular images all manifested sharp peaks corresponding to blood vessels intersected by the lines, with the Gaussian-fitted vessel widths well matching the expected diameters (Fig. 5.1h–j, bottom). It is noteworthy that it was impossible to measure vessel widths from the NIR-I images, where on average a 2~3-fold broadening of the original vessel width was observed.

Micro-CT is a widely-used imaging modality with superb penetration depth for visualizing anatomical features deep inside the body, but possesses its own limitations such as the sub-optimal spatial resolution (~100 μm) and long scanning and post-processing times (minutes to hours).27,28 To show the strengths of NIR-II fluorescence imaging over micro-CT, we quantitatively compared the spatial resolution of the same
proximal femoral vessels in a mouse, imaged by both NIR-II and micro-CT methods (Fig. 5.3a&b). A line was drawn in both images to intersect the proximal femoral artery and vein perpendicularly, and the line cross-sectional intensity profiles shown in Fig. 5.3c&d both revealed two peaks corresponding to the two femoral vessels with very similar vessel widths extracted from Gaussian fit to these profiles. Therefore, the vessel width analysis based on the micro-CT images (0.292 mm and 0.247 mm, Fig. 5.3d) validated the accuracy of NIR-II fluorescence based imaging and measurement (0.284 mm and 0.255 mm, Fig. 5.3c), suggesting both techniques were comparable in visualizing vascular features with sizes >100 μm.

We then compared the spatial resolution limits for both NIR-II and micro-CT methods by determining the smallest discernible vessels that could be resolved by these two imaging modalities. We imaged the distal region of the same mouse hind limb using both techniques, and observed a greater number of vascular branches in the NIR-II image (Fig. 5.3e) than in the micro-CT image (Fig. 5.3f). Line intensity profile intersecting a small vessel in the NIR-II image clearly revealed a Gaussian fit diameter of ~35.4 μm (Fig. 5.3g), approximately 3× smaller than the smallest discernible vessel in the micro-CT image with a Gaussian-fit width of ~104 μm (Fig. 5.3h).

To show that NIR-II fluorescence imaging was able to penetrate millimeters depth inside the turbid biological tissue without losing the image sharpness, we carried out experiment to find out the depth of the imaged femoral vessels underneath the skin. We intravenously injected a nude mouse with a 200 μL solution containing 0.10 mg/mL SWNTs and then imaged the mouse at 1.5 h post injection (p.i.). The NIR-II
images clearly resolved both femoral vessels and the femur in the two hind limbs (Fig. 5.4a&b), suggesting the SWNTs still circulating inside the blood vessels at the time of imaging. Then this mouse was sacrificed and frozen in liquid nitrogen to fix the SWNTs inside the femoral vessels. We first dissected the left hind limb by cutting the limb perpendicularly to the direction of the femur and the femoral vessels. By transection of the limb in the middle of the thigh, the cross-section was imaged in the NIR-II window with both the femoral vessels and the femur showing up in the image (Fig. 5.4c&d). We measured the distance from the femoral vessels to its closest skin surface in the image (Fig. 5.4c) and found the femoral vessels were located at a depth of ~1.5 mm underneath the skin. In a similar way the depth of the femur was determined as ~2.9 mm underneath the skin. The depth measurement based on transection images was also confirmed by dissecting the right hind limb parallel to the direction of the femur, and we found that the features corresponding to the femoral vessels disappeared after removing four slices of 0.5 mm thickness (Fig. 5.4f), verifying the depth of the femoral vessels located between 1.5 mm (three slices) and 2.0 mm (four slices).

Besides the higher spatial resolution, NIR-II fluorescence imaging also generated these static vascular images with much shorter acquisition time (~300 ms) than micro-CT (~2 h). The bright NIR-II fluorescence of SWNTs under an increased excitation power density of 140 mW·cm$^{-2}$ allowed us to record blood flow in real time with even shorter exposure time of 100 ms, affording dynamic imaging of vasculature immediately after injection of SWNT fluorophores into the tail vein. When a 200 µL bolus of SWNTs at a concentration of 0.10 mg/mL was injected into a nude mouse
(Mouse C1), an NIR-II signal was first observed in the proximal femoral artery within 5 s after injection (Fig. 5.5a), before more signals showed up in the entire femoral artery and part of the proximal musculature subserved by the femoral artery at ~8 s p.i. (Fig. 5.5b). We then observed the outflow of the SWNT fluorophores into the femoral vein, which was evidenced by the increased width of the vascular feature in the middle of the limb corresponding to the vascular bundle in the femoral sheath at a later time point of ~37 s (Fig. 5.5c). The close proximity between the femoral artery and vein inside the femoral sheath made it difficult to distinguish one from the other, but we exploited their temporal difference revealed by the dynamic imaging that the appearance of the femoral artery preceded that of the femoral vein, to discriminate the two types of vessels based on PCA.\(^{17,24}\) PCA is a useful mathematical tool that assigns all pixels in an image into different groups (i.e., the principal components or PCs) with all pixels in each group possessing similar variance in time. Fig. 5.5d revealed an overlaid image for the arterial component (red) and the venous component (blue) extracted from the PCA analysis, with clear differentiation of the two vessel types. The video-rate dynamic imaging and PCA analysis were reproduced on three other mice (Mouse C2-4) and the results are shown in Fig. 5.6a-f.

We applied the dynamic NIR-II fluorescence imaging in mice with surgically induced acute unilateral hind limb ischemia (named ‘ischemic mice’, Mouse I1–3). In this mouse ischemia model for PAD, the mouse proximal superficial femoral artery was ligated and excised, while the deep femoral artery was only ligated, leading to a reduction of hind limb blood perfusion by ~80% immediately postoperatively.\(^{21}\) We injected these ischemic mice with SWNT fluorophores on the first postoperative day.
of the surgery, and observed a substantial delay of the arterial inflow into the hind limb, evidenced by the much later appearance of the NIR-II signal in the femoral artery (Fig. 5.5e-g) than that in the control, healthy mice (Mouse C1-4). In a similar manner to the control, healthy femoral blood flow, we also performed PCA on this ischemic hind limb and only observed a dominant red color corresponding to arterial flow within the analyzed time of the first ~37.5 s p.i., during which time the venous return had not occurred owing to the marked delay of blood perfusion (Fig. 5.5h). We allowed the InGaAs camera to continue capturing images for this ischemic hind limb over a much longer period of > 4 min and observed the blood return through the femoral vein at a much later time (~2 min p.i.) than in the control hind limb, suggesting the acute hind limb ischemia indeed caused significant delay of the blood perfusion (Fig. 5.7). The dynamic NIR-II fluorescence imaging was also repeated on an additional two mice with induced hind limb ischemia (Fig. 5.6g-j).

In addition to dynamic NIR-II imaging of a small region covering the hind limb only, we also performed the video-rate imaging of a larger area of the mouse body including both the femoral and part of the abdominal regions. The time course NIR-II fluorescence images showed the appearance of the aorta, as well as the inferior epigastric, external iliac and femoral arteries within 5 s p.i. of the SWNT fluorophores (Fig. 5.8a&b). Following the arterial inflow of the blood carrying the SWNTs, signals were observed in the surrounding tissues subserved by these arterial vessels (Fig. 5.8c), and later in the femoral and inferior epigastric veins that drained these regions (Fig. 5.8d). These arterial and venous conduits observed sequentially in the dynamic NIR-II imaging were clearly differentiated in the PCA overlaid image owing to their temporal
difference (Fig. 5.8e). It is noteworthy that the aorta was positioned at a depth of >5 mm in a 1-cm-thick mouse in its supine position (Fig. 5.8f), and the fact that we could resolve the aorta from the early frames of the dynamic imaging (Fig. 5.8b) suggested a penetration depth of >5 mm by NIR-II fluorescence imaging.

The fast, video-rate NIR-II fluorescence imaging allowed us to quantitate blood velocity and volumetric flow in the femoral artery. In a time series of the NIR-II fluorescence images taken on the ischemic Mouse I, owing to the markedly reduced blood perfusion, the propagation of the signal marking the flow front of the blood inflow could be clearly resolved from 12 s to 19.5 s p.i. of SWNTs (Fig. 5.9a). Then we extracted the location of the flow front from each frame and plotted it against time p.i., which showed a linear increase of the traveled distance of the signal as a function of time. The raw data was fit into a linear equation with the slope corresponding to a blood velocity of 0.163 cm/s inside the ischemic femoral artery (Fig. 5.9b). The volumetric blood flow was related to the linear blood velocity by the cross-sectional area of the blood vessel (see Section 5.2 for more information). The mean diameter of the femoral artery was measured as 174 μm from the NIR-II images, which resulted in a femoral arterial blood flow of 2.33x10⁻³ mL/min.

Unlike the reduced blood velocity in the ischemic femoral artery, we found that the progression of the flow front labeled by the NIR-II signal through the normal, healthy femoral artery was too rapid with a speed beyond the temporal resolution of our current dynamic imaging capability. Therefore, we resorted to an alternative method to evaluate the fast blood velocity in the control, healthy hind limb by
correlating the average NIR-II intensity increase with the blood velocity in a narrow ROI corresponding to the femoral artery. To compensate for the variation in the brightness of the injected NIR-II fluorophore and the actual injected dose, the average NIR-II fluorescence intensity was normalized against the maximum in the entire time course and plotted against the p.i. time (see Section 5.2 for more details). We first established the relationship between the NIR-II intensity increase with the femoral arterial blood velocity on the ischemic Mouse I1, by extracting an average NIR-II intensity increase of 2.18%\/s (Fig. 5.9c) corresponding to an average blood velocity of 0.163 cm/s. Then we plotted the traveled distance of the flow front against the NIR-II intensity and observed a linear relationship between these two, with a fitted slope of ~0.0737 cm/% (Fig. 5.9d). This slope allowed us to convert the percent NIR-II intensity increase per unit time to the travelled distance of blood per unit time, which was the blood velocity by definition. We validated the accuracy and reproducibility of this conversion coefficient with an additional two ischemic mice (Mouse I2 and I3) and obtained an average conversion coefficient of 0.0747±0.0019 cm/% (Fig. 5.10 and Table 5.1).

We applied the as-derived intensity-to-velocity conversion coefficient to the rapid blood flow of femoral artery in a healthy, control mouse (Mouse C1). Time course NIR-II fluorescence images in Fig. 5.9e revealed a very fast propagation of the blood flow without showing discrete signal front as observed in the slow blood flow case. To circumvent this problem we extracted the average NIR-II intensity within the ROI of the femoral artery, and derived the rate of the normalized NIR-II intensity increase as 68.7±5.2%/s from the linear rise region of the curve (Fig. 5.9f). With the
conversion coefficient of 0.0747 cm/%, one immediately obtained the velocity of 5.13±0.39 cm/s by multiplying the normalized NIR-II intensity increase rate by the coefficient. This measured femoral blood velocity was validated against a standard method, Doppler ultrasound run by a blinded operator on the same femoral artery of this mouse. For the same Mouse C1, the ultrasound method measured a Doppler-derived femoral blood velocity of 4.97±0.17 cm/s (Fig. 5.9g), in good agreement with the result derived from the dynamic NIR-II imaging method with a deviation of merely ~3%.

We further validated our intensity-to-velocity conversion coefficient in a simplified tubing flow experiment, where the SWNT solution was pumped into a thin catheter tubing pre-filled with pure water (Fig. 5.11a-c), and derived the conversion coefficient based on the measured rate of NIR-II intensity increase and the preset fluid velocity (see Section 5.2). By varying the experimental parameters one at a time, we found the coefficient (0.0764±0.0025 cm/%) was independent of the SWNT concentration, SWNT QY, tubing diameter, ROI length and velocity (Fig. 5.11d-i), but was only dependent on the pre-ROI length (i.e., the distance between the injection site and the start of the ROI) (Fig. 5.11j and Table 5.2). We also confirmed this finding by numerical simulations (Fig. 5.11k-s and Table 5.2) based on a linear flow model with axial mixing in a tubing system.25 For blood velocity quantification in the mouse femoral artery, the pre-ROI length should be invariant for the same type of animals since it simply reflected the distance the blood needed to travel from the injection site (the tail vein) to the femoral artery of interest. Therefore the intensity-to-
velocity coefficient was also the same among experiments and should be applicable from the ischemic mice to the control, healthy mice.

We then applied the validated intensity-to-velocity conversion coefficient of $0.0747 \pm 0.0019$ cm/\% to an additional three control mice (Mouse C2-4, Fig. 5.12) and compared the femoral velocities derived from NIR-II imaging to those from Doppler ultrasound technique with excellent agreement found between the two methods (Fig. 5.9h). We also carried out the femoral velocity measurements on three ischemic mice, and observed a substantial reduction in blood velocity in ischemic limbs by $\sim 30$ times (Fig. 5.9i), which was consistent with previous publications.\textsuperscript{21,22,29} It was also worth noting that the markedly reduced femoral blood velocity in the ischemic hind limb was very low and beyond the dynamic range of the measurable velocities by Doppler ultrasound, while our NIR-II imaging method was able to provide a much broader dynamic range for \textit{in vivo} blood velocity measurement.

NIR-II fluorescence was also employed to study the recovery of blood perfusion in the ischemic hind limb over the period of 10 days. We performed dynamic NIR-II fluorescence imaging and laser Doppler blood spectroscopy on a group of 6 mice on postoperative day 0, 3, 7 and 10 after the induction of acute hind limb ischemia, and found a clear recovery of blood perfusion in the ischemic hind limb from day 3 to day 10 after surgery (Fig. 5.13). One salient advantage of NIR-II fluorescence imaging over laser Doppler was the much crisper vascular images with higher spatial resolution that resembled angiograms, while the image quality of laser Doppler suffered from severe interference of the speckle artifacts.
To quantitate the tissue perfusion in the ischemic hind limb over time, we analyzed the normalized NIR-II intensity within symmetric ROIs in the control and ischemic hind limbs as a function of time immediately after injection on postoperative days 0, 3, 7, and 10 (Fig. 5.14a-d), by exploiting the high temporal resolution of dynamic NIR-II fluorescence imaging. The linear rising edge in each plot was extracted and fitted into a linear equation with its slope proportional to the average blood velocity within that specific ROI, according to the previously derived intensity-to-velocity conversion coefficient. Therefore, the blood perfusion in the ischemic hind limb could be quantified by internal comparison with the control limb on the same postoperative day, and expressed as the ratio of the linear-fitted slope of the ischemic limb to that of the control limb. The bar chart shown in Fig. 5.14e clearly revealed a statistically significant increase in blood perfusion in the ischemic hind limb tissue over a course of 10 days post-surgery (blue bars), along with similar trends demonstrated by laser Doppler spectroscopy (red bars) and fluorescent microbead based perfusion analysis (green bars).

It was noteworthy that there were consistent differences between these three methods, where the laser Doppler method exhibited the highest tissue perfusion values while the microbead method showed the lowest. We attributed these systematic differences of blood perfusion measurements to the different tissue sampling depths of these methods: laser Doppler had the most superficial penetration depth due to the use of red laser light with center wavelength of 650-690 nm, NIR-II fluorescence imaging probed tissue perfusion at deeper penetration depths than laser Doppler by employing longer-wavelength, NIR-II photons in the range of 1100-1400 nm with reduced
scattering,\textsuperscript{16-18} and the microbead method was able to measure tissue perfusion at any depth underneath the skin at the cost of sacrificing the animal and excising the desired tissue. As a result, both laser Doppler and NIR-II imaging were surface-weighted imaging techniques although a more volumetric assessment of tissue perfusion up to a few millimeters under the skin could be provided by NIR-II fluorescence imaging. The difference in tissue probing depth explained the trend observed for these three techniques, where the NIR-II fluorescence-based tissue perfusion measurement lied between laser Doppler and the microbead method. Moreover, this observation suggested NIR-II as a good compromise between the other two methods in that it can assess blood perfusion at greater depth into the tissue than laser Doppler, while allowing serial imaging of the same animal over time, which was not attainable by the invasive microbead method.

Besides the dynamic imaging capability of NIR-II to provide real-time tissue perfusion quantification, we have also applied static NIR-II fluorescence imaging to visualize and track the changes in the vascular anatomy in the ischemic hind limb as a response to the femoral artery ligation over a period of 10 days post-surgery. A large number of new collateral vessels were revealed by NIR-II fluorescence imaging in the ischemic hind limb on day 7 versus day 3 post-surgery, while the vascular anatomy in the control hind limb remained essentially unchanged over time (Fig. 5.15a,b,e,f). The cross-sectional line intensity profiles intersecting the femoral vessels confirmed the observation of newly recruited vascular branches represented by a greater number of individual peaks for the ischemic hind limb on day 7 than on day 3. More NIR-II fluorescence images taken on day 7 are shown in Fig. 5.16 for two ischemic mice,
revealing many neovessels formed during the revascularization process from both the medial and lateral sides of the hind limb. The NIR-II fluorescence images were also compared with micro-CT images for the same mouse on both day 3 and day 7 post-surgery. Micro-CT imaging was considered the standard for vascular imaging with the use of blood-pool contrast agent to enhance the contrast-to-noise ratio. However, the micro-CT images (Fig. 5.15c,d,g,h) all exhibited much fewer vascular structures than NIR-II, and the trend of vascular regeneration from day 3 to day 7 was not as clearly demonstrated by micro-CT as by the NIR-II imaging method, presumably due to the limited spatial resolution and the huge background interference from the bone.

Besides the qualitative imaging showing the microvascular regeneration from day 3 to day 7 in the ischemic hind limb (Fig. 5.15 & 5.16), we counted the number of vessels including the femoral and the surrounding collateral vessels in the mouse hind limbs and quantified relative vessel numbers (defined as ischemic/control) based on the NIR-II images, to demonstrate the temporal kinetics of microvascular regeneration. The trends revealed in Fig. 5.17a&b for a short-term study of 10 days and a long-term study of 26 days both suggested a statistically significant increase ($P < 0.005$) in relative vessel numbers between postoperative days 7 and 12, with an increase of up to ~6 times in comparison to day 0. Interestingly, this temporal increase of relative vessel numbers was followed by a gradual decline to normal level by day 26 possibly due to the regression of neovessels.\textsuperscript{30-32} We also employed immunohistochemical staining to verify the relative vessel numbers measured by NIR-II imaging, with good agreement between the two methods showing the transient increase of microvascular density.
exceeded the normal level in the ischemic hind limb on day 7 post-surgery (Fig. 5.17c&d).

5.4 Conclusion

In summary, we have shown in this chapter that the NIR-II fluorescence of SWNTs could be exploited to image the mouse hind limb vasculature with much improved spatial resolution and image sharpness than the traditional fluorescence imaging in the NIR-I window. The ~3-fold higher spatial resolution of NIR-II fluorescence imaging than micro-CT allowed us to resolve smaller blood vessels in the mouse hind limb, in particular for visualizing microvascular regeneration in a non-invasive way after acute hind limb ischemia. The high temporal resolution afforded by dynamic NIR-II fluorescence imaging allowed for vessel type differentiation based on the different hemodynamics between the arterial and venous vessels. Moreover, the video-rate recording of NIR-II fluorescence images provided an alternative way to Doppler ultrasound of measuring fast blood velocity in the femoral artery, but with a broader dynamic range of measurable velocity than ultrasound. For tissue blood perfusion measurement, NIR-II fluorescence imaging outperformed laser Doppler spectroscopy by sampling at an increased tissue depth, and had salient advantages over fluorescent microbead method owing to the minimum invasiveness that allowed for serial imaging.
5.5 Figures

Figure 5.1 | NIR-I and NIR-II Fluorescence Imaging of Mouse Vasculature. (a) A schematic drawing showing the SWNT-IRDye-800 conjugate with fluorescence
emission in the NIR-I region (~800 nm) from IRDye-800 label (red star) and in the NIR-II region (1.1–1.4 \( \mu \text{m} \)) from the SWNT backbone (black tube) when excited by a 785-nm laser. (b) The UV-Vis-NIR absorption spectrum of the SWNT-IRDye-800 conjugate in an aqueous solution (black dashed curve), along with the fluorescence emission spectra of IRDye-800 (green solid curve) and the SWNT (red solid curve). (c) A white light digital photograph (left) and the corresponding NIR-II fluorescence image (right) of the SWNT-IRDye-800 solution at a concentration of 0.10 mg/ml for SWNTs. (d) A schematic drawing showing the simultaneous imaging setup of the mouse in the NIR-I and NIR-II windows with zoomable magnifications. (e-g) NIR-I fluorescence images (top) and corresponding line intensity profiles (black solid curves, bottom) with Gaussian fit (red dashed curves, bottom) along the red dashed bars in the top images of a mouse injected with SWNT-IRDye-800 at 1× (e), 2.5× (f) and 7× (g) magnifications. (h-j) NIR-II fluorescence images (top) and corresponding line intensity profiles (black solid curves, bottom) with Gaussian fit (red dashed curves, bottom) along the red dashed bars in the top images of the same mouse at 1× (h), 2.5× (i) and 7× (j) magnifications.
Figure 5.2 | Cytotoxicity of SWNTs. The IC50 of SWNTs on microvascular endothelial cells was determined by fitting the cell viability data at different concentrations (black squares) into a sigmoidal function (red curve), which revealed the IC50 value of 0.1778±0.0237 g/L.
Figure 5.3 | Comparison of NIR-II Fluorescence Imaging and Micro-CT Imaging.

(a) An NIR-II fluorescence image of the proximal femoral vessels in the mouse thigh.

(b) A micro-CT image of the same proximal femoral vessels. (c) The line profile of NIR-II fluorescence intensity (black curve) along the green dashed bar in a, with the
two peaks fit into Gaussian functions (red curves). (d) The line intensity profile of X-ray contrast (black curve) along the green dashed bar in b, with the two peaks fit into Gaussian functions (red curves). (e) An NIR-II fluorescence image of the distal femoral vessels and vascular branches in the gastrocnemius. (f) A micro-CT image of the same vessels in the gastrocnemius. (g) The line profile of NIR-II fluorescence intensity (black curve) along the green dashed bar in e, with the peak fit into a Gaussian function (red curve). (h) The line intensity profile of X-ray contrast (black curve) along the green dashed bar in f, with the peak fit into a Gaussian function (red curve). The scale bar indicates 2 mm in all images.
**Figure 5.4 | Determination of the Depth of Femoral Vessels.** (a&b) NIR-II fluorescence images of the two hind limbs of a nude mouse at 1.5 h post injection of SWNTs. (c) A white light digital camera photograph showing the transection image of the left hind limb, where the femur could be seen. (d) An NIR-II fluorescence image of the transected hind limb as shown in c, where the femoral vessels and the femur could be seen with strong NIR-II fluorescence. (e) Depth measurements of the femoral
vessels and the femur. (f) A series of NIR-II fluorescence images of the right hind limb after removal of 0.5-mm thick slices from on top of the femoral vessels.
Figure 5.5 | Dynamic NIR-II Fluorescence Imaging of Mouse Hind Limb Blood Flow. (a-c) Time course NIR-II fluorescence images of a healthy mouse hind limb after tail-vein injection of SWNT fluorophores. (d) A PCA overlaid image showing the arterial (red) and venous (blue) vessels based on the first 200 video-rate NIR-II fluorescence images after injection. (e-g) Time course NIR-II fluorescence images of an ischemic mouse hind limb after tail-vein injection of SWNT fluorophores. (h) A PCA overlaid image showing only the arterial vessels (red) based on the first 200 video-rate NIR-II fluorescence images after injection. The scale bar indicates 2 mm in all images.
Figure 5.6 | Reproduced Dynamic NIR-II Imaging of Mouse Hind Limb Blood Flow. (a,c,e) Time course NIR-II fluorescence images of hind limb blood flow in an additional three healthy mice (Mouse C2-4, respectively) after tail-vein injection of SWNT fluorophores. (b,d,f) PCA overlaid images showing the differentiation of the arterial (red) and venous (blue) vessels for Mouse C2-4, respectively. (g&i) Time course NIR-II fluorescence images of hind limb blood flow in an additional two ischemic mice (Mouse I2-3, respectively) after tail-vein injection of SWNT fluorophores. (h&j) PCA overlaid images showing the differentiation of the arterial
(red) and venous (blue) vessels for Mouse I2-3, respectively. The scale bar indicates 2 mm in all images.
Figure 5.7 | Dynamic NIR-II Imaging of Ischemic Blood Flow Over a Longer Time. (a-h) Time course NIR-II fluorescence images of an ischemic mouse (Mouse I1) hind limb imaged over a period of >4 min after tail-vein injection of SWNT fluorophores, showing the blood return through the femoral vein at >2 min p.i. due to the ischemia-induced reduction in blood perfusion. (i) PCA overlaid images showing the differentiation of the arterial (red) and venous (blue) vessels for Mouse I1 based on the dynamic NIR-II images over >4 min after injection. The scale bar indicates 2 mm.
Figure 5.8 | Dynamic NIR-II Fluorescence Imaging of Mouse Blood Flow in Femoral and Abdominal Regions. (a-d) Time course NIR-II fluorescence images of the femoral and abdominal regions of a healthy mouse. (e) PCA overlaid images showing the differentiation of the arterial (red) and venous (blue) conduits based on the time-course NIR-II images shown in a-d. (f) A white light digital photograph showing the mouse imaged in its supine position beside a ruler.
Figure 5.9 | Blood Velocity Quantification in the Femoral Artery. (a) Time course NIR-II fluorescence images of an ischemic mouse (Mouse I1) hind limb, showing the
progression of the flow front (indicated by a red arrow). The site of arterial occlusion was indicated by a yellow arrow. (b) Traveled distance of the flow front plotted against time. (c) Normalized NIR-II fluorescence intensity in the femoral artery plotted against time. (d) The traveled distance of the flow front plotted against the normalized NIR-II fluorescence intensity in the femoral artery, showing a linear relationship between the blood velocity and the NIR-II fluorescence increase. (e) Time course NIR-II fluorescence images of a control, healthy mouse (Mouse C1) hind limb, showing rapid propagation of blood flow. (f) Normalized NIR-II fluorescence intensity in the femoral artery plotted against time. (g) Ultrasound blood velocity measurement of the femoral artery (red structure in the top image) based on analysis of three cardiac cycles (cyan curves in the bottom image). (h) Comparison of NIR-II-derived arterial blood velocity (black) and ultrasound-measured blood velocity (gray) based on the measurements of four control, healthy mice. (i) Comparison of the average femoral artery blood velocity of the control group (n=4) and the ischemic group (n=3), with the data acquired by the NIR-II method (black bars) and the ultrasound technique (gray bars). Please be noted that the femoral artery blood velocity of the ischemic group was too slow and unmeasurable by ultrasound. The scale bar indicates 5 mm in a and e, and the intensity scale bar on the right-hand side of the figure ranges from 0 to 1 for normalized NIR-II intensity.
Figure 5.10 | Reproduction of the Intensity-to-Velocity Conversion Coefficient.

(a&e) Time course NIR-II fluorescence images of the hind limb of ischemic Mouse I2 and I3, showing the progression of the flow front. (b&f) The traveled distance of the...
flow front plotted against time for these two additional ischemic mice. (c&g) The normalized NIR-II fluorescence intensity in the femoral artery plotted against time for these two additional ischemic mice. (d&h) The traveled distance of the flow front plotted against the normalized NIR-II fluorescence intensity in the femoral artery, showing a linear relationship between the blood velocity and the NIR-II fluorescence increase.
Figure 5.11 | Validation of the Intensity-to-Velocity Conversion Coefficient. (a-c) Dynamic NIR-II fluorescence images of an SWNT solution pumped into a thin catheter tubing pre-filled with pure water. (d-j) Normalized NIR-II fluorescence intensity within a certain ROI of the catheter tubing plotted against time p.i., for the standard condition (d), 4× lower SWNT concentration (e), 2× higher SWNT QY (f), 2× smaller tubing diameter (g), 2× shorter ROI length (h), 10× slower fluid velocity (i),...
and 2× longer pre-ROI length (j). (k) A schematic drawing showing the linear flow model with axial mixing, which is used for the simulation. (l-r) Normalized NIR-II fluorescence intensity within a certain ROI of the linear flow system plotted against time p.i., for the standard condition (l), 4× lower SWNT concentration (m), 2× higher SWNT QY (n), 2× smaller tubing diameter (o), 2× shorter ROI length (p), 10× slower fluid velocity (q), and 2× longer pre-ROI length (r). (s) A bar chart showing all conversion coefficients derived from the syringe pump experiments (red) and the linear flow model simulation (blue), and comparing these coefficients with that derived from the animal experiments in Table 1 (green dashed line).
Figure 5.12 | Reproduced Blood Velocity Quantification in the Femoral Artery of Control, Healthy Mice. (a,c,e) Normalized NIR-II fluorescence intensity in the femoral artery plotted as a function of p.i. time for three control, healthy mice C2-4, respectively. (b,d,f) Ultrasound blood velocity measurement of the femoral artery (red structure in the top image) based on analysis of three cardiac cycles (cyan curves in the bottom image) for these three mice.
**Figure 5.13 | Tissue Perfusion Imaging on an Ischemic Mouse.** (a&b) NIR-II fluorescence images of the same ischemic mouse taken at 20 s.p.i. of SWNTs on day 3 (a) and day 10 (b) post-surgery. The white arrows indicate the ischemic hind limb. (c&d) Laser Doppler perfusion images of the same ischemic mouse on day 3 (c) and day 10 (d) post-surgery. The black arrows indicate the ischemic hind limb.
Figure 5.14 | Tissue Perfusion Quantification on Ischemic Mice. (a-d) Normalized NIR-II fluorescence intensity extracted from the control (black) and ischemic hindlimbs (red) and plotted against p.i. time on post-operative days 0, 3, 7 and 10, respectively. (e) A bar chart showing the RTP values (ischemic/control) on post-operative days 0, 3, 7 and 10, measured by laser Doppler spectroscopy (red bars), NIR-II fluorescence imaging (blue bars) and fluorescent microbead based perfusion measurement (green bars). Statistically significant differences in RTP were found for all methods between day 0 and day 10 (*$P < 0.05$ and **$P < 0.005$).
Figure 5.15 | Revascularization Imaging Using NIR-II and micro-CT. (a,b,e,f) NIR-II fluorescence images (top) and line intensity profiles (black curve, bottom) with Gaussian-fitted vessel width analysis (red dashed curves, bottom) of the control (a,e) and ischemic (b,f) mouse hind limbs on day 3 (a,b) and day 7 (e,f) after the induction of acute hind limb ischemia. (c,d,g,h) Micro-CT images (top) and line intensity profiles (black curve, bottom) with Gaussian-fitted vessel width analysis (red dashed curves, bottom) of the control (c,g) and ischemic (d,h) mouse hind limbs on day 3 (c,d) and day 7 (g,h) after the induction of acute hind limb ischemia.
Figure 5.16 | NIR-II Fluorescence Imaging of Neovessels During Revascularization. (a-d) Medial view of both control (a,c) and ischemic (b,d) hind limbs of two mice (a,b for one mouse and c,d for the other) on day 7 after the induction of acute hind limb ischemia. (e-h) Lateral view of both control (e,g) and ischemic (f,h) hind limbs of the same two mice (e,f for one mouse and g,h for the other) on day 7 after the induction of acute hind limb ischemia.
Figure 5.17 | Quantification of Revascularization on Ischemic Mice. (a) A bar chart summarizing the relative vessel numbers (ischemic/control) measured by the NIR-II fluorescence method and plotted as a function of postoperative day in a short-term study of 10 days. (b) A bar chart summarizing the relative vessel numbers (ischemic/control) measured by the NIR-II fluorescence method and plotted as a function of postoperative day in a long-term study of 26 days. (c) A bar chart summarizing the relative microvascular density (ischemic/control) measured by immunofluorescence imaging and plotted as a function of postoperative day in a short-term study of 10 days. (d) Representative immunofluorescence images of hind limb tissue slices resected on day 3 and day 7 post-surgery, where the CD31 staining is shown in green and nucleus staining is in blue. Statistically significant difference was found for days 7-12 in comparison to day 0 and shown as *P < 0.05 and **P < 0.005.
5.6 Tables

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Table 5.1 | The Intensity-to-Velocity Conversion Coefficient Derived from Three Ischemic Mice. We reproduced the conversion coefficient measurement on three mice with acute hind limb ischemia (Mouse I1–3) and derived the average coefficient based on the three replicates.
### Variable Control

<table>
<thead>
<tr>
<th>Variable Control</th>
<th>Experimental Results [cm/%]</th>
<th>Simulation Results [cm/%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Settings</td>
<td>0.0778</td>
<td>0.0730</td>
</tr>
<tr>
<td>Change SWNT concentration</td>
<td>0.0739</td>
<td>0.0730</td>
</tr>
<tr>
<td>Change SWNT quantum yield</td>
<td>0.0756</td>
<td>0.0730</td>
</tr>
<tr>
<td>Change tubing diameter</td>
<td>0.0766</td>
<td>0.0730</td>
</tr>
<tr>
<td>Change ROI length</td>
<td>0.0789</td>
<td>0.0780</td>
</tr>
<tr>
<td>Change fluid velocity</td>
<td>0.0757</td>
<td>0.0722</td>
</tr>
<tr>
<td>Change pre-ROI length</td>
<td>0.1648</td>
<td>0.1701</td>
</tr>
</tbody>
</table>

**Table 5.2 | Variable Dependency Study of the Intensity-to-Velocity Conversion Coefficient.** The conversion coefficients derived from the syringe pump experiments (left column) and the simulation (right column) are tabulated for the standard settings and other conditions with one variable changed at a time.
5.7 References


Gounis, M. J., Spiga, M. G., Graham, R. M., Wilson, A., Haliko, S., Lieber, B. B., Wakhloo, A. K. & Webster, K. A. Angiogenesis is confined to the transient period of
Chapter 6. Non-Invasive Brain Vascular Imaging with Sub-10 μm Resolution

Through Intact Scalp and Skull Using 1300-1400 nm Fluorescence

* The work in this chapter is under review for publication as:


6.1 Introduction

Brain is the most vital organ of the human body and any dysfunction or disease to the brain (such as stroke) could be lethal. Tight protection of the brain from the outside makes brain particularly difficult to image for deciphering brain functions and abnormalities. Cerebrovascular diseases are a leading cause of death in most developed countries,¹ and understanding the diseases with animal models has mostly relied on tomographic imaging modalities such as X-ray computed tomography (CT) and magnetic resonance imaging (MRI).²⁻⁴ Although X-ray CT and MRI are able to imaging the entire brain vasculature with almost unlimited penetration depth, the spatial resolution is usually limited to sub-millimeters,⁵,⁶ incapable of visualizing cerebral capillaries with sub-10 μm resolutions.

On the other hand, in vivo fluorescence imaging has inherent benefits compared to other non-optical imaging modalities, including diffraction-limited spatial resolution and the immediate feedback. However, fluorescence imaging of the brain has been only pursued in the visible (400-750 nm) and traditional near-infrared (NIR-I, 750-900 nm) windows, and the strong scattering of fluorescence photons in these short-wavelength windows requires invasive skull thinning and removal procedures to
eliminate the scattering of photons through the opaque extracerebral tissues.\textsuperscript{7-10} Moreover, the penetration depth even with craniotomy is still limited to 1-2 mm\textsuperscript{7,11-13} by strong light scattering in the turbid brain tissues.\textsuperscript{14,15}

It has been shown in the previous chapters that \textit{in vivo} biomedical imaging with single-walled carbon nanotubes (SWNTs) in the second near-infrared region (NIR-II region, 1.0-1.7 \textmu m) can provide crisp imaging resolution at deep tissue penetration owing to the much reduced light scattering and autofluorescence.\textsuperscript{16-19} The deep location of the brain with tight protection of the skull and scalp makes the brain vasculature even more difficult to image than the hind limb vasculatures as shown in \textbf{Chapter 5}, and to optimize the SWNTs with the greatest penetration depth for the more demanding brain imaging, we utilized the intrinsic photoluminescence of SWNTs in the 1.3-1.4 \textmu m near infrared (NIR-IIa) window, which is the longest fluorescence wavelength one can obtain from the high pressure carbon monoxide (HiPco) SWNTs. We will show in the following paragraphs that the NIR-IIa fluorescence allowed for through-scalp and through-skull imaging of mouse cerebral vasculature without the need of craniotomy in an epifluorescence setup, achieving an unprecedented resolution of < 10 \textmu m at a depth of up to 3 mm underneath the scalp. Moreover, the dynamic NIR-IIa fluorescence imaging allowed us to track the cerebral blood perfusion in real time and pinpoint the location of arterial occlusion in an animal model of stroke.
6.2 Materials and Methods

Preparation of water soluble and biocompatible SWNT-IRDye-800 conjugate. Raw HiPco SWNTs (Unidym) were bath sonicated in an aqueous solution containing 1 wt% sodium deoxycholate for 1 h, and ultracentrifuged at 300,000 g for 1 h to remove bundles and other large aggregates of SWNTs in the suspension. The supernatant was carefully collected and a mixture of DSPE-mPEG(5k) (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol, 5000)], Laysan Bio) along with DSPE-PEG(5k)-NH₂ (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)5,000], Laysan Bio) was added to the solution to reach a final DSPE-mPEG(5k) concentration of 0.75 mg/mL and a final DSPE-PEG(5k)-NH₂ concentration of 0.25 mg/mL. Note that to make biocompatible SWNTs without the IRDye-800 labels, 100% of the DSPE-mPEG(5k) was added to the supernatant instead to reach a final concentration of 1.0 mg/mL. Then the suspension was bath sonicated for 5 min, dialyzed against 1x PBS in a 3500MWCO dialysis membrane (Fisher) to remove sodium deoxycholate, and ultracentrifuged again at 300,000 g for 0.5 h to remove bundles and other aggregates formed during dialysis. The amine-functionalized SWNTs were conjugated further with IRDye-800 according to a previous protocol. In brief, the SWNT solution after dialysis and ultracentrifugation was washed with DIUF water in a 30k NMWL centrifugal filter unit to remove excess surfactant and concentrated to ~2 μM. 10× PBS was added to this solution to reach a 1× PBS solution with pH 7.4, and then the solution was mixed with a DMSO solution of IRDye-800 NHS ester (LI-COR) at a concentration of 0.1 mM. The mixture was allowed to sit for 1 h at room temperature before the reaction.
reached completion. Excess IRDye-800 was removed by filtration through a 100k NMWL centrifugal filter, and the as-prepared SWNT-IRDye-800 conjugate solution was stored at 4 °C and away from any light illumination to avoid photobleaching of IRDye-800.

**Separation of the large-diameter semiconducting (LS) nanotubes.** We used a 1-cm diameter column filled with ally dextran-based size-exclusion gel (Sephacryl S-200, GE Healthcare) as the filtration medium to carry out diameter separation of the raw HiPco SWNTs. In brief, HiPco SWNTs (Unidym) were bath sonicated in an aqueous solution containing 1 wt% sodium cholate (SC) for 1 h, and ultracentrifuged at 300,000 g for 0.5 h to remove bundles and other large aggregates of SWNTs in the suspension. Then the supernatant after ultracentrifugation was retained and diluted with the same volume of 1 wt% sodium dodecyl sulfate (SDS) solution, resulting in a mixed solution of SC at 0.5 wt% and SDS at 0.5 wt%. This solution was added to the aforementioned gel column, where the semiconducting SWNTs were trapped in the filtration medium. To obtain the LS SWNTs, a mixed surfactant solution containing 0.6 wt% SC and 0.4 wt% SDS was added to the column to elute the LS nanotubes.

**UV-Vis-NIR absorbance measurement.** UV-Vis-NIR absorbance spectra of pure water, 1% Intralipid, the mouse skull and scalp were measured in the range of 600-1800 nm by a Cary 6000i UV-Vis-NIR spectrophotometer, background-subtracted for any absorbance contribution from the solvent whenever a solvent was used.

**NIR fluorescence spectroscopy of the SWNT-IRDye-800 solution.** The NIR fluorescence spectrum of the SWNT-IRDye-800 solution was taken on a home-built
NIR spectroscopy setup. The excitation was provided by an 808-nm laser diode (RMPC lasers) at a total output power of 160 mW and filtered by an 850-nm short-pass filter, a 1000-nm short-pass filter, an 1100-nm short-pass filter and a 1300-nm short-pass filter. The excitation light cleaned by these filters was then focused onto a solution containing the SWNT-IRDye-800 conjugate in a 1-mm path cuvette (Starna Cells, Inc.). The emitted fluorescence of the solution was collected in a transmission geometry at an angle of 180° to the incident excitation light. The transmitted excitation light was rejected using an 850-nm long-pass filter and fluorescence was collected by the spectrometer (Acton SP2300i) equipped with a one-dimensional (1D) indium-gallium-arsenide (InGaAs) linear array detector (Princeton OMA-V). The raw fluorescence spectrum was corrected post-collection to account for the bleed-through of the laser excitation, the extinction profile of the emission filter and the sensitivity of the detector using the MATLAB software.

**Laboratory mouse handling and surgery.** All experiments involving the use of laboratory animals were carried out under the approval of Stanford University’s Administrative Panel on Laboratory Animal Care. C57Bl/6 mice (Taconic Farms) were used in this study. Induction of cerebral hypoperfusion and middle cerebral artery occlusion (MCAO) was performed according to previously published protocols. In brief, the animals were anesthetized using a mixture of 30% O₂, 70% N₂O, and 2% isoflurane before surgery was performed. For induction of MCAO, an incision was made on the left common carotid artery (CCA), and a silicon-coated nylon filament (Doccol Co., CA) was inserted through the left CCA into internal carotid artery (ICA) until the tip of the filament occluded the origin of the middle
cerebral artery (MCA). For induction of cerebral hypoperfusion, only the left external carotid artery (ECA) and the CCA were ligated. A group of 3 control mice without receiving the surgery, a group of 4 mice with induced MCAO, and another group of 4 mice with induced cerebral hypoperfusion were used in this study. The hair on the scalp was carefully removed using Nair prior to tail-vein injection and imaging. All mice were anesthetized before injection and imaging in a rodent anesthesia machine supplying a mixed gas flow of 2 L/min O\textsubscript{2} and 3% Isoflurane. For injection of the NIR-II contrast agent, a 28 gauge syringe needle was inserted into the lateral tail vein, and a bolus of 200 μL SWNT solution was allowed to be injected into the tail vein during the first frames of the dynamic NIR-II fluorescence imaging. For brain vascular imaging in different sub-regions of the NIR, a 200 μL bolus of 0.43 mg/mL SWNT-IRDye-800 bioconjugate was injected at approximately 5 min before the animal was transferred to the imaging stage and imaged for brain vasculature. For dynamic NIR-IIa fluorescence imaging, a 200 μL bolus of 0.43 mg/mL SWNT-DSPE-mPEG without IRDye-800 was injected in the dark and the InGaAs camera started continuous image acquisition immediately after the SWNT solution was injected. To keep the mouse anesthetized during imaging, a nose cone was used to cover the face of the mouse and deliver a mixed gas flow of 1.5 L/min O\textsubscript{2} and 3% Isoflurane.

**In vivo static fluorescence imaging in the NIR windows.** For static fluorescence imaging of the phantom-embedded capillary tube and live animals in the NIR-I and NIR-II windows, the excitation was provided by an 808-nm laser diode, fiber-coupled to a collimator with a focal length of 4.5 mm, cleaned by an 850-nm short-pass filter and a 1000-nm short-pass filter to remove the long-wavelength photons, and directed
to sample stage with a power density of 140 mW/cm$^2$. For fluorescence imaging in the NIR-I window, the emitted fluorescence photons were filtered through an 850-nm long-pass filter and a 900-nm short-pass filter to collect NIR-I fluorescence in the 850-900 nm region. For fluorescence imaging in the NIR-II window, the emitted photons were filtered through a 910-nm long-pass filter and a 1000-nm long-pass filter to collect NIR-II fluorescence in the 1000-1700 nm region (note the upper bound of detection range was determined by the sensitivity profile of the camera). For fluorescence imaging in the NIR-IIa window, the emitted photons were filtered through a 1000-nm long-pass filter, a 1300-nm long-pass filter and a 1400-nm short-pass filter to confine the fluorescence in the 1300-1400 nm region. A two-dimensional (2D) InGaAs array of 320 × 256 pixels (Princeton Instruments) was used to form NIR images in all sub-regions. A lens pair consisting of a 200 mm achromat and a 75 mm achromat with a distance of ~200 mm in between was used to image with a field of view of 25 mm × 20 mm, and a lens pair consisting of a 150 mm achromat and a 200 mm achromat separated by ~150 mm was used for a higher magnification with a field of view of 8 mm × 6.4 mm. The NIR fluorescence images of the phantom-embedded capillary tube were flat-field corrected post acquisition.

For high resolution microscopic imaging of brain microvasculature in the NIR-IIa region, the same 808-nm laser diode was used for excitation in a microscopic imaging setup with objective lens of different magnifications (4x and 10x, Bausch & Lomb). The excitation light was cleaned by an 850-nm short-pass filter, a 1000-nm short-pass filter, an 1100-nm short-pass filter and a 1300-nm short-pass filter to remove the long-wavelength photons. The C57Bl/6 mouse with the hair shaved off
was injected with a 200 μL bolus of 0.22 mg/mL LS nanotubes and placed in the prone position on a home-made stereotactic imaging stage. The stage was fixed on a motorized three-dimensional (3D) translation stage (Newport) that allowed for the position adjustment of the mouse relative to the objective. Two dovetail manual translation stages with two posts fixed on them were used for fixing the breathing motion of the mouse head with fine adjustments. The emitted fluorescence was collected in the 1300-1400 nm NIR-IIa window using the same filter set as in the low-magnification setup under an exposure time of 300 ms to 1 s. To determine the depth of the imaged brain vessels, we first focused the imaging plane to the surface of the scalp, set this depth as zero, moved the mouse head closer to the objective until a sharp image was obtained, and recorded the distance the stage had travelled relative to the objective from the zero depth by the 3D translation stage. Since an air-immersed objective was used, while the imaged vascular structures were inside the brain tissue, correction was needed to obtain the actual depths of the imaged vessels based on the refractive indices of air (1.00), scalp skin (1.38), cranial bone (1.56) and brain tissue (1.35).

**In vivo dynamic fluorescence imaging in the NIR-IIa window.** The video-rate dynamic fluorescence imaging was carried out on the same imaging system as in the static fluorescence imaging, employing the InGaAs camera only in the 1300-1400 nm NIR-IIa window. A lens set that gave a field of view of 25 mm × 20 mm was used for the dynamic imaging. To capture video-rate images, the 2D InGaAs detector was allowed to expose continuously with an exposure time of 100 ms immediately after a bolus injection of 200 μL of the SWNTs at 0.43 mg/mL, and the images were acquired.
by a user-written LabVIEW software with an additional overhead time of 87.5 ms between two consecutive frames. The exposure time and the overhead time comprised a complete cycle of 187.5 ms for each image acquisition with a frame rate of 5.3 frames/s. To make the following cerebral blood perfusion measurement easier, a MATLAB built-in function `imrotate` was used to rotate the mouse head in the time-course frames if the mouse head was tilted over the course of dynamic imaging.

The video-rate images taken in the aforementioned manner were used for dynamic contrast-enhanced visualization using principal component analysis (PCA), in a similar way to previous publications by the Hillman group\textsuperscript{26} and our group.\textsuperscript{19} In a typical image processing task by the MATLAB software, the first 100 frames immediately after SWNT injection were loaded into a 3D array and reshaped into a 2D array, which was fed into the MATLAB built-in `princomp` function for PCA analysis. From the output of the `princomp` function, we wrote a program in MATLAB to automatically combine the components of pixels showing up earlier in the video and assign them the arterial features (color-coded in red), while combining the components of pixels showing up later in the video and assign them the venous features (color-coded in blue).

**Quantification of brain blood perfusion based on dynamic NIR-IIa fluorescence imaging.** The time-course NIR-IIa fluorescence images obtained from the dynamic NIR-II fluorescence imaging were first loaded into the MATLAB software, and the average NIR-IIa fluorescence intensity within symmetric ROIs located at the lateral sulcus region in both cerebral hemispheres was extracted from the time-course NIR-II images. The average NIR-II intensity was normalized against the maximum intensity
of the control cerebral hemisphere and plotted against time from 0 s to 3.94 s post injection (p.i.), in order to compensate for the difference in the amount and the brightness of the injected SWNTs. The normalized NIR-II intensity was expressed in units of %. The intensity-time plot usually featured a linear rise phase starting at ~2 s p.i., followed by a plateau region. We used the linear rise phase to fit into a linear function with its slope in units of %/s, which could be translated into the corresponding blood velocity in units of cm/s by applying the intensity-to-velocity conversion coefficient in units of cm/%\textsuperscript{17,18} The relative perfusion was derived from the linear-fit slopes of the control cerebral hemisphere (slope\textsubscript{control}) and the surgerized cerebral hemisphere (slope\textsubscript{surgerized}) as follows,

\[ \text{Relative Perfusion} = \frac{\text{slope}_{\text{surgerized}}}{\text{slope}_{\text{control}}} \]

**Brain blood perfusion measurement based on laser Doppler.** The blood perfusion of mouse brain was measured with laser Doppler spectroscopy. Before measurement, the mice were anesthetized under mixed gases of 1.5% Isoflurane, 30% O\textsubscript{2} and 70% N\textsubscript{2}O. The animals were maintained with a body temperature at 36-37 °C. A flexible fiber optic probe was affixed to the mouse skull over the MCA (2 mm posterior and 6.5 mm lateral to bregma) after removal of scalp skin for cerebral blood flow (CBF) quantification. The baseline CBF values were acquired before any vascular intervention and considered as the 100% flow.
6.3 Results and Discussion

To mimic the turbid and highly scattering biological tissue, we used a phantom medium (Intralipid®, 1 wt% aqueous solution) to study the penetration depths of fluorescence imaging in different sub-regions of the NIR.\textsuperscript{19} The SWNT-IRDye-800 fluorophore conjugate was used to afford fluorescence emission in a wide range from 800 nm to 1700 nm upon an 808-nm excitation, in order to compare image sharpness in the NIR-I (<900 nm, from the IRDye-800 fluorescence), NIR-II (1.0-1.7 μm, from the SWNT fluorescence) and NIR-IIa (1.3-1.4 μm, from SWNT fluorescence) regions.\textsuperscript{27,28} The emission spectrum of SWNT-IRDye-800 conjugate is shown in Fig. 6.1a, allowing us to select desired wavelength ranges using different optical filter sets. We then filled a capillary tube with the SWNT-IRDye-800 fluorophore conjugate to mimic the blood vessel, and immersed the capillary tube in the 1 wt% Intralipid solution, which was used to mimic the highly scattering biological tissue.

When the capillary tube was immersed at a depth of 1 mm inside the Intralipid phantom, all spectral sub-regions of NIR showed sharp images of the capillary tube without significant difference between different wavelengths, owing to the shallow immersion depth of the capillary tube in the phantom (Fig. 6.1b, top). In strong contrast, the images taken for the same capillary tube at an immersion depth of 1 cm exhibited highly smeared features that caused the capillary tube to become invisible in the NIR-I region and the entire NIR-II region, while the image taken in the NIR-IIa region with the longest wavelengths still resolved the sharp edges of the tube (Fig. 6.1b, bottom), owing to the lowest scattering of photons among all three windows.
This observation was rationalized by inspecting the measured scattering curve versus wavelength of the Intralipid phantom (Fig. 6.1c, red curve, measured by the method shown in Fig. 6.2), which clearly showed the photon scattering scaled inversely proportional to the wavelength, following a relationship of \( \mu'_s = 1.6 \lambda^{-2.4} \) (Fig. 6.1c, blue curve).\(^{29}\) Note that the scattering coefficient \( \mu'_s \) has a unit of \( \text{mm}^{-1} \), and the wavelength \( \lambda \) has a unit of \( \mu\text{m} \). Therefore, fluorescence imaging in the 1.3-1.4 \( \mu\text{m} \) NIR-IIa window improved the feature sharpness and spatial resolution by rejecting shorter wavelength photons below 1.3 \( \mu\text{m} \) with greater scattering of photons. It is also noteworthy that the attempt to utilize even longer wavelengths for NIR-II fluorescence imaging was prohibited by the strong absorption in the >1.4 mm region due to the first overtone vibration of water molecules (Fig. 6.1c, black curve).\(^{30}\)

Using the same SWNT-IRDye-800 fluorophore conjugate injected intravenously, we carried out brain vascular imaging in different sub-regions of NIR of a healthy C57Bl/6 mouse with its hair over the scalp skin shaved off (Fig. 6.3a). The shaved mouse head was imaged under a magnification where each pixel corresponded to \( \sim 78 \ \mu\text{m} \) in real space. Under the excitation of an 808-nm laser irradiation, the mouse head emitted fluorescence in the broad 850-1650 nm region as revealed in Fig. 6.1a, and fluorescence photons with different wavelengths were collected to form images of the brain in different sub-regions of NIR. When the NIR-I fluorescence with <900 nm wavelengths was collected to form the image, barely any vessels in the brain could be observed due to the significant scattering by the extracerebral tissues (Fig. 6.3b). Some major blood vessels started showing up in the
entire NIR-II region (Fig. 6.3c), while the sharpest cerebrovascular image was obtained using fluorescence in the 1.3-1.4 \( \mu \text{m} \) NIR-IIa region (Fig. 6.3d). This observation confirmed the trend of wavelength-dependent phantom imaging, suggesting the background signal and feature blurriness could be much reduced by rejecting the shorter-wavelength photons in the NIR-II region, which had a higher degree of scattering in animal tissues. It is noteworthy that a number of cerebral vessels could be observed in the image shown in Fig. 6.3d, including the inferior cerebral vein (labeled ‘1’), the superior sagittal sinus (labeled ‘2’), the transverse sinus (labeled ‘3’) and many other smaller vessel branches, suggesting an imaging penetration depth of 1~2 mm underneath the scalp skin (Fig. 6.4).

Similar to the extinction spectrum of the Intralipid phantom shown in Fig. 6.1c, the extinction spectra of the scalp skin and the cranial bone both featured a water absorption peak due to overtone vibration (1.4-1.5 \( \mu \text{m} \)), and a declining baseline corresponding to photon scattering (Fig. 6.3e). Prior literature has reported the relationship between wavelength and the photon scattering in different tissues including scalp, skull and the brain tissue,\(^{7,31,32}\) as follows,

\[
\mu_s'(\text{scalp})/\text{mm}^{-1} = 0.11(\lambda/\mu \text{m})^{-4} + 1.61(\lambda/\mu \text{m})^{-0.22} \quad (\text{where the two terms are attributed to Rayleigh scattering and Mie scattering, respectively})
\]

\[
\mu_s'(\text{skull})/\text{mm}^{-1} = 1.72(\lambda/\mu \text{m})^{-0.65}
\]

\[
\mu_s'(\text{brain tissue})/\text{mm}^{-1} = 4.72(\lambda/\mu \text{m})^{-2.07}
\]
The reduction of scattered photons from the shorter-wavelength NIR-I window to the longer-wavelength NIR-IIa window can be estimated as follows. For a wavelength of 800 nm in the traditional NIR-I window, the reduced scattering coefficient is 1.96 mm$^{-1}$ for scalp and 1.99 mm$^{-1}$ for skull, while for a wavelength of 1350 nm in the NIR-IIa window, this coefficient is 1.54 mm$^{-1}$ for scalp and 1.42 mm$^{-1}$ for skull. Given a measured thickness of 0.7 mm for the scalp skin and that of 0.6 mm for the cranial bone, the NIR-IIa fluorescence at 1350 nm are estimated to have ~47% fewer scattered photons than the NIR-I fluorescence at 800 nm as they travel through the skull and scalp. The much reduced photon scattering in the NIR-IIa window led to much sharper images and higher signal-to-background ratio (SBR) for visualizing cerebrovasculature through the highly scattering extracerebral tissues (Fig. 6.3b vs. Fig. 6.3d).

To resolve the cerebral capillary network inside the brain, we used a home-made stereotactic stage to fix the mouse head and reduce the motion of the mouse (Fig. 6.5a), and performed microscopic imaging of the brain microvasculature through intact scalp and skull (Fig. 6.5b). Objectives with different magnifying powers were used in the stereotactic imaging setup, and a 3D translation stage with digital position control and readout allowed us to move the mouse head relative to the objective and measure the focal depth of brain vascular imaging. On the other hand, to enrich the emitted photons from SWNTs in the 1.3-1.4 μm NIR-IIa region, we performed chemical separation of the starting HiPco SWNTs through gel chromatography to obtain LS nanotubes with a greater percentage of NIR-IIa fluorescence on the per unit mass basis (Fig. 6.6). Using the separated LS nanotubes, we first imaged the entire
mouse brain vasculature (Fig. 6.5c), zoomed into the left cerebral hemisphere to visualize more branched vessels (Fig. 6.5d), and further zoomed into an even smaller region near the superior sagittal sinus using a 4× microscopic objective (Fig. 6.5e). Many small cerebral capillaries were observed in Fig. 6.5e at a focal depth of ~2.6 mm underneath the surface of the scalp skin (i.e., ~1.3 mm deep inside the brain tissue given a total thickness of 1.3 mm of all extracerebral tissues, Fig. 6.4), which was determined by the travelled distance of the mouse head fixed on a 3D translation stage relative to the objective (see Section 6.2 for more details). One of the cerebral capillaries imaged at an even higher magnification using a 10× objective revealed a Gaussian-fitted width of 6.6 μm (Fig. 6.5f), which represented the highest resolution imaging of the brain capillary vessels in an in vivo, live animal setting thus far. More brain capillary images are taken on 3 different mice in the depth range of 1-3 mm using NIR-IIa fluorescence and shown in Fig. 6.7. From the NIR-IIa cerebrovascular images, we measured the widths of a total of 63 different capillaries and plotted their width distribution in the histogram graph (Fig. 6.8). An average vessel width of 9.4 μm with a standard deviation of 2.5 μm was found for these analyzed capillary vessels in a width range of 5 μm to 15 μm and a depth range of 1 mm to 3 mm underneath the scalp skin. However, the attempt to resolve any vascular structure at a depth of > 3 mm was not successful even with the long-wavelength fluorescence in the 1.3-1.4 μm region, most likely due to the interference from out-of-focus, foreground signals.33 We envisage this problem being solved by an NIR-IIa confocal microscope, which should be able to perform optical sectioning of the brain tissue and reconstruct the 3D cerebrovascular network to a deeper depth than the widefield imaging setup.
The NIR-IIa fluorescence imaging has salient advantages in the context of brain vascular imaging, including high spatial resolution (down to < 10 μm), deep tissue penetration (up to 3 mm) and non-invasive imaging without the need of craniotomy. Previously, fluorescence imaging in the traditional NIR-I region with shorter photon wavelengths of 750-900 nm could only resolve capillary vessels at a maximum depth of ~350 μm, even with the help of craniotomy to remove the highly scattering tissues over the brain. This striking difference is attributed to the highly scattering nature and the large wavelength dependence of photon scattering (which scales with $\lambda^{-2.07}$) of the brain tissue, which has a large scattering coefficient of ~7.49 mm$^{-1}$ at 800 nm in the NIR-I window, versus ~2.54 mm$^{-1}$ at 1350 nm in the NIR-IIa window (Fig. 6.3f), suggesting $\sim10^4\times$ greater photon scattering in the NIR-I window than in the NIR-IIa when photons are traveling through a ~2 mm thick brain tissue. Non-linear fluorescence imaging techniques, such as the two- and multi-photon fluorescence microscopy, have been reported to achieve a deep penetration of 1-2 mm for brain imaging, owing to the highly localized excitation and emission of fluorescence. However, to achieve such a deep penetration depth inside the brain, the invasive removal of the scalp is always involved, while the skull either needs to be replaced with a cranial window, or polished and thinned to afford greater transparency. Similar invasive treatment to the extracerebral tissues is needed for brain vascular imaging with laser speckle contrast imaging (LSCI) and optical frequency domain imaging (OFDI), which have been reported to achieve a spatial resolution of ~10 μm at the cost of removing both the scalp and skull. It has been reported that invasive treatment to the extracerebral tissues is usually associated with
inflammatory responses, such as altered pial blood vessels, which could affect the validity of the imaging results. To avoid such invasive treatment, optical coherence tomography (OCT) and photoacoustic microscopy (PAM) have both shown the ability to visualize cortical vessels through intact scalp and skull, but the limited imaging resolution of both techniques (10-100 μm) have prevented them from resolving sub-10 μm capillary vessels.\textsuperscript{39,40} The tradeoff between imaging resolution and invasiveness has been a long-existing problem for all other brain imaging modalities, and our NIR-IIa fluorescence imaging technique for brain angiography has provided a completely non-invasive approach of visualizing the brain microvasculature with unprecedented sub-10 μm resolution.

In addition to anatomical brain angiography with NIR-IIa fluorescence, we also performed dynamic fluorescence imaging in the NIR-IIa window to track cerebral blood flow in real time. Immediately following the intravenous injection of a solution of SWNTs into the tail vein of a healthy, control mouse (Mouse C1), we observed a signal appearing in the lateral sulcus region on both sides of the cerebrum within merely 3 s (Fig. 6.9a), succeeded by the outflow of blood carrying SWNT fluorophores that led to the appearance of the interior cerebral vein, the superior sagittal sinus and the transverse sinus (Fig. 6.9b\&c). We then applied PCA to the video frames over a time course of ~20 s p.i. and successfully differentiated the arterial vessels (Fig. 6.9d) from the venous vessels (Fig. 6.9e) based on their hemodynamic difference (Fig. 6.9f).\textsuperscript{17,19} After injection and dynamic imaging, the intravenously administered SWNTs remained circulating in the blood vessels for at least 3 h p.i., which set the time window for static imaging of brain vasculatures under
desired magnifications (Fig. 6.10). Similar results of NIR-IIa dynamic imaging were reproduced with an additional two healthy mice (Mouse C2-3, Fig. 6.11a-h).

We then repeated the cerebral hemodynamic imaging using NIR-IIa fluorescence in mice with surgically induced MCAO as an animal model for stroke in the left cerebral hemisphere. After an SWNT solution was intravenously injected into an MCAO mouse (Mouse M1), the change of NIR-IIa fluorescence signal in the right hemisphere showed a very similar blood flow pattern to the control, healthy Mouse C1. In striking contrast, a marked delay of blood perfusion was observed in the left cerebral hemisphere, owing to the induced arterial occlusion that blocked blood inflow (Fig. 6.9g-i). We performed PCA analysis in a similar way to the control Mouse C1, and found the absence of arterial vessels (color coded in red) in the diseased, left hemisphere, in comparison to the more balanced venous vessels (color coded in blue) in both hemispheres (Fig. 6.9j-l). This finding confirmed the blood flow occlusion in the left middle cerebral artery and suggested dynamic NIR-IIa fluorescence imaging as a useful tool for locating the site of stroke in real time and real space. We also repeated the dynamic NIR-IIa fluorescence imaging on an additional three mice with MCAO (Mouse M2-4) and observed similar results (Fig. 6.12a-l).

Besides the qualitative imaging of cerebral blood perfusion in real time using dynamic NIR-IIa fluorescence technique, we also quantified the relative blood perfusion in the diseases, left hemisphere against the healthy, right hemisphere by analyzing the average fluorescence signals in the symmetric MCA regions of both cerebral hemispheres. In the healthy, control Mouse C1, the linear rise of NIR-IIa fluorescence intensity in the left hemisphere matched that in the right with almost
identical slopes, suggesting an expected relative blood perfusion of ~1 since both hemispheres should be healthy in this case (Fig. 6.9m, Fig. 6.11i-j). When a diseased Mouse M1 with MCAO was analyzed for the cerebral blood perfusion, a much reduced perfusion value of 0.159 was found in the left hemisphere due to the occluded left MCA (Fig. 6.9n & Fig. 6.12m-o). We also included another group of 4 mice with induced cerebral hypoperfusion, which was used as an animal model for circulatory shock in the brain, and found a slight decrease of blood perfusion by ~20% (Fig. 6.13). The cerebral blood perfusion measurement with NIR-IIa fluorescence was compared with the gold standard method, laser Doppler blood spectroscopy, and a good agreement was found between the two methods (Fig. 6.9o). It is noteworthy that laser Doppler is an invasive technique that requires the removal of scalp skin for cortical blood perfusion measurement, while NIR-IIa probes deep tissue blood perfusion without any invasive treatment to the scalp/skull. Another benefit of NIR-IIa brain imaging is that it can simultaneously track the blood flow in multiple vessels owing to a widefield imaging setup, which is not attainable by dynamic two-photon fluorescence microscopy that scans one single vessel at a time to obtain the blood velocity in that specific vessel.\textsuperscript{41-43}

\textbf{6.4 Conclusion}

In this chapter, we have shown a sub-region of the NIR-II window, termed NIR-IIa window in the range of 1.3-1.4 \(\mu\text{m}\), allowed us to perform vascular imaging inside the mouse brain through intact scalp and skull. This sub-region of NIR-II
window was enabled by the broad emission band of SWNTs in the 1.0-1.7 μm window, and was further optimized by enriching SWNTs with fluorescence emitted in this region. With even reduced scattering of photons in this new, NIR-IIa window, we have achieved sub-10 μm cerebrovascular imaging at a maximum penetration depth of ~3 mm underneath the scalp skin, and dynamic blood perfusion imaging in the brain with a temporal resolution of >5 frames per second. The results of NIR-IIa brain imaging shown in this chapter represent the highest resolution of in vivo cerebrovascular imaging with minimum invasion of the extracerebral tissues, which can potentially serve as a useful biomedical imaging tool helping understand the complex mechanisms of neurovascular regulation in small animal model studies.
Figure 6.1 | Phantom Imaging in Different NIR Sub-Regions. (a) A fluorescence emission spectrum of SWNT-IRDye-800 fluorophore conjugate under excitation of an 808-nm laser. The different sub-regions of NIR are color-shaded in blue (NIR-I), green (NIR-II) and red (NIR-IIa). (b) NIR fluorescence images taken in the NIR-I (left column), NIR-II (middle column) and NIR-IIa (right column) windows of a capillary tube filled with the SWNT-IRDye-800 fluorophore conjugate and immersed at a depth of 1 mm (top row) and 10 mm (bottom row). (c) The extinction (black curve) and scattering spectra (red curve: from experiment; blue curve: from literature\textsuperscript{29}) of the 1% Intralipid phantom used in this study.
Figure 6.2 | Scattering Spectrum of 1% Intralipid in Water. The scattering spectrum of 1% Intralipid in water (red curve) was measured by subtracting the absorption spectrum of water (green curve) and the absorption spectrum of 1% Intralipid in a good solvent (blue curve; measured in acetone) from the extinction spectrum of 1% Intralipid in water (black curve).
Figure 6.3 | Mouse Brain Imaging in Different NIR Sub-Regions. (a) A white light, digital photograph of a mouse head with hair shaved off the scalp. (b-d) NIR fluorescence images of the same mouse head shown in a, by detecting fluorescence in the 850-900 nm NIR-I window (b), the 1000-1700 nm NIR-II window (c) and the 1300-1400 nm NIR-IIa window (d). Note that the major cerebral vessels are labeled in d as 1 (the inferior cerebral vein), 2 (the superior sagittal sinus) and 3 (the transverse sinus). (e) The extinction spectra of the scalp skin (red) and the cranial bone (blue).
plotted for unit tissue thickness (per millimeter), along with the absorption spectrum of water (black). (f) The scattering spectra of the scalp skin (red), cranial bone (blue) and brain tissue (black), based on the previously reported formula of reduced scattering coefficient versus wavelength for these tissues.\textsuperscript{7,31,32}
Figure 6.4 | Thickness Measurement of Extracerebral Tissues. (a) A white light digital photograph showing the head dissected from a C57Bl/6 mouse, which was perfused with 5% Evans Blue to render the blood vessels blue before this mouse was sacrificed. The extracerebral tissues, including the scalp skin, the cranial bone and the meninges were completely removed over the right cerebral hemisphere, while those over the left hemisphere still remained intact. (b-d) Triplicate measurements of the total thickness of all extracerebral tissues by digital calipers. The thickness measurement was performed at three different locations of the dissected extracerebral tissues, with an average thickness of 1.37 mm.
Figure 6.5 | High Resolution Imaging of Mouse Cerebral Vasculature. (a) A white light digital photograph of a home-made stereotactic microscopic imaging setup. A red laser is used to show the location of the excitation laser beam on the scalp. (b) A schematic drawing showing NIR-IIa fluorescence imaging of the brain microvasculature through intact scalp skin, skull and deep into the brain tissue. (c) A low-magnification NIR-IIa fluorescence image of a shaved mouse head. The field of view is 25 mm × 20 mm with a pixel resolution of 78 μm. (d) An NIR-IIa
fluorescence image showing the zoomed-in view of the mouse head in c. The field of view is 8 mm × 6.4 mm with a pixel resolution of 25 μm. (e) A microscopic NIR-IIa fluorescence image showing a further zoomed-in view of the mouse cerebrovascular image in d. The field of view is 1.7 mm × 1.4 mm with a pixel resolution of 5 μm, and the depth of the vessels visualized in this image was found as ~2.6 mm. (f) Another microscopic NIR-IIa fluorescence image taken under an even higher magnification than the image shown in e. The field of view is 0.80 mm × 0.64 mm with a pixel resolution of 2.5 μm. The cross-sectional intensity profile (black) and its Gaussian fit (red) are shown in the inset for the yellow dashed line in the image.
Figure 6.6 | Enrichment of NIR-IIa Fluorescence. (a) A photoluminescence versus excitation (PLE) spectrum of an aqueous solution of the unseparated HiPco nanotubes. (b) A PLE spectrum of an aqueous solution of the separated LS nanotubes. The 1.3-1.4 μm region is shaded red to highlight the location of the NIR-IIa window in the PLE map.
Figure 6.7 | High Resolution Cerebral Capillary Images in the NIR-IIa Window. (a-h) More high resolution cerebral vascular images taken in the NIR-IIa window at depths ranging from 1 to 3 mm below the surface of the scalp skin. A total of 3 mice were used for obtaining these images. A 4× objective with a field of view of 1.7 mm × 1.4 mm was used for images a & b, while a 10× objective with a field of view of 0.80 mm × 0.64 mm was used for images c-h. For each image, its corresponding cross-sectional fluorescence intensity profile along the green dashed line is shown as the black curve under the image, which is fitted to Gaussian functions shown as the red curves.
Figure 6.8 | Statistical Analysis of Cerebral Capillary Widths. (a-f) Microscopic NIR-IIa fluorescence images of the cerebral vasculature, showing capillary vessels used for statistical analysis (intersected by green dashed lines). (g) A histogram plot
showing the width distribution of the analyzed cerebral capillaries. A Gaussian fit (black curve) to the bar chart revealed an average vessel width of $9.4 \pm 2.5 \mu m$. 
Figure 6.9 | Dynamic NIR-IIa Fluorescence Imaging of Mouse Cerebral Blood Flow. (a-c) Time course NIR-IIa fluorescence images of a control, healthy mouse brain (Mouse C1). (d-f) PCA overlaid images showing arteries (red) and veins (blue) in the brain of Mouse C1 based on dynamic NIR-IIa video frames. (g-i) Time course NIR-IIa fluorescence images of a stroke mouse brain with induced MCAO in the left
cerebral hemisphere (Mouse M1). (j-l) PCA overlaid images showing arteries (red) and veins (blue) in the brain of Mouse M1, where the absence of arterial vessels in the left hemisphere can be seen due to MCAO. (m-n) Normalized average NIR-IIa signal in the left (red) and right (black) cerebral hemispheres of Mouse C1 (m) and M1 (n), plotted as a function of p.i. time. (o) Average relative blood perfusion in the left cerebral hemisphere, measured by NIR-IIa fluorescence imaging (red bars) and laser Doppler blood flowmetry (blue bars), for the control group ($n = 3$), MCAO group ($n = 4$) and cerebral hypoperfusion group ($n = 4$). Errors bars reflected the standard deviation of each group.
Figure 6.10 | Time Window of NIR-IIa Brain Vascular Imaging. NIR-IIa fluorescence images of a shaved mouse head taken at 1 min, 25 min, 60 min, 3 h and 5.5 h p.i., suggesting a time window of ~3 h p.i. for static brain vessel imaging, within which there was minimum change in the brain vascular image. Note that a black tape was placed to cover a wound on the head of this mouse for the images taken at 3 h and 5.5 h p.i.
Figure 6.11 | Reproduction of Dynamic NIR-IIa Brain Imaging in Control Mice.

(a-c) Time course NIR-IIa fluorescence images of a control, healthy Mouse C2. (d) A PCA overlaid image showing arteries (red) and veins (blue) in the brain of Mouse C2. (e-g) Time course NIR-IIa fluorescence images of a control, healthy Mouse C3. (h) A PCA overlaid image showing arteries (red) and veins (blue) in the brain of Mouse C3. (i-j) Normalized average NIR-IIa signal in the left (red) and right (black) cerebral hemispheres of Mouse C2 (i) and C3 (j), plotted as a function of p.i. time.
Figure 6.12 | Reproduction of Dynamic NIR-IIa Brain Imaging in MCAO Mice.

(a-c) Time course NIR-IIa fluorescence images of the brain of Mouse M2 with induced MCAO in the left cerebral hemisphere. (d) A PCA overlaid image showing arteries (red) and veins (blue) in the brain of Mouse M2. (e-g) Time course NIR-IIa fluorescence images of the brain of Mouse M3 with induced MCAO in the left cerebral hemisphere. (h) A PCA overlaid image showing arteries (red) and veins (blue) in the brain of Mouse M3. (i-k) Time course NIR-IIa fluorescence images of the brain of Mouse M4 with induced MCAO in the left cerebral hemisphere. (l) A PCA overlaid image showing arteries (red) and veins (blue) in the brain of Mouse M4. (m-o) Normalized average NIR-IIa signal in the left (red) and right (black) cerebral hemispheres of Mouse M2 (m), M3 (n) and M4 (o), plotted as a function of p.i. time.
Figure 6.13 | Reproduction of Dynamic NIR-IIa Brain Imaging in Mice with Cerebral Hypoperfusion. (a-c) Time course NIR-IIa fluorescence images of the brain of Mouse H1 with induced cerebral hypoperfusion in the left cerebral hemisphere. (d)
A PCA overlaid image showing arteries (red) and veins (blue) in the brain of Mouse H1. (e-g) Time course NIR-IIa fluorescence images of the brain of Mouse H2 with induced cerebral hypoperfusion in the left cerebral hemisphere. (h) A PCA overlaid image showing arteries (red) and veins (blue) in the brain of Mouse H2. (i-k) Time course NIR-IIa fluorescence images of the brain of Mouse H3 with induced cerebral hypoperfusion in the left cerebral hemisphere. (l) A PCA overlaid image showing arteries (red) and veins (blue) in the brain of Mouse H3. (m-o) Time course NIR-IIa fluorescence images of the brain of Mouse H4 with induced cerebral hypoperfusion in the left cerebral hemisphere. (p) A PCA overlaid image showing arteries (red) and veins (blue) in the brain of Mouse H4. (q-t) Normalized average NIR-IIa signal in the left (red) and right (black) cerebral hemispheres of Mouse H1 (q), H2 (r), H3 (s) and H4 (t), plotted as a function of p.i. time.
6.6 References


Chapter 7. In Vitro and In Vivo Fluorescence Imaging with Ag₂S Quantum Dots in the Second Near-Infrared Window

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7.1 Introduction

In the previous chapters, we have shown in vitro and in vivo fluorescence imaging in the second near-infrared window (NIR-II window, 1.0-1.7 μm) using single-walled carbon nanotubes (SWNTs) as biocompatible fluorophores. We have shown that, owing to the much lower photon scattering and tissue autofluorescence at longer wavelengths, 1-4 biological imaging in the NIR-II window has benefited from deeper tissue penetration, higher signal-to-background ratio (SBR) and better image fidelity, allowing us to image sub-10 μm features at millimeter depths inside the highly scattering animal tissue. 5,6 However, one of the major limitations of NIR-II fluorescence imaging remains the few choices of available NIR-II fluorophores that are biocompatible for biological imaging. A handful of inorganic and organic materials, such as SWNTs, 7 quantum dots (QDs), 8,9 and polymethine dyes 10,11 have been reported to have fluorescence emission in the NIR-II window, but so far only SWNTs have been made water soluble and biocompatible for in vitro cell imaging and in vivo animal imaging applications. Moreover, another issue of NIR-II fluorophores is their relatively low fluorescence quantum yields (QYs) in comparison with their
counterparts in the shorter-wavelength visible window (400-750 nm) and the traditional near-infrared window (NIR-I window, 750-900 nm).\(^7,\!12,\!13\) The sub-optimal QYs of NIR-II fluorophores, in particular SWNTs, limit their performance for \textit{in vivo} NIR-II fluorescence imaging with sufficient temporal resolution.\(^14\)

Due to the insufficient fluorescence brightness, the difficulty of biofunctionalization and the limited selection of current NIR-II fluorophores, there has always been an urgent need of developing new NIR-II fluorescent probes with higher fluorescence quantum yield and good biocompatibility. In this chapter, we will describe the synthesis and biofunctionalization of a new type of semiconducting QDs, Ag\(_2\)S QDs, for \textit{in vitro} and \textit{in vivo} fluorescence imaging at an emission wavelength of \(~1200\) nm in the NIR-II window. Semiconducting QDs, such as PbS,\(^9\) PbSe\(^{15}\) and CdHgTe,\(^{16}\) have been reported with fluorescence in the NIR-II window. However, the highly toxic nature of heavy metal elements such as Pb, Cd and Hg and the difficulty of surface functionalization of these QDs with biocompatible coatings have prohibited their \textit{in vivo} applications.\(^{17}\) Our Ag\(_2\)S QDs, on the other hand, do not contain any toxic metal element such as Cd, Pd or Hg, and have demonstrated in this chapter good biocompatibility and facile surface functionalization of polyethylene glycol (PEG) and various targeting ligands. The bright NIR-II fluorescence emission, low toxicity and small size of Ag\(_2\)S QDs have enabled us to perform selective molecular imaging of cancer cells \textit{in vitro} and tumor specific imaging with high tumor-to-background ratio (TBR) \textit{in vivo}.  

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7.2 Materials and Methods

Synthesis of water-soluble Ag$_2$S QDs. We synthesized hydrophobic Ag$_2$S QDs first in the organic phase and then transferred the as-synthesized Ag$_2$S QDs to an aqueous solution according to a previous publication.$^8$ In brief, 0.1 mmol of silver diethylidithiocarbamate [(C$_2$H$_5$)$_2$NCS$_2$Ag] and 10 g of 1-dodecanethiol (DT) were both added into a 100-mL three-necked flask at room temperature (RT). The mixture was stirred and vacuumed for approximately 5 min to remove any residual oxygen in the slurry. At a heating rate of 15 °C/min, the reactants were heated up to 210 °C, and allowed to stay at 210 °C for 1 h under nitrogen atmosphere. Then the mixture was cooled down naturally to RT, before a 50-mL solution of ethanol was added to the mixture to precipitate the product. The product of Ag$_2$S QDs was centrifuged at 6729 g for 20 min, allowing us to collect the precipitate from the pellet. DT-coated, hydrophobic Ag$_2$S QDs were obtained from the precipitates with average diameter of ~5.4 nm. We then transferred the hydrophilic Ag$_2$S QDs to aqueous phase and made them hydrophilic with surface coating of dihydrolipoic acid (DHLA). 0.05 mol of the as-synthesized hydrophobic Ag$_2$S QDs was mixed with 15 mL of cyclohexane, 15 mL of ethanol and 0.15 g of DHLA, and stirred at RT for 48 h. Then the mixture was centrifuged at 26916 g for 20 min, thoroughly washed with deionized (DI) water, and then redispersed in DI water. This procedure resulted in hydrophilic and water-soluble Ag$_2$S QDs with a surface coating of DHLA and sufficient –COOH groups.

Conjugation of DHLA-Ag$_2$S QDs with targeting ligands. 0.1 mg of the as-made water-soluble Ag$_2$S QDs with DHLA coating was dissolved in 200 µL of dimethyl sulfoxide (DMSO), while 1.15 mg (0.01mmol) of N-hydroxysuccinimide (NHS) was
dissolved in 50 µL of DMSO and was added to the Ag₂S QD solution under vigorous magnetic stirring. On the other hand, 1.91 mg (0.01 mmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was dissolved in 50 µL of DMSO and was added into the mixed DMSO solution of Ag₂S QDs and NHS. The reaction was allowed to proceed for 1 h in the dark under magnetic stirring. Then the Ag₂S QDs with activated surface groups were centrifuged, thoroughly washed with DMSO twice and redispersed in DMSO. To conjugate the surface activated Ag₂S QDs with Erbitux (Cetuximab), which was a targeting ligand for epidermal growth factor receptors (EGFR) on the membrane of MDA-MB-468 cells, 2 × 10⁻⁹ mol of Erbitux protein dissolved in 1× PBS was added to the Ag₂S QDs with a minimum amount of DMSO. To conjugate the surface activated Ag₂S QDs with RGD, which was a targeting ligand for αᵥβ₃-integrin on the membrane of U87-MG cells, 2 × 10⁻⁹ mol of cyclo-RGDfK (RGD-lysine) dissolved in 1× PBS was added to the Ag₂S QDs with a minimum amount of DMSO.

**Conjugation of DHLA-Ag₂S QDs with PEG.** The conjugation of DHLA-Ag₂S QDs with six-armed PEG was done in a similar way to conjugation with the targeting ligands via EDC/NHS chemistry. Briefly, 24 mg of six-armed PEG (6PEG, 10 kDa, Sunbio Inc.) and 120 µL of EDC/NHS solution at a concentration of 20 mg/mL were added into a 1.2 mL suspension of DHLA-Ag₂S in water at 0.5 mg/mL and sonicated for 30 min. After the sonication, another 360 µL of EDC/NHS solution at a concentration of 20 mg/mL were added, and vigorously stirred for 8 h. The solution was centrifuged at 26916 g for 20 min and thoroughly washed in 1× PBS buffer, and then redispersed in 1× PBS buffer to give the 6PEG-Ag₂S QD solution.
**UV-Vis-NIR absorbance measurement.** UV-Vis-NIR absorbance spectrum of the 6PEG-Ag$_2$S QD solution in the range of 400-900 nm was measured by a Cary 6000i UV-Vis-NIR spectrophotometer, background-subtracted for any absorbance contribution from the solvent water. A mass extinction coefficient of 2.728 L·g$^{-1}$·cm$^{-1}$ at 600 nm was used to determine the mass concentration of the Ag$_2$S QD solution based on the UV-Vis-NIR absorbance spectrum.

**Photoluminescence versus excitation (PLE) spectroscopy.** The PLE spectrum of the 6PEG-Ag$_2$S QD solution was taken on a home-built NIR-II spectroscopy setup. The excitation was provided by a white-light source of an ozone-free mercury/xenon lamp (Oriel) with a total power of 200 W, which was filtered by a UV filter to remove the ultraviolet light, a water filter to remove the short-wavelength infrared (SWIR) light (>1400 nm), and an 850 nm short-pass filter to remove the NIR light. The excitation light cleaned by these filters was then dispersed by a monochromator (Oriel) to generate single-wavelength excitation lines with a bandwidth of 15 nm, focused onto a 1 mm path cuvette with the 6PEG-Ag$_2$S QD solution at 1.34 mg/mL. The emitted fluorescence of SWNTs was collected in the transmission geometry. The transmitted excitation light from the sample was rejected using an 850-nm long-pass filter and collected by the spectrometer equipped with a one-dimensional (1D) indium-gallium-arsenide (InGaAs) linear array detector. The raw PLE spectra were corrected post-collection to account for the excitation power difference at different wavelengths, the extinction profile of the emission filter, and the sensitivity of the detector using the MATLAB software.
Transmission electron microscopy (TEM) imaging. The 6PEG-Ag$_2$S QD sample for TEM imaging was prepared by drop-drying the 6PEG-Ag$_2$S QD aqueous solution on a copper grid coated with amorphous carbon. Imaging and size measurement were performed with a Tecnai G2 F20 S-Twin TEM (FEI, USA) operated at a voltage of 200 kV. To obtain the statistical size distribution of the 6PEG-Ag$_2$S QDs, >100 individual QDs were analyzed from the TEM images.

Cell culture and staining. All cell culture media used in this work were supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin and L-glutamine. The U87-MG brain glioblastoma cells were cultured in Low Glucose Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 1 g/L D-glucose and 110 mg/L sodium pyruvate. The MDA-MB-468 breast cancer cells were grown in Leibovitz’s L-15 medium. U87-MG cells were maintained in cell culture flasks (Corning) with vented caps and placed in a 37 °C humidified incubator with 5% CO$_2$, while the MDA-MB-468 cells were maintained in cell culture flasks (Corning) with unvented caps and placed in the same incubator since CO$_2$ is detrimental to the growth of MDA-MB-468 cells.

For cell staining, both cell lines growing in the flasks were trypsinized at 37 °C for 5-10 min, before the Erbitux-Ag$_2$S QDs or the RGD-Ag$_2$S QDs were added to the suspensions of U87-MG (α$_v$β$_3$- positive and EGFR-negative) and MDA-MB-468 (α$_v$β$_3$- negative and EGFR-positive) cells. The final staining concentration of the Ag$_2$S QDs in the cell suspension was ~100 µg/mL. The staining was carried out at 4 °C for 2 h, followed by washing the cells thoroughly with cold 1× PBS three times to remove
the free, unbound QD conjugates in the suspension. The cells were always kept at 4 °C before imaging.

**High-magnification NIR-II fluorescence microscopy imaging.** Microscopic cell imaging in the NIR-II window was performed on a homemade widefield microscope setup coupled to a two-dimensional (2D) InGaAs camera (Princeton 2D OMA-V) to look at the fluorescence of Ag$_2$S QDs. A 658-nm laser diode (100 mW, Thorlabs) cleaned through a 750 nm short-pass filter (Omega) was used as the excitation source. The 658-nm laser was focused to an 80 μm diameter spot on the sample by a 100× objective lens (Olympus), and the emitted NIR-II photoluminescence from the Ag$_2$S QDs was allowed to pass through a 900 nm long-pass filter and an 1100 nm long-pass filter (Thorlabs) and focused onto the 2D InGaAs camera, with an exposure time of 3 s. For bright field white light images of the same field of view, a fiber optic illuminator (Fiber-Lite) was used to illuminate the cell sample in the transmission mode and the white light images were taken in the same setup with the same filters at an exposure time of 2 ms.

**Laboratory mouse handling.** All experiments involving the use of laboratory animals were carried out under the approval of Stanford University’s Administrative Panel on Laboratory Animal Care. Female Balb/c mice (Charles Rivers) were used in this study. A group of 7 mice were used for NIR-II fluorescence imaging with 4T1 tumor model, and they were inoculated with ~1 million 4T1 murine breast cancer cells on either the left or right hindlimb, or on both hindlimbs. The inoculated tumors were allowed to grow for 7 days before they reached 5-20 mm$^3$ in volume. The mice were
completely shaven prior to imaging. All mice were anesthetized before injection and imaging in a rodent anesthesia machine supplying a mixed gas flow of 2 L/min O₂ and 3% Isoflurane. For injection of the NIR-II contrast agent, a 28 gauge syringe needle was inserted into the lateral tail vein, and a bolus of 200 µL 6PEG-Ag₂S QD solution at a mass concentration of 1.34 mg/mL was injected into the tail vein in the dark during the first frames of the dynamic NIR-II fluorescence imaging. The injected dose was equivalent to ~0.75 nmol per mouse.

In vivo static fluorescence imaging in the NIR-II window. For static tumor imaging of live animals in the NIR-II window, the excitation was provided by an 808-nm laser diode, fiber-coupled to a collimator with a focal length of 4.5 mm, cleaned by an 850-nm short-pass filter and a 1000-nm short-pass filter to remove the long-wavelength photons, and directed to sample stage with a power density of 140 mW/cm². The tumor-bearing mouse was placed on the imaging stage in the prone position one at a time. The emitted fluorescence photons were filtered through a 900-nm long-pass filter and an 1100-nm long-pass filter to collect NIR-II fluorescence in the 1100-1700 nm region (note the upper bound of detection range was determined by the sensitivity profile of the camera). The 2D InGaAs camera of 320 × 256 pixels (Princeton Instruments) was used to form NIR-II fluorescence images. A lens pair consisting of two touching achromats (focal lengths of 200 mm and 75 mm) was used to image with a field of view covering the whole body of the mouse.

In vivo dynamic fluorescence imaging in the NIR-II window. The video-rate dynamic fluorescence imaging was carried out on the same imaging system as in the
static fluorescence imaging. To capture video-rate images, the 2D InGaAs detector was allowed to expose continuously with an exposure time of 100 ms immediately after a bolus injection of 200 μL 6PEG-Ag$_2$S QD solution at 1.34 mg/mL, and the images were acquired by a user-written LabVIEW software with an additional overhead time of 19 ms between two consecutive frames. The exposure time and the overhead time comprised a complete cycle of 119 ms for each image acquisition with a frame rate of 8.4 frames/s.

The video-rate images taken in the aforementioned manner were used for dynamic contrast-enhanced visualization using principal component analysis (PCA), in a similar way to previous publications by the Hillman group$^{18}$ and our group.$^{19}$ In a typical image processing task by the MATLAB software for tumor identification, the first 100 frames immediately after injection of Ag$_2$S QDs were loaded into a 3D array and reshaped into a 2D array, which was fed into the MATLAB built-in *princomp* function for PCA analysis. The 2$^{nd}$, 3$^{rd}$ and 4$^{th}$ principle components were color-coded in red, green and blue in the PCA overlaid image.

**Quantification of blood circulation half-life.** At various time points post injection (p.i.) of the 6PEG-Ag$_2$S QDs, 5-10 μL of arterial blood was drawn from the tail artery of 3 injected mice. NIR-II fluorescence images were taken for blood samples drawn from the mice injected with 6PEG-Ag$_2$S QDs and compared with a QD-free blood sample drawn from a control mouse not injected with QDs (for determining the blood autofluorescence, which was found to be minimal), and the QD-free blood spiked with a known concentration of 6PEG-Ag$_2$S QDs. The % injected dose (ID) of 6PEG-Ag$_2$S
QDs/gram of blood was analyzed by subtracting the blood autofluorescence and comparing with the sample with known concentration of 6PEG-Ag₂S QDs. The % ID/gram was plotted as a function of p.i. time and fitted into a first order exponential decay, from which the blood circulation half-life of 6PEG-Ag₂S QDs was derived.

**Quantification of organ biodistribution.** At 72 h p.i. of 6PEG-Ag₂S QDs, six mice injected with QDs were sacrificed, along with one control mouse that had not received any injection of the 6PEG-Ag₂S QDs. The mouse organs were collected post-mortem and weighed.

For NIR-II fluorescence-based semi-quantitative biodistribution measurement, the organs from three injected mice were mixed with 1 mL of the tissue lysis buffer containing 1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 40 mM tris acetate, 10 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM dithiothreitol (DTT) and ground using a hand-blender. The mixture was then heated to 70 °C for 2 h, resulting in a homogenous suspension. The organs of the control mouse were collected and treated in the same way, allowing us to quantify the autofluorescence level for each organ. The control organ suspensions were then spiked with a known concentration of 6PEG-Ag₂S QDs, allowing us to quantify the amount of QDs in any unknown sample based on its NIR-II fluorescence. NIR-II fluorescence images were taken for all organ suspensions with quantitative fluorescence intensity and normalized against the control mouse organs, and the biodistribution of 6PEG-Ag₂S QDs was quantified in units of % ID/gram for each organ as a semi-quantitative measure, except for the liver which caused significant quenching of the NIR-II fluorescence of QDs over time.
For the quantitative biodistribution measurement based on inductively-coupled plasmon-mass spectrometry (ICP-MS), the organs from the other three injected mice were mixed with 35% HNO₃ and heated up to 70 °C for 12 h, resulting in a homogenous solution. The organ solutions were diluted 2000 times in DI water and submitted for ICP-MS measurements at the Environmental Measurement I: Gas-Solution Analytical Center at Stanford University. The Ag concentration in each organ solution measured by ICP-MS was compared with that in the injected solution to derive the % ID/gram value of Ag₂S QDs in that specific organ.

**Short term excretion study of 6PEG-Ag₂S QDs.** Female Balb/c mice without any tumor (n = 4) were injected with 200 µL of the 6PEG-Ag₂S QD solution at 1.34 mg/mL and housed in metabolic cages. Urine and feces were collected and weighed for the first 24 h p.i. for all mice, after which two of them were sacrificed for ICP-MS based biodistribution according to the aforementioned protocol, while the other two were maintained and collected for urine and feces until 7 days p.i.. The urine and feces samples were dissolved in 35% HNO₃ at 70 °C for 12 h, diluted in DI water and analyzed by ICP-MS. The Ag concentration in each urine/feces sample measured by ICP-MS was compared with that in the injected solution to derive the % ID/gram value of Ag₂S QDs in that specific excretion sample.

**Dynamic light scattering (DLS) measurement of 6PEG-Ag₂S QDs.** An aqueous solution of 6PEG-Ag₂S QDs at a mass concentration of 0.05 mg/mL was used for DLS measurement. The solution was loaded in a 1-cm quartz cuvette with four
transparent walls and DLS was performed using a Brookhaven Instruments 90Plus Particle Size Analyzer.

### 7.3 Results and Discussion

We first synthesized the dodecanethiol-coated Ag₂S QDs in organic phase using the method described by a previously published protocol, and then transferred the hydrophobic Ag₂S QDs to aqueous phase by exchanging the dodecanethiol coating to dihydrolipoic acid. The DHLA-coated Ag₂S QDs were water soluble and had abundant –COOH functional groups allowing us to covalently attach different targeting ligands and PEG chains to the QDs (see Fig. 7.1a for the structure of six-armed PEG, 6PEG). Taking the 6PEG-Ag₂S QDs with an average size of ~5.4 nm (Fig. 7.1f) as an example, the brownish colored solution (Fig. 7.1b) featured strong fluorescence emission in the NIR-II window upon an 808-nm laser excitation (Fig. 7.1c), while the NIR-II fluorescence remained highly stable in different media such as saline buffer, serum and whole blood (Fig. 7.2a-c) and under prolonged laser excitation at a power density of 0.14 W/cm² (Fig. 7.2d). The absorption spectrum of the 6PEG-Ag₂S QD solution in the 400-900 nm range exhibited declining absorbance at longer wavelengths (Fig. 7.1d), which was consistent with the absorption measurement of Ag₂S QDs in a previous publication. The PLE spectrum revealed the emission peak of 6PEG-Ag₂S QDs at approximately 1200 nm (Fig. 7.1e), which was located in the NIR-II window and independent of excitation wavelength.

We carried out selective molecular imaging of live cancer cells with Ag₂S QDs
to show the capability of these brightly fluorescent QDs as effective NIR-II fluorescent labels. Two different cell lines, the human glioblastoma U87-MG cells and the human breast cancer MDA-MB-468 cells were used for selective cell targeting experiments, because these two cell lines represented distinct expression levels of two important membrane receptors, \( \alpha_v \beta_3 \) integrin (higher expression in U87-MG cells and lower expression in MDA-MB-468 cells) and EGFR (higher expression in MDA-MB-468 cells and lower expression in U87-MG cells). To specifically target these two receptors with Ag\(_2\)S QDs, we covalently attached Erbitux (Cetuximab) protein and RGD peptide to the –COOH groups of the DHLA-coated Ag\(_2\)S QDs\(^{14,20}\) and made two bioconjugates with different targeting ligands respectively (see Section 7.2 for more details). Orthogonal combinations of the two cell lines and the two Ag\(_2\)S QD bioconjugates resulted in four cell staining experiments, which were conducted at 4 °C for 2 h. The NIR-II fluorescence images of the cells after staining and washing clearly revealed a much higher NIR-II signal from the Ag\(_2\)S QDs in the EGFR-positive MDA-MB-468 cells than the EGFR-negative U87-MG cells when both were stained with Ag\(_2\)S QD-Erbitux, while a higher NIR-II signal was observed for the \( \alpha_v \beta_3 \)-positive U87-MG cells than the \( \alpha_v \beta_3 \)-negative MDA-MB-468 cells when stained with Ag\(_2\)S QD-RGD (Fig. 7.3). Similar to selective cell staining and imaging using SWNTs as described in Chapter 3, the cells appeared round-shaped in the NIR-II fluorescence images and their corresponding white light optical images, owing to trypsinization to remove the extracellular matrix (ECM) and staining at a low temperature of 4 °C. We have reversed the change of cell morphology by increasing the incubation temperature to 37 °C and observed elongation and expansion of the U87-MG cells, showing the
proof of live cells during staining and imaging. The selective targeting experiments suggested Ag$_2$S QDs as a new fluorescent label for molecular imaging in the NIR-II window, and that the surface functionalization of Ag$_2$S QDs could be engineered to afford either biological affinitive or inert QDs for specific cells and tissues.

We then used the 6PEG-Ag$_2$S QDs with PEGylated, inert surface as a blood pool contrast agent for in vivo NIR-II fluorescence imaging in real time. A 200 µL solution of the 6PEG-Ag$_2$S QDs at a mass concentration of 1.34 mg/mL was injected into the tail vein of a tumor bearing Balb/c mouse, with the xenograft 4T1 tumor inoculated subcutaneously near the right hind limb. The injected solution corresponded to an equivalent dose of ~0.75 nmol per mouse, which was on the low side compared to other types of QDs injected for in vivo fluorescence imaging in shorter-wavelength windows (0.2~6 nmol per mouse). The NIR-II fluorescence images taken immediately after injection of 6PEG-Ag$_2$S QDs revealed the lungs at merely ~2 s p.i., corresponding to the pulmonary circulation of venous blood to get oxygenated in the lungs (Fig. 7.4a-c). At ~4 s p.i., the systemic circulation brought the blood with the Ag$_2$S QDs into the kidneys (Fig. 7.4d), and the tumor tissue started showing up in the NIR-II images after 15 s p.i. as the injected 6PEG-Ag$_2$S QDs entered the local microvasculature inside the tumor (Fig. 7.4e). The tortuous vasculature inside the tumor tended to ‘trap’ the circulation of 6PEG-Ag$_2$S QDs, leading to a continuous increase of NIR-II fluorescence signal and better visibility of the vessels in the tumor region over time (Fig. 7.4f&g). Therefore, dynamic NIR-II fluorescence imaging with 6PEG-Ag$_2$S QDs allowed us to pinpoint the location of the tumor immediately after injection of the QD contrast agent.
We have shown in Chapters 5 and 6 that PCA was a very useful mathematically tool allowing us to differentiate the faster arterial blood flow from the slower venous flow based on their hemodynamic difference. Here PCA was also used to differentiate different organs based on their appearance in the dynamic imaging, and to discriminate the cancerous tissue from the normal tissue owing to the distinct vascular morphology that led to distinct flow patterns. Dynamic contrast was generated by PCA on the first 100 video frames taken from the dynamic NIR-II fluorescence imaging (0~11.9 s p.i.), and revealed clear distinction between the lungs (color coded in blue) and the kidneys (color coded in red), as well as distinguished the tumor (color coded in green) from other organs. PCA worked by grouping the many pixels in an image into a few components, where the pixels assigned to one component shared similar time variance, and thus turning the dynamic contrast (temporal contrast) into spatial contrast to enhance the image visibility. Owing to the enhanced image quality, we found that PCA provided a more sensitive way of pinpointing the tumor location by distinguishing the tumor from normal tissue within a much shorter time (~12 s p.i.) than inspecting the real-time video alone (~2min p.i.).

After dynamic NIR-II fluorescence imaging that tracked the blood flow in real time, we also monitored the distribution of the injected 6PEG-Ag$_2$S QDs inside the mouse in a longer time frame up to 24 h p.i. (Fig. 7.5a-e & Fig. 7.6). Owing to the enhanced permeability and retention (EPR) effect of the tortuous tumor vasculature, we observed a steady increase of NIR-II fluorescence indicating continuous accumulation of 6PEG-Ag$_2$S QDs in the tumor region, alongside with a monotonic decrease of NIR-II signal in the skin and other organs, from 30 min p.i. to 24 h p.i.
The two opposite trends led to an increase of the tumor-to-background ratio (TBR) from 30 min p.i. to 10 h p.i., followed by a slight decrease of TBR from 10 h p.i. to 24 h p.i. (Fig. 7.5g). It is noteworthy that after 4 h p.i., the TBR rose above the threshold of 5 set by Rose’s criterion for tumor identification with 100% certainty, and peaked with a TBR of ~15 at 10 h p.i., which was the optimum time point for static tumor imaging and detection with Ag₂S QDs. The exceptionally high tumor accumulation of injected 6PEG-Ag₂S QDs even led to visibly darkened tumor mass at 24 h p.i. (Fig. 7.5f).

To glean the effect of the PEGylated surface coating to the pharmacokinetics of intravenously administered Ag₂S QDs, we measured the blood circulation half-life of 6PEG-Ag₂S QDs by fitting the concentration of Ag₂S QDs in the blood at different time points after injection into a first-order exponential decay (see Section 7.2 for more details). We found a circulation half-life of 4.37±0.75 h (Fig. 7.5h) for the mouse imaged in Fig. 7.4 & 7.5, and repeated the measurement on an additional two mice with a half-life of 3.98±1.16 h and 3.51±0.86 h, respectively (Fig. 7.7). The measured circulation half-life of 6PEG-Ag₂S QDs with a branched PEG coating was significantly longer than previously reported QDs coated with linear PEG, such as CdSe/ZnS-PEG₇₅₀ with a \( t_{1/2} \) of <12 min, and CdSe/ZnS-PEG₅₀₀₀ with a \( t_{1/2} \) of 2 h.\(^{23,26}\). This longer circulation half-life suggested a better surface passivation and higher biocompatibility, resulting in slower uptake and clearance of the injected Ag₂S QDs by the reticuloendothelial system (RES).

We also evaluated the biodistribution of the injected 6PEG-Ag₂S QDs in various mouse organs at 72 h p.i., when the majority of the administered QDs should
have stopped circulating in blood given a circulation half-life of 3~4 h. Two independent methods of measuring the amount of Ag₂S QDs were employed, including the NIR-II fluorescence-based semi-quantitative measurement (Fig. 7.8) and ICP-MS based quantitative measurement (Fig. 7.5i). Both methods suggested high uptake of the 6PEG-Ag₂S QDs inside the liver and spleen, which are part of the RES system and responsible for metabolism and excretion of the injected Ag₂S QDs. The ICP-MS method, which was considered as more accurate since it measured the absolute Ag content in the organ samples, found a tumor uptake of ~10% ID/gram for non-targeted, passive accumulation of 6PEG-Ag₂S QDs. The ~10% ID/gram passive tumor uptake was among the highest values reported so far for intravenously administered QDs, and was comparable to a previous work where a ~20% ID/gram tumor uptake was found at 4 h p.i. and followed by a drop to ~7% by 24 h p.i. based on fluorescence intensity measurement. The high tumor uptake was a direct result of the relatively long blood circulation half-life, and confirmed the successful surface passivation with the branched 6PEG coating and minimized opsonization for RES uptake in vivo.

As with any new materials introduced for in vivo uses, the potential toxicity to the organism and long term fate in the body usually need thorough evaluation. Our Ag₂S QDs was made of a material with ultralow solubility product constant in water [K_{sp}(Ag₂S) = 6.3 \times 10^{-50}], much lower than other metal chalcogenide materials, ensuring the minimum amount of Ag ions dissolved and released into the biological surroundings. Through careful in vivo toxicology studies including blood biochemistry, hematological analysis and histological examinations performed by our collaborators,
the 6PEG-Ag$_2$S QDs demonstrated minimal toxicity over a period of 2 months at an intravenously administered dose up to 1.68 nmol per mouse, which was >2-fold higher than used for imaging in this work.$^{28}$ Nonetheless, we also carried out an independent short-term study of the retention and excretion after injection of 6PEG-Ag$_2$S QDs on a group of 4 mice, to glean the clearance pathway of this novel NIR-II fluorescent nanomaterial from the body. Urine and feces were collected for ICP-MS based quantification of Ag$_2$S QDs on a daily basis, revealing the excretion of intravenously administered 6PEG-Ag$_2$S QDs mainly via the biliary pathway with a steady clearance rate (Fig. 7.9a), while a small amount of the injected QDs was excreted through the urine via renal clearance (Fig. 7.9b). This excretion pattern was rationalized by measuring the hydrodynamic radius of the 6PEG-Ag$_2$S QDs (~26.8 nm), which was significantly larger than the renal filtration cutoff size of ~5.5 nm (Fig. 7.9d).$^{29}$ We also examined the retention of the injected 6PEG-Ag$_2$S QDs in various organs at 24 h and 168 h p.i., and found the majority of Ag$_2$S QDs cleared out from most organs except the liver and the spleen (Fig. 7.9c).

7.4 Conclusion

In conclusion, we have developed water soluble and biocompatible Ag$_2$S QDs with different surface functional groups by covalently linking targeting ligands and branched PEG chains to the carboxyl residues on the surface of the Ag$_2$S QDs. The toxic heavy metal free Ag$_2$S QDs have proved to be a novel NIR-II fluorescent label for in vitro cell molecular imaging and in vivo tumor imaging. The ~1200 nm emission
of Ag$_2$S QDs upon excitation of 808 nm laser allowed for deep tissue imaging and fast tumor diagnosis, with an unprecedented high tumor uptake of >10% ID/gram through non-specific EPR effect of 6PEG-Ag$_2$S QDs owing to the branched PEGylation. The short-term excretion study revealed a mixed clearance pathway through both urine and feces, where the biliary excretion was found as the dominant way of clearance.
7.5 Figures

Figure 7.1 | Characterizations of 6PEG-Ag$_2$S QDs. (a) A schematic drawing of the 6PEG-Ag$_2$S QDs, with the inset showing the chemical structure of the six-arm branched PEG molecule. The 6PEG-Ag$_2$S QDs emit fluorescence at ~1200 nm upon an 808-nm excitation. (b) A white-light digital camera photograph of the 6PEG-Ag$_2$S QDs dispersed in an aqueous solution. (c) An NIR-II fluorescence image of the solution of 6PEG-Ag$_2$S QDs shown in b. (d) A UV-Vis-NIR absorption spectrum of the 6PEG-Ag$_2$S QD solution in the range of 400-900 nm. (e) A PLE spectrum of the 6PEG-Ag$_2$S QD solution showing the emission centered at ~1200 nm with a broad excitation band ranging from 550 nm to 820 nm. (f) A TEM photograph of the 6PEG-Ag$_2$S QDs with an average diameter of 5.4 nm. Scale bar: 20 nm.
Figure 7.2 | Stability of 6PEG-Ag₂S QDs. (a) A white-light digital camera photograph showing the 6PEG-Ag₂S QDs spiked in 1× PBS, fetal bovine serum (FBS) and mouse whole blood at a mass concentration of 0.134 mg/mL. (b) A corresponding NIR-II fluorescence image for the white-light image in a. (c) A bar chart showing the mean NIR-II fluorescence of 6PEG-Ag₂S QDs in 1× PBS, FBS and mouse whole blood, showing no quenching of fluorescence in biological media. (d) The NIR-II photoluminescence (PL) intensity of 6PEG-Ag₂S QDs plotted as a function of time under continuous laser irradiation, showing <50% decay of fluorescence over 0.5 h.
**Figure 7.3 | Selective Cell Staining with Ag$_2$S QDs.** Microscopic NIR-II fluorescence images of the EGFR-positive MDA-MB-468 cells (a&c) and $\alpha_v\beta_3$-positive U87-MG cells (b&d) stained with Ag$_2$S QD-Erbitux (a&b) and Ag$_2$S QD-RGD (c&d) bioconjugates are shown with their corresponding white light optical images in the insets.
Figure 7.4 | Video-Rate NIR-II Imaging of a Tumor Bearing Mouse with 6PEG-Ag$_2$S QDs. (a-g) Time course NIR-II fluorescence images of a 4T1 tumor bearing mouse extracted from a video taken immediately after intravenous injection of 6PEG-Ag$_2$S QDs. (h) A PCA overlaid image based on the video frames of dynamic NIR-II fluorescence imaging, showing different organs and tissues assigned into distinct principal components and color coded in red (kidneys), green (tumor) and lungs (blue).
Figure 7.5 | Post Injection Static NIR-II Imaging of a Tumor Bearing Mouse with 6PEG-Ag$_2$S QDs. (a-e) Time course static NIR-II fluorescence images of a 4T1 tumor bearing mouse taken at different time points after injection of 6PEG-Ag$_2$S QDs. The increasing accumulation of NIR-II fluorescence in the tumor region can be seen over time. (f) A white light digital camera photograph of the same mouse taken at 24 h p.i., showing the visibly darkened tumor mass due to the exceptionally high tumor uptake of the 6PEG-Ag$_2$S QDs. (g) TBR plotted as a function of time p.i., indicating a 100% certainty of tumor identification from static NIR-II imaging after 4 h p.i., based on the Rose criterion. (h) A graph showing the % ID/gram of 6PEG-Ag$_2$S QDs in the blood circulation measured by NIR-II fluorescence and plotted against time p.i., which was
fitted into a first order exponential decay with a circulation half-life of 4.37±0.75 h for the 6PEG-Ag$_2$S QDs. (i) A graph showing the % ID/gram of 6PEG-Ag$_2$S QDs in different organs and the tumor mass at 72 h p.i., measured by ICP-MS.
Figure 7.6 | Post Injection Static NIR-II Imaging Reproduced on an Additional Two Tumor Bearing Mice. (a-h) Time course static NIR-II fluorescence images of the 4T1 tumor bearing Mouse 2 (a-d) and Mouse 3 (e-h) taken at different time points after injection of 6PEG-Ag2S QDs.
Figure 7.7 | Blood Circulation Half-life Measurement Reproduced on an Additional Two Mice. A blood circulation half-life of $3.98 \pm 1.16$ h and $3.51 \pm 0.86$ h was found for the two mice, respectively, based on fitting the NIR-II fluorescence measurements of mouse blood samples to a first-order exponential decay.
Figure 7.8 | Biodistribution of Ag$_2$S QDs Based on NIR-II Fluorescence Measurement. This graph shows the % ID/gram of 6PEG-Ag$_2$S QDs in different organs and the tumor mass at 72 h p.i., measured by NIR-II fluorescence imaging.
Figure 7.9 | Short-Term Excretion and Retention Study of 6PEG-Ag$_2$S QDs. (a) A graph showing the % ID/gram of 6PEG-Ag$_2$S QDs in the feces collected and measured by ICP-MS on a daily basis. (b) A graph showing the % ID/gram of 6PEG-Ag$_2$S QDs in the urine collected and measured by ICP-MS on a daily basis. (c) A graph showing the % ID/gram of 6PEG-Ag$_2$S QDs in various organs at 24 h and 168 h p.i., measured by ICP-MS. (d) A bar chart showing the distribution of hydrodynamic radius of 6PEG-Ag$_2$S QDs in water measured by DLS.
7.6 References

25 Bushberg, J. T. *The essential physics of medical imaging*. (Lippincott Williams & Wilkins, 2002).
Chapter 8. Ultrafast *In Vivo* Fluorescence Imaging of Blood Flow with Conjugated Polymers in the Second Near-Infrared Window

* The work in this chapter is accepted for publication as:


8.1 Introduction

Owing to the many benefits of the second near-infrared (NIR-II, 1.0-1.7 μm) window for *in vivo* fluorescence imaging, a number of nanomaterials with fluorescence in this window have been developed in the past five years, including the single-walled carbon nanotubes (SWNTs)$^{1-7}$ and semiconducting quantum dots (QDs)$^{8-12}$ as described in Chapters 2–7, as well as a handful of other NIR-II fluorophores reported by us and others, such as the rare-earth-doped nanoparticles$^{13}$ and small organic fluorophore incorporated nanoparticles.$^{14}$ Although these NIR-II fluorescent nanomaterials can be surface functionalized to afford good biocompatibility and minimum toxicity for *in vivo* administration,$^{12,15,16}$ there remain a few drawbacks of existing NIR-II fluorophores, such as the unfavorably low fluorescence quantum yield (QY),$^{4,13,17}$ potential toxicity due to heavy metals in the chemical composition,$^{8-13}$ and the relatively short fluorescence emission wavelengths associated with lingering scattering.$^{14}$ Therefore, it is desired to develop a much wider selection of NIR-II fluorophores to overcome these shortcomings. Ideally, the optimal NIR-II fluorophores should allow for tunable fluorescence emission wavelengths in the 1.0-1.7 μm NIR-II window with high QYs and good biocompatibility.
It has been reported that, via alternating copolymerization of electron-donating molecules with electron-withdrawing molecules, the bandgap energy can be reduced to longer wavelengths owing to the formation of charge-transfer structure between the neighboring donor and acceptor units.\textsuperscript{18,19} The bandgap energy can be engineered by synthesizing new structures of the donor and acceptor monomers, resulting in low bandgap copolymers with tunable absorption bands for efficient light harvesting.\textsuperscript{20-25} However, the fluorescence properties of these low bandgap copolymers have been rarely studied and none of these polymers has ever been used as fluorophores for \textit{in vitro} or \textit{in vivo} biomedical imaging in the NIR-II window. In this chapter, we will describe the synthesis of such a poly(donor-acceptor) (pDA) molecule with exceptionally bright fluorescence emission in the NIR-II window, and the application of the PEGylated pDA nanoparticles for \textit{in vitro} targeted cell imaging and \textit{in vivo} hemodynamic imaging. The strong NIR-II fluorescence emission of the pDA molecules has allowed us to track the blood flow pattern in cardiogram waveform with unprecedented fast temporal resolution.

\textbf{8.2 Materials and Methods}

\textbf{Synthesis of the pDA copolymer.} Scheme 8.1 shows the chemical structures of the two monomers M1 and M2, which can be synthesized according to previously published protocols.\textsuperscript{26-28} The pDA polymer was synthesized by alternating copolymerization of the two monomers M1 and M2, as shown in the chemical equation of Scheme 8.1. In brief, 0.091 g of the monomer M1 (0.2 mmol), 0.148 g of
the monomer M2 (0.2 mmol) and 15 mL of anhydrous toluene were mixed in a two-neck round-bottom flask. The mixture was flushed with nitrogen gas for 10 min, before 15 mg of Pd(PPh₃)₄ was added into the flask. Then the mixture was flushed with nitrogen gas for another 25 min. The two-neck round-bottom flask was placed in an oil bath and heated up to 110 °C carefully. Then the reactant mixture was allowed to stay at 110°C for 24 h with magnetic stirring under the inert atmosphere of nitrogen gas. After the reaction reached completion, the flask was allowed to cool down naturally to room temperature (RT), and 100 mL of methanol was added to the mixture to precipitate the polymer product. The mixture was filtered through a Soxhlet thimble, where Soxhlet extraction was carried out using methanol, hexanes and chloroform. Rotary evaporation of the chloroform fraction from Soxhlet extraction resulted in a green solid of the pDA polymer. The green solid was allowed to dry under vacuum overnight, and 80 mg of the pDA polymer was obtained with a yield of 56%.

**Nuclear magnetic resonance (NMR) spectroscopy of monomers and polymer.** The ¹H NMR spectra were measured for the two monomers and the pDA polymer, and ¹⁹F NMR spectrum was measured for the fluorinated monomer M1 only. The NMR spectroscopy was performed on a Bruker AV-400 spectrometer at RT, where chemical shifts were described as δ values in units of ppm, and tetramethylsilane (TMS) was used as an internal reference. The splitting patterns in the NMR spectra were labeled as s (singlet), d (doublet), t (triplet), m (multiplet) and br (broad). We have assigned all peaks in the NMR spectrum of the pDA polymer and compared them to the peaks in the NMR spectra of the monomers M1 and M2 as follows,
6.80 (br, 2H): aromatic H on the benzenedifuran, corresponding to 7.06 (s, 2H) of M2.

4.31 (br, 4H): methylene H adjacent to O of the phenol ester, corresponding to 4.35 (d, 4H) of M2.

3.06 (br, 2H), methylene H adjacent to the carbonyl group (α-hydrogen of the ketone), corresponding to 2.95 (t, 2H) of M1.

2.01-1.21 (br, 30H): all other H’s on the methylene groups, corresponding to 1.76 (m, 2H) plus 1.39-1.30 (m, 10H) of monomer M1, and 1.78 (m, 2H) plus 1.70-1.37 (m, 16 H) of monomer M2.

0.81-1.21 (br, 15H): all H’s on the terminal methyl groups (5 terminal methyl groups per unit), corresponding to 0.91 (t, 3H) of monomer M1 and 0.99 (m, 12 H) of monomer M2.

**Elemental analysis of the polymer.** Elemental analysis was performed on a Flash EA 1112 elemental analyzer. We calculated the elemental composition of pDA copolymer based on the molecular formula of \((C_{41}H_{53}FO_{5}S_{2})_n\) as C: 69.26%; H: 7.80%; O: 11.25%, and compared the calculated values to the measured elemental composition: C: 70.08%; H: 7.73%; O: 11.09%, where a good agreement between the calculation and measurement was found.

**Gel permeation chromatography (GPC) of the polymer.** We performed GPC to measure the molecular weight and polydispersity of the as-synthesized pDA copolymer. Polystyrene was used as the reference for the number-average molecular weight \((M_n)\), weight-average molecular weight \((M_w)\) and polydispersity index (PDI, \(M_w/M_n\)). GPC measurements were performed using a Waters 515 HPLC pump, a
Waters 2414 differential refractometer, and three Waters Styragel columns (HT2, HT3 and HT4). Tetrahydrofuran (THF) was used as the eluent at a flow rate of 1.0 mL/min at 35 °C.

**Preparation of pDA-PEG and pDA-PEG-NH₂.** The pDA copolymer was first dissolved in THF to reach a concentration of 0.075 mg/mL, and DSPE-mPEG(5k) (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol, 5000)], Laysan Bio) or DSPE-PEG(5k)-NH₂ (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)5,000], Laysan Bio) was dissolved in deionized ultra-filtered (DIUF) water to reach a concentration of 1.1 mg/mL. Then the THF solution of pDA polymer and the aqueous solution of DSPE-mPEG(5k) or DSPE-PEG(5k)-NH₂ were mixed with a volume ratio of 1:9 and vortexed. If DSPE-mPEG(5k) was used for mixing, pDA-PEG was made; if DSPE-PEG(5k)-NH₂ was used for mixing, pDA-PEG-NH₂ was made. The pDA polymer should maintain soluble without any visible precipitation in the mixed solvent of water and THF. Note that no sonication should be involved in this mixing step, otherwise some visible precipitates would form after sonication.

The mixture was then allowed to dialyze against water in a 3500MWCO dialysis membrane (Fisher) to remove THF, resulting in a clear aqueous solution of pDA-PEG or pDA-PEG-NH₂ nanoparticles. The solution was ultracentrifuged at 300,000 g for 0.5 h to remove aggregates formed during dialysis. Only the supernatant was collected after ultracentrifugation, and was washed with DIUF water in a 30k NMWL centrifugal filter (Amicon) unit to remove excess surfactant. No precipitation was observed during the centrifugal filtration step, suggesting a strongly held complex
was formed through supramolecular interactions between the hydrophobic pDA and the phospholipid chain of DSPE-mPEG or DSPE-PEG-NH₂. The pDA-PEG or pDA-PEG-NH₂ nanoparticles can be concentrated down to an equivalent pDA concentration of ~1.5 mg/mL by the 30k NMWL centrifugal filter.

**Preparation of pDA-PEG-Erbitux bioconjugate.** The as-made pDA-PEG-NH₂ solution with excess surfactant removed through centrifugal filtration was concentrated down to an equivalent pDA concentration of 0.32 mg/mL, which was determined by applying a mass extinction coefficient of 30.4 L·g⁻¹·cm⁻¹ for pDA at its absorption peak in the UV-Vis-NIR absorption spectrum. 200 μL of this solution was mixed with 1 mM sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) at pH 7.4, and allowed to react for 1 h on an orbital shaker at RT. In the meantime, a 12.3 μL solution of Erbitux (Cetuximab) at 2 mg/mL (~13.3 μM) along with a 1.64 μL solution of Traut’s reagent at 1 mM was added to an 86 μL solution of 1× phosphate buffered saline (PBS). The mixture containing Erbitux and Traut’s reagent with a molar ratio of 1:10 was allowed to react for 1.5 h at RT. The mixture of pDA and sulfo-SMCC was washed with DIUF water in a 30k NMWL centrifugal filter (Amicon) unit to remove excess sulfo-SMCC, and the mixture of Erbitux and Traut’s reagent was washed with 1× PBS in a 30k NMWL centrifugal filter unit to remove excess Traut’s reagent. Then the two solutions were mixed together and allowed to react at pH 7.4 for 2 days at 4 °C, resulting in a solution of pDA-PEG-Erbitux bioconjugate ready for selective staining and molecular imaging experiments later.
**UV-Vis-NIR absorbance measurement.** UV-Vis-NIR absorbance spectrum of the pDA-PEG aqueous solution in the range of 400-900 nm was measured by a Cary 6000i UV-Vis-NIR spectrophotometer, background-subtracted for any absorbance contribution from the solvent water. A mass extinction coefficient of 30.4 L·g⁻¹·cm⁻¹ at the peak absorption was used to determine the concentration of the pDA-PEG solution in terms of the equivalent mass of pDA, based on the UV-Vis-NIR absorbance spectrum.

**NIR-II fluorescence spectroscopy of the pDA-PEG solution.** The NIR-II fluorescence spectrum of the pDA-PEG solution was taken on a home-built NIR spectroscopy setup. The excitation was provided by an 808-nm laser diode (RMPC lasers) at a total output power of 160 mW and filtered by an 850-nm short-pass filter, a 1000-nm short-pass filter, an 1100-nm short-pass filter and a 1300-nm short-pass filter. The excitation light cleaned by these filters was then focused onto an aqueous solution of pDA-PEG in a 1-mm path cuvette (Starna Cells, Inc.). The emitted fluorescence of the solution was collected in a transmission geometry at an angle of 180° to the incident excitation light. The transmitted excitation light was rejected using a 900-nm long-pass filter and the fluorescence was collected by the spectrometer (Acton SP2300i) equipped with a one-dimensional (1D) indium-gallium-arsenide (InGaAs) linear array detector (Princeton OMA-V). The raw fluorescence spectrum in the range of 900-1500 nm was corrected post-collection to account for the extinction profile of the emission filter and the sensitivity of the detector using the MATLAB software.

**Fluorescence QY measurement of pDA-PEG.** Fluorescence QY of pDA-PEG in an aqueous solution was measured in a similar manner to a previous paper,²⁹ employing
the NIR-II fluorescent IR-26 dye as the reference (QY = 0.5%). To calibrate the fluorescence intensity versus concentration for the reference molecule in our setup, we dissolved IR-26 in 1,2-dichloroethane (DCE) to make a stock solution at 1 mg/mL, and diluted the stock solution in pure DCE to obtain a series of IR-26 solutions with absorbance of ~0.10, ~0.08, ~0.06, ~0.04 and ~0.02 at 808 nm, which was the wavelength of the laser excitation. The use of highly diluted solutions of the fluorophore was to minimize secondary optical processes such as self-quenching effect and the reabsorption/reemission effect. Then these five solutions of IR-26 dye in DCE were loaded in a 1-cm path cuvette (Starna Cells, Inc.) for fluorescence spectral measurement one at a time.

Fluorescence spectroscopy was carried out in a similar way to the previous “NIR-II fluorescence spectroscopy of the pDA-PEG solution” section. In brief, the excitation was provided by an 808-nm laser diode (RMPC lasers) with a linewidth FWHM of 1.89 nm at a total output power of 160 mW and filtered by an 850-nm short-pass filter, a 1000-nm short-pass filter, an 1100-nm short-pass filter and a 1300-nm short-pass filter. The emitted fluorescence of the solution in the 1-cm path cuvette was collected in a transmission geometry using a 900-nm long-pass filter as the emission filter.

The same UV-Vis-NIR absorbance and fluorescence emission measurements were conducted for pDA-PEG and SWNTs in aqueous solutions too. Then all raw fluorescence spectra of the reference and the samples in the range of 900-1500 nm were corrected post-collection using the MATLAB software, and integrated over all acquired wavelengths. According to the definition of QY, all emitted fluorescence
photons needed to be integrated for QY measurement.\textsuperscript{31} Due to the non-ideal cutoff profiles of the filters we used for fluorescence spectroscopy, the integrated fluorescence emission in the range of 900-1500 nm was only a subset of all emitted fluorescence photons, which should start from 809 nm (given an excitation source at 808 nm). Therefore we anticipated an underestimated QY measurement for pDA-PEG in our setup. We then plotted the integrated total fluorescence intensity against absorbance at 808 nm for each sample at each concentration, and fitted the plot into a linear function with its slope extracted. The slope derived from the reference (\textit{slope}_\textit{ref}) with a known QY of 0.5\% (\textit{QY}_\textit{ref}) and the slope from the sample (\textit{slope}_\textit{sample}) with unknown QY were used for the calculation of the QY of the sample (\textit{QY}_\textit{sample}):\[
QY_{\text{sample}} = QY_{\text{ref}} \cdot \frac{slope_{\text{sample}}}{slope_{\text{ref}}} \cdot \left(\frac{n_{\text{sample}}}{n_{\text{ref}}}\right)^2
\]
where \textit{n}_{\text{sample}} and \textit{n}_{\text{ref}} indicated the refractive indices of water and DCE, respectively.

\textbf{Atomic force microscopy (AFM) imaging of pDA-PEG.} AFM imaging of the pDA-PEG nanoparticles was acquired with a Nanoscope IIIa multimode AFM performing in the tapping mode. The sample for AFM imaging was prepared by drop-drying 0.5 \(\mu\)L of a very diluted aqueous solution at an equivalent pDA concentration of 750 ng/L on the SiO\(_2\) coated Si substrate. No post-processing steps were needed after drop-drying for AFM imaging. The histogram showing the size distribution of the pDA-PEG nanoparticles was obtained by measuring the heights of 100 nanoparticles from the AFM images.
Dynamic light scattering (DLS) measurement of pDA-PEG. An aqueous solution of pDA-PEG in 1× PBS at a mass concentration of 0.75 µg/ml for pDA was used for DLS measurement. The solution was loaded in a 1-cm quartz cuvette with four transparent walls and DLS was performed using a Brookhaven Instruments 90Plus Particle Size Analyzer.

Cell culture and staining. All cell culture media used in this work were supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin and L-glutamine. The U87-MG brain glioblastoma cells were cultured in Low Glucose Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 1 g/L D-glucose and 110 mg/L sodium pyruvate. The MDA-MB-468 breast cancer cells were grown in Leibovitz’s L-15 medium. U87-MG cells were maintained in cell culture flasks (Corning) with vented caps and placed in a 37 °C humidified incubator with 5% CO₂, while the MDA-MB-468 cells were maintained in cell culture flasks (Corning) with unvented caps and placed in the same incubator since CO₂ is detrimental to the growth of MDA-MB-468 cells. These two cell lines were selected for cell imaging because of their different expression levels of epidermal growth factor receptors (EGFR) on the cell membranes.

For cell staining, both cell lines growing in the flasks were trypsinized at 37 °C for 5-10 min, before the pDA-PEG-Erbitux bioconjugate was added to the suspensions of U87-MG (EGFR-negative) and MDA-MB-468 (EGFR-positive) cells. The final staining concentration of pDA-PEG-Erbitux in the cell suspension was ~5 µg/mL for pDA. The staining was carried out at 4 °C for 1 h, followed by washing the cells
thoroughly with cold 1× PBS three times to remove the free, unbound pDA-PEG-Erbitux conjugates in the suspension. The cells were always kept at 4 °C before imaging.

**High-magnification NIR-II fluorescence microscopy imaging.** Microscopic cell imaging in the NIR-II window was performed on a homemade widefield microscope setup coupled to a two-dimensional (2D) InGaAs camera (Princeton 2D OMA-V) to look at the fluorescence of pDA polymer. A 658-nm laser diode (100 mW, Thorlabs) cleaned through a 750 nm short-pass filter (Omega) was used as the excitation source. The 658-nm laser was focused to a 150 μm diameter spot on the sample by a 50× objective lens (Olympus), and the emitted NIR-II photoluminescence from the Ag2S QDs was allowed to pass through a 900 nm long-pass filter and a 1000 nm long-pass filter (Thorlabs) and focused onto the 2D InGaAs camera, with an exposure time of 3 s. For bright field white light images of the same field of view, a fiber optic illuminator (Fiber-Lite) was used to illuminate the cell sample in the transmission mode and the white light images were taken in the same setup with the same filters at an exposure time of 2 ms.

**In vivo ultrafast fluorescence imaging of blood flow in the NIR-II window.** For ultrafast dynamic NIR-II fluorescence imaging in the NIR-II window, the excitation was provided by an 808-nm laser diode, fiber-coupled to a collimator with a focal length of 4.5 mm, cleaned by an 850-nm short-pass filter and a 1000-nm short-pass filter to remove the long-wavelength photons, and directed to the imaging plane with a power density of 140 mW/cm². A female Balb/c mouse with hair shaved off the hind
limb skin was placed on the imaging stage in the supine position. The emitted fluorescence photons were filtered through a 900-nm long-pass filter and a 1000-nm long-pass filter to collect NIR-II fluorescence in the 1000-1700 nm region. A 2D InGaAs array of 320 × 256 pixels (Princeton Instruments) was used to form NIR-II fluorescence images. A lens pair consisting of two achromats (focal lengths of 200 mm and 75 mm) with a distance of ~200 mm between the two lenses was used to image with a field of view covering the hind limb area only. To capture video-rate images, the 2D InGaAs detector was allowed to expose continuously with an exposure time of 20 ms immediately after a bolus injection of 200 µL pDA-PEG solution at 0.25 mg/mL, and the images were acquired by a user-written LabVIEW software with an additional overhead time of 19 ms between two consecutive frames. The exposure time and the overhead time comprised a complete cycle of 39 ms for each image acquisition with a frame rate of 25.6 frames per second (fps).

8.3 Results and Discussion

We synthesized the pDA polymer, poly(benzo[1,2-b:3,4-b']difuran-alt-fluorothieno-[3,4-b]thiophene), through an alternating copolymerization reaction of two monomers, the electron-withdrawing 1-(4,6-dibromo-3-fluorothieno[3,4-b]thiophen-2-yl)nonan-1-one (named Monomer 1) and the electron donating 2, 6-bis(trimethyltin)-4,8-bis(2-ethylhexyloxy)benzo[1,2-b:3,4-b']difuran (named Monomer 2). A fluorine atom was introduced to the thienothiophene conjugated system to tune the bandgap energy of the pDA molecule and thus shift its fluorescence
emission to longer wavelengths (Scheme 8.1). The two monomers and the pDA polymer were characterized by $^1$H and $^{19}$F NMR spectroscopy (see Section 8.2 and Fig. 8.1-8.4), and the pDA polymer was also characterized by GPC, revealing a number-average molecular weight ($M_n$) of $\sim$16 kDa (see Section 8.2 and Fig. 8.5).

To make the hydrophobic pDA polymer soluble in water, we coated the pDA molecules with the amphiphilic PEGylated phospholipid [DSPE-mPEG (5k)], a biocompatible surfactant that had been widely used to disperse SWNTs in water and biological environment$^{4,15}$ (see Section 8.2 for experimental details). This solubilization process resulted in a supramolecular conjugate consisting of a hydrophobic pDA core and a hydrophilic, PEGylated shell that allowed the complex to be stably dispersed in an aqueous solution (Fig. 8.6a). The pDA-PEG complex was found to have an average dried size of $\sim$2.9 nm based on the AFM height measurement (Fig. 8.6b & red bars in 8.7a), and an average hydrodynamic size of $\sim$17.8 nm based on the DLS measurement (blue bars, Fig. 8.7a). This difference in size measurement was attributed to the hydrated and extended PEG chain with an average root mean square length of $\sim$7 nm for 5k PEG,$^{32}$ which coats on the surface of each pDA core of $\sim$3 nm to increase the overall hydrodynamic size (Fig. 8.7b).

The absorption spectrum of the pDA-PEG aqueous solution featured an absorbance peak at $\sim$654 nm, while the main emission peak at $\sim$1047 nm in the fluorescence spectrum was separated from the absorbance peak by a $\sim$400 nm, large Stokes shift (Fig. 8.6c). A schematic of the proposed band diagram of the pDA polymer is shown in Fig. 8.8 to account for the absorption and fluorescence emission properties. As discussed in Section 8.1, this significantly red-shifted emission was
attributed to the formation of the charge-transfer structure between the alternating electron donor and acceptor units, resulting in lower bandgap energy than the corresponding homopolymers.\textsuperscript{18,23,33} Using the standard NIR-II fluorophore IR-26 dye as the reference with a fluorescence QY of 0.5%,\textsuperscript{30} we measured the fluorescence QY of the pDA-PEG aqueous solution as \(~1.7\)%, substantially higher than the QY of SWNTs (\(~0.4\)% measured in the same way (Fig. 8.9). High fluorescence stability and photostability were found for the pDA-PEG in water, evidenced by negligible quenching by salt and biological molecules in PBS and fetal bovine serum (Fig. 8.6e and Fig. 8.10a-c), and minimum photobleaching under continuous laser irradiation (Fig. 8.6f and Fig. 8.10d-f). Therefore, by coating the pDA polymer with a PEGylated surfactant, we made a water soluble and photostable NIR-II fluorophore with exceptionally bright fluorescence emission suitable for \textit{in vivo} fluorescence imaging.

We then performed selective molecular imaging of cancer cells using the NIR-II fluorescence of pDA polymer. As described in detail in Section 8.2, an amine-terminated surfactant, DSPE-PEG-NH\textsubscript{2} was used to coat the pDA polymer, allowing for further functionalization with the targeting ligand, Erbitux (Cetuximab) antibody via the SMCC-mediated crosslinking reactions between amine and thiol groups.\textsuperscript{34,35} The as-made pDA-PEG-Erbitux bioconjugate was applied to the suspensions of MDA-MB-468 cells and U87-MG cells for selective staining and targeting of the EGFR membrane receptors (Fig. 8.11a). Microscopic NIR-II fluorescence imaging of the EGFR-positive MDA-MB-468 cells (Fig. 8.11b\&c) showed on average \(~5.8\) times higher NIR-II signal (Fig. 8.11f) than the EGFR-negative U87-MG cells (Fig.
suggesting the pDA-PEG-Erbitux bioconjugate as a specific NIR-II fluorescent label affording molecular imaging capability of certain biomarkers.

Owing to the exceptionally high fluorescence QY of the pDA-PEG fluorescence (~1.7%), we performed ultrafast blood flow tracking in the NIR-II window with an unprecedented frame rate. The aqueous solution of pDA-PEG in the presence of PBS was intravenously injected into the tail vein of a Balb/c mouse, and the blood flow carrying the injected NIR-II fluorophores inside the femoral artery was dynamically imaged and tracked at a frame rate of ~25.6 fps with an exposure time of 20 ms for each frame. It is noteworthy that SWNTs were used for femoral vessel blood flow imaging in Chapter 5, where a low frame rate of ~5.3 fps with a single exposure time of 100 ms was used due to the much lower QY of SWNTs (~0.4%).

Immediately after injection of the pDA-PEG solution, a fast-moving blood flow front was observed as it travelled down the femoral artery (Fig. 8.12a). Due to the fast arterial blood velocity, the blood travelled through the entire 2 cm length of the femoral artery within merely 300 ms, making the flow pattern almost unresolvable with the previous frame rate of ~5.3 fps using SWNTs. In strong contrast, the high frame rate of ~25.6 fps using the pDA-PEG fluorophore allowed us to accurately quantify the blood velocity in the femoral artery, by plotting the travelled distance of the blood flow front against time and fitting the plot to a linear function (Fig. 8.12b). The slope was extracted from linear fitting to derive an average blood velocity of 4.36 cm/s in the femoral artery, in good agreement with ultrasound measurements on different mice in Chapter 5 (4~7 cm/s). Unlike the blood velocity measurement described in Chapter 5 where an intensity-to-velocity conversion coefficient was
involved due to the limited temporal resolution of dynamic imaging with SWNTs, blood flow tracking with pDA-PEG represented the first example of directly tracking the fast arterial flow front on the millisecond time scale.

Interestingly, the high temporal resolution of blood flow tracking using pDA-PEG even allowed us to analyze the change of blood velocity over time. By taking the 1st derivative of the distance vs. time curve in Fig. 8.12b, we plotted the instantaneous velocity as a function of time in Fig. 8.12c, where a periodic variation of blood velocity over time was found. This periodic variation of instantaneous blood velocity featured a period of 150~200 ms with a peak velocity of ~8 cm/s and a valley velocity of ~2 cm/s. This observed oscillation of blood velocity was attributed to the systolic (ventricular ejection) and diastolic (ventricular relaxation) phases of each cardiac cycle, which was made temporally resolvable entirely by the high frame rate of ~25.6 fps, much faster than the heart rate of ~ 5 beats/s (a cardiac cycle of ~200 ms). However, since the arterial blood flow with a fast velocity of ~4.36 cm/s passed through the entire femoral artery (~2 cm) within only ~400 ms, the analysis of instantaneous velocity based on flow front tracking only allowed us to record the oscillation up to 2 cardiac cycles. Then the entire femoral vessels were filled with the injected pDA-PEG fluorophore, as shown in the NIR-II fluorescence image taken at 39 s post injection (p.i.) of the same mouse (Fig. 8.12d).

In addition to the oscillating velocity of the blood flow front, the periodic cardiac cycles were also reflected in the change of NIR-II fluorescence intensity in a selected region of interest (ROI) of the femoral artery (Fig. 8.13a). By plotting the average NIR-II intensity in the ROI versus time, evenly spaced humps were observed
in the plot, on top of a linearly increasing baseline (Fig. 8.13b). We attributed the rise of each hump to the systolic phase (ventricular ejection) of each cardiac cycle, while the increasing baseline was owing to an increasing level of axial mixing between the injected pDA-PEG solution with the fluorophore-free blood, similar to what we had observed in the SWNT case (see Fig. 5.11 in Chapter 5). After the linearly increasing baseline was subtracted from the plot in Fig. 8.13b, 5 consecutive spikes were clearly observed over a time course of ~1 s (Fig. 8.13c), showing a similar cardiogram waveform to Doppler ultrasound measurement in previous publications. A linearly increasing background was also subtracted from the video frames of the dynamic NIR-II fluorescence imaging of the blood flow, revealing the individual pulses of the pDA-PEG fluorophore flowing through the vessel as a result of periodic heart beat (Fig. 8.13d-j). Unlike the blood velocity oscillation based on flow front tracking, the NIR-II fluorescence intensity within a certain ROI allowed us to track up to 5 cardiac cycles.

It is noteworthy that a few local intensity maxima were observed as bright ‘dots’ along the femoral artery in some of the baseline-subtracted NIR-II fluorescence images (Fig. 8.13f-h). More interestingly, these local intensity maxima were fixed in location and did not change their positions over time. An analysis of the fluorescence intensity along with the local vessel width measured from the NIR-II images for 11 evenly spaced points along the femoral artery revealed the slightly larger local diameter of the lumen was the cause of these local intensity maxima. In other words, a larger lumen of the femoral vessel contained more pDA-PEG molecules in the blood and thus had higher NIR-II fluorescence intensity (Fig. 8.14). We also analyzed the
NIR-II fluorescence oscillations within the ROI and extracted an average cardiac cycle of 206.7 ms by fitting the appearance times of the 5 pulses to a linear function (Fig. 8.13k). The measured cardiac cycle of 206.7 ms corresponded to a heart rate of 290 beats/min, consistent with previous reports for mice via cardiac gating.\(^\text{37}\) Thus, the high-temporal resolution NIR-II imaging afforded by the high fluorescence QY of the pDA polymer eliminated the need of cardiac gating during imaging, which was typically required for measuring fast dynamics of the cardiovascular system using slower imaging techniques.\(^\text{37,38}\)

We have shown in Chapters 5 and 6 that \textit{in vivo} fluorescence imaging could be greatly facilitated by detecting fluorescence in the long-wavelength, NIR-II window, which allowed for millimeters penetration depth owing to the reduced photon scattering and tissue autofluorescence.\(^\text{5,7}\) Although conjugated copolymers have found wide applications in organic solar cells,\(^\text{23,39}\) light-emitting diodes (LEDs)\(^\text{40,41}\) and organic electronics,\(^\text{42,43}\) fluorescent imaging with these polymers has been only limited to the visible (400-750 nm) and traditional near-infrared (NIR-I, 750-900 nm) windows with much shorter emission wavelengths.\(^\text{44-46}\) The work described in this chapter represented the first biocompatible formulation of a conjugated copolymer with NIR-II fluorescence emission for \textit{in vitro} and \textit{in vivo} imaging applications. By modifying the chemical structures of the donor and acceptor monomers, we have synthesized a library of conjugated copolymers with tunable excitation and emission wavelengths (Fig. 8.15), which could allow for multicolor molecular imaging with even deeper tissue penetration in the NIR-II window.
Coherent imaging techniques, such as ultrasonography and optical coherence tomography (OCT) have shown the capability of measuring hemodynamics and cardiac cycles in a non-invasive way without any gating devices involved, owing to the deep penetration of ultrasound wave, rejection of incoherent scattered signal, and high operating frequencies (kHz~MHz). However, one of the limitations of the coherent imaging techniques remains the low spatial resolution (10 μm ~ 1 mm) and poor contrast, due to the use of long wavelength ultrasound and speckle artifacts.

In addition to what we have shown in this chapter for NIR-II fluorescence based fast blood tracking, we also attempted to track capillary blood flow within sub-10 μm vessels and obtained the average blood velocity in a particular capillary as ~55.2 μm/s (Fig. 8.16), which was consistent with previous studies. It is noteworthy that blood flow within such a small capillary was not resolvable and measurable by ultrasonography and OCT due to the resolution limit. In addition to structural and hemodynamic imaging, the pDA-PEG fluorophores have also proved to be useful for functional imaging of the increased metabolic demand and regional blood flow as a result of heat-induced tissue inflammation (Fig. 8.17), allowing us to directly visualize the difference in metabolic demand under various medical circumstances.

Smaller size of the intravenously administered nanomaterials favored reduced toxicity and more rapid clearance from the body. To this end, we carried out a systematic study to investigate the hydrodynamic size dependence of pDA-PEG on the initial pDA concentration in THF and the molecular weight of PEG in the surfactant. Fig. 8.18 revealed that, both a lower initial pDA concentration in THF and a smaller molecular weight of PEG favored the formation of pDA-PEG nanoparticles with a
smaller hydrodynamic diameter in an aqueous solution. The dependence of PEG molecular weight could be easily rationalized by the size dependent radius of gyration for different PEG chains,\textsuperscript{56} while the dependence on the initial pDA concentrations was attributed to the different pDA/PEG ratios during the reaction between the hydrophobic pDA and the surfactant, which affected the average number of pDA polymers in the core of each nanoparticle. Therefore the pDA-2k-PEG with a lower initial pDA concentration and thus a smaller pDA/PEG ratio had fewer pDA molecules per nanoparticle and smaller shell due to the 2k PEG, resulting in the smallest hydrodynamic diameter among all synthetic conditions.

8.4 Conclusion

In a brief summary, we synthesized a toolbox of low-bandgap conjugated polymers (i.e., pDA polymers) with tunable fluorescence wavelengths from 1050 nm to 1350 nm in the NIR-II window via alternating copolymerization reaction of electron donating and withdrawing monomers. The pDA polymers could be solubilized in water by the amphiphilic DSPE-PEG surfactants, resulting in biocompatible fluorescent nanoparticles. The surfactant coating on the pDA allowed for further functionalization with a targeting ligand, Erbitux antibody, resulting in the pDA-PEG-Erbitux bioconjugate with selective molecular imaging capability of cancer cells with overexpressed EGFR receptors. The exceptionally high fluorescence QY of the pDA-PEG, which was measured as \(~1.7\%\), allowed for dynamic NIR-II imaging of ultrafast
arterial blood flow and clear resolution of cardiogram waveform at an unprecedented frame rate of ~25.6 fps.
8.5 Schemes

Scheme 8.1 | Synthesis of pDA Polymer. This scheme shows the synthesis of poly(benzo[1,2-b:3,4-b']difuran-alt-fluorothieno-[3,4-b]thiophene) (pDA) from the two monomers, a fluorothieno-[3,4-b]thiophene derivative M1, and a benzo[1,2-b:3,4-b']difuran derivative M2.
8.6 Figures

Figure 8.1 | $^1$H NMR spectrum of the monomer M1. The peaks are assigned as follows: 2.95 (t, 2H), 1.76 (m, 2H), 1.39-1.30 (m, 10H), 0.91 (t, 3H).
Figure 8.2 | $^{19}$F NMR spectrum of the monomer M1. The peak in the spectrum is assigned as follows: -129 ppm (s, Ar-F)
Figure 8.3 | $^1$H NMR spectrum of the monomer M2. The peaks are assigned as follows: 7.06 (s, 2H), 4.35 (d, 4H), 1.78 (m, 2H), 1.70-1.37 (m, 16 H), 0.99 (m, 12 H), 0.44 (s, 18 H).
Figure 8.4 | $^1$H NMR spectrum of the pDA polymer. The peaks are assigned as follows: 6.80 (br, 2H), 4.31 (br, 4H), 3.06 (br, 2H), 2.01-1.21 (br, 30H), 0.81-1.21 (br, 15H). The inset is a zoomed-in view of the original spectrum in the region of 2.5-7.3 ppm.
Figure 8.5 | GPC spectrum of the pH polymer. The molecular weights and polydispersity of the pH polymer are listed as follows: $M_n$ (number-average molecular weight): 16,192; $M_w$ (weight-average molecular weight): 30,991; $M_p$ (peak molecular weight): 24,941; PDI (polydispersity index): 1.91.
Figure 8.6 | Basic Characterizations of pDA-PEG. (a) A schematic drawing of the pDA-PEG conjugate showing the composition of a hydrophobic core of pDA polymer and a hydrophilic shell of PEG chains. (b) An AFM image of the pDA-PEG nanoparticles drop-dried on a SiO₂/Si substrate. Note that the height in the AFM image, rather than the lateral distance, gave a better measure of the dried size of the pDA-PEG nanoparticles, owing to the tip size convolution of AFM imaging.⁵⁷ (c) UV-Vis-NIR absorption and fluorescence emission spectra of the pDA-PEG aqueous solution. (d) A typical NIR-II fluorescence image of the pDA-PEG aqueous solution taken in the 1.0-1.7 μm NIR-II window under an 808-nm laser excitation. (e) A bar chart showing the average NIR-II fluorescence intensity of pDA-PEG in water, PBS and serum, suggesting good fluorescence stability in different media. (f) Normalized NIR-II fluorescence intensity of pDA-PEG in water (black), PBS (blue) and serum...
(red) plotted as a function of time of continuous irradiation of an 808-nm laser excitation, showing good photostability of the pDA-PEG fluorophore.
Figure 8.7 | Size Measurement of pDA-PEG(5k). (a) A bar chart showing the dried size distribution of pDA-PEG(5k) nanoparticles based on the AFM height measurement (red bars), and the hydrodynamic size distribution of pDA-PEG(5k) in a 1× PBS aqueous solution based on DLS measurement (blue bars). (b) A schematic diagram showing the size breakdown of the pDA-PEG(5k) nanoparticle, which was comprised of a polymer core of ~3 nm and a hydrated PEG shell of ~7 nm.
Figure 8.8 | Band Diagram of Conjugated Polymer. This simplified schematic shows the evolution of the band structure of conjugated polymer from smaller conjugated oligomers. The excitation path is shown as the blue arrows, the non-radiative decay as the black dashed arrows, and the fluorescence emission as the red arrows.
Figure 8.9 | Quantum Yield Measurement of pDA-PEG and SWNT. (a) UV-Vis-NIR absorption spectra of the IR26 dye in DCE with linearly spaced concentrations. (b) Fluorescence emission spectra of the same DCE solutions of IR26 dye in a. Excitation was provided by an 808-nm laser diode. (c) The total fluorescence intensity integrated in the 900-1500 nm range of the IR26 dye in DCE, plotted against the absorbance at 808 nm. (d) UV-Vis-NIR absorption spectra of pDA-PEG in water with linearly spaced concentrations. (e) Fluorescence emission spectra of the same aqueous solutions of pDA-PEG in d. Excitation was provided by an 808-nm laser diode. (f) The total fluorescence intensity integrated in the 900-1500 nm range of pDA-PEG in
water, plotted against the absorbance at 808 nm. The linearly fitted slope resulted in a measured QY of 1.7 ± 0.11% for pDA-PEG. (g) UV-Vis-NIR absorption spectra of SWNTs in water with linearly spaced concentrations. (h) Fluorescence emission spectra of the same aqueous solutions of SWNTs in g. Excitation was provided by an 808-nm laser diode. (i) The total fluorescence intensity integrated in the 900-1500 nm range of SWNTs in water, plotted against the absorbance at 808 nm. The linearly fitted slope resulted in a measured QY of 0.4 ± 0.01% for SWNTs.
Figure 8.10 | Photostability of pDA-PEG in Different Media. (a-c) NIR-II fluorescence images of pDA-PEG spiked in water (a), PBS (b) and serum (c), at the beginning ($t = 0$ s) of the continuous laser irradiation of 808 nm. A concentration of 7.5 µg/mL was used for all samples. (d-f) NIR-II fluorescence images of the same pDA-PEG samples in water (d), PBS (e) and serum (f), at the end ($t = 3600$ s) of the continuous laser irradiation of 808 nm.
Figure 8.11 | Selective Cell Imaging with pDA-PEG-Erbitux. (a) A schematic drawing showing the pDA-PEG-Erbitux bioconjugate targets the EGFR receptors on the MDA-MB-468 cell membrane through the Erbitux targeting ligand. (b-e) White light optical images (b&d) and their corresponding NIR-II fluorescence images (c&e) of the EGFR-positive MDA-MB-468 cells (b&c) and the EGFR-negative U87-MG cells (d&e) incubated with the pDA-PEG-Erbitux bioconjugate. (f) A bar chart showing the average NIR-II fluorescence on both cell lines with a positive/negative ratio of ~5.8.
Figure 8.12 | Ultrafast NIR-II Imaging of Arterial Blood Flow with pDA-PEG. (a) Time course NIR-II fluorescence images of a mouse hindlimb immediately after injection of the pDA-PEG fluorophore. The blood flow fronts were labeled as the red arrows. (b) The travelled distance of the blood flow front, which was determined by NIR-II fluorescence imaging in a, plotted as a function of time. The linear fit to the plot revealed an average blood velocity of 4.36 cm/s. (c) The instantaneous blood
velocity in the femoral artery plotted as a function of time, showing the periodic oscillation corresponding to cardiac cycles. The instantaneous blood velocity was derived by taking the 1st derivative of the distance-time curve in b. (d) An NIR-II fluorescence image showing the same mouse hindlimb at 39 s p.i., when both femoral vessels and other small vascular branches were filled with the pDA-PEG fluorophore at a relative constant concentration and brightness.
**Figure 8.13 | Blood Flow Pattern Based on ROI Intensity Analysis.** (a) An NIR-II fluorescence image showing the mouse hindlimb, where a red box was drawn to indicate the selection of ROI. (b) The average ROI intensity plotted as a function of time, showing consecutive humps (black arrows) on top of a linearly increasing baseline (blue dashes line). (c) A plot showing the average ROI intensity versus time after subtracting the baseline, where the consecutive spikes became more obvious. (d-j) A time course of NIR-II fluorescence images of the mouse hind limb, after subtraction of a time-dependent background from each frame. (k) Appearance time of the spikes
shown in e plotted for a series of 5 pulses and fitted into a linear function to extract an average cardiac cycle of 206.7 ms/pulse and a heart rate of 290 beats/min.
Figure 8.14 | The Origin of Dots in Baseline Subtracted NIR-II Images. (a) An NIR-II fluorescence image taken at 390 ms p.i. for the mouse hind limb after subtracting the baseline shown in Fig. 8.13b, showing only the ROI region in Fig. 8.13a. (b) The same image as in a, with red circles labeling the intensity maxima (the dots) in the image and the green circles labeling the intensity minima between two neighboring maxima. (c) The NIR-II fluorescence intensity and the measured local vessel diameter plotted for all labeled points in b, showing a positive correlation between the two curves.
Figure 8.15 | A Library of pDA Polymers. (a-d) The chemical structures of 4 pDA conjugated copolymers synthesized thus far, which were termed pDA-1 (a), pDA-2 (b), pDA-3 (c) and pDA-4 (d). (e) UV-Vis-NIR absorption spectra of these 4 pDA molecules in the range of 450-1600 nm. (f) Fluorescence emission spectra of these 4 pDA molecules in the range of 900-1600 nm.
Figure 8.16 | Dynamic NIR-II Imaging of Capillary Blood Flow with pDA-PEG.

(a) A time course of NIR-II fluorescence images of the pDA-PEG fluorophores flowing with blood inside a capillary vessel with width of ~6 μm. (b) The travelled distance of blood labeled by the NIR-II fluorescence of pDA-PEG, plotted as a function of time. The linear fit to the plot revealed an average blood velocity of 55.2 μm/s.
Figure 8.17 | NIR-II Imaging of Regional Blood Distribution with pDA-PEG. (a)
A white light, digital camera photograph of a nude mouse with heat-induced tissue inflammation in its right hindlimb (on the left side to the viewer). This photograph was
taken at approximately 5 h after inflammation was induced. (b-h) A time course of NIR-II fluorescence images of the mouse shown in a, taken at 10 min (b), 5 h (c), 17 h (d), 35 h (e), 43 h (f), 96 h (g) and 118 h (h) after inflammation was induced. (i) Average NIR-II fluorescence intensity in the left, control hind limb (black) and the right, inflamed hind limb (red), plotted as a function of time after inflammation was induced. (j) A plot showing the ratio of the NIR-II intensity in the right, inflamed hind limb over that in the left, control hind limb, as a function of time after inflammation was induced.
Figure 8.18 | Size Tuning of pDA-PEG. This plot shows how hydrodynamic diameter of the pDA-PEG nanoparticles measured by DLS evolves as a function of the initial pDA concentration in THF and the molecular weight of PEG in the DSPE-mPEG surfactant. It is noteworthy that the smallest measured size of 2~6 nm for pDA-PEG(2k) at an initial pDA concentration of 0.025 mg/mL might contain empty micelles comprised of the surfactants only, which were expected to have a hydrodynamic diameter of 2~4 nm.
8.7 References


Chapter 9. Conclusion and Future Directions

The aim of this PhD dissertation is to optimize the brightness (Chapters 2&3) and emission wavelengths (Chapter 6) of single-walled carbon nanotubes (SWNTs) as fluorescent labels in the second near-infrared window (NIR-II window, 1.0-1.7 μm) for real world biomedical problems (Chapters 3-6), as well as to develop new NIR-II fluorescent materials including Ag₂S quantum dots (QDs) and conjugated copolymer nanoparticles for *in vitro* and *in vivo* fluorescence imaging in the NIR-II window (Chapters 7-8). The work described in this PhD dissertation has advanced the field of NIR-II fluorescence imaging by showing the salient benefits of the longer-wavelength NIR-II window for *in vivo* imaging in animal models of cancer, cardiovascular disease and cerebrovascular disease, as well as expanding the toolbox of available NIR-II fluorophores for biomedical researchers.

The work demonstrated in Chapters 2-4 focused on the NIR-II fluorescence enhancement of SWNTs in an *in vitro* setting. We have shown in Chapter 2 the first observation of metal enhancement fluorescence (MEF) of SWNTs,¹ which was also the first observation of MEF in the 1.0-1.7 μm NIR-II window, given that all previous reports on MEF were for fluorescence with wavelengths in the <900 nm region.²⁻⁷ An enhancement factor (EF) of up to approximately 10-fold was found for surfactant-coated SWNTs deposited on the solution-phase grown gold colloidal film (named ‘Au/Au film’) with the highest surface coverage of Au colloids, significantly facilitating single nanotube imaging and spectroscopy of SWNTs embedded in a polymer matrix. The distance dependent profile of MEF was studied for SWNTs on
the Au/Au film based on ensemble measurement, revealing a monotonically decreasing EF of surfactant-coated SWNTs at increasing distance between SWNTs and the Au surface, as well as a quenching distance of SWNTs shorter than the size of a 5kDa polyethylene glycol (PEG) chain. Exploiting the fluorescence enhancement of SWNTs, we have then demonstrated the Au/Au film as a useful platform for improving the signal and sensitivity of molecular imaging on individual cancer cells in Chapter 3.\(^8\) We found that the receptor-bound SWNTs had to stay on the outside of the cell membranes to get efficiently enhanced by up to \(\sim9\)-fold, further confirming the distance dependent enhancement profile for SWNTs as found in Chapter 2. The plasmonic Au/Au film allowed us to detect and map the distribution of surface receptors such as the \(\alpha_5\beta_3\) integrin at ultra-low abundance of \(\sim680\) integrins per cell, owing to the fluorescence enhancement of SWNT molecular tags. The ultra-sensitive enhancement profile of SWNTs versus distance to the Au surface acted as a ‘nanoscopic ruler’ that allowed us to probe the transmembrane motion of single carbon nanotube molecules in real time and real space, as we have shown in Chapter 4.\(^9\) This useful imaging tool with nanometer sensitivity revealed the internalization process of SWNTs as receptor-mediated endocytosis, evidenced by temperature-dependent profile of the transmembrane motion, and the dependence on the formation of clathrin-coated pits.\(^10\) As a brief summary, in these three chapters, we first studied the interaction between SWNTs and Au nanoparticles from a physicochemical perspective, and then applied the findings for solving biological problems through NIR-II fluorescence imaging.
The work demonstrated in Chapters 5-6 applied the beneficial NIR-II fluorescence of SWNTs in an in vivo setting. Chapter 5 represents the first example of employing SWNTs and NIR-II fluorescence imaging for solving a real-world medical problem, lower limb ischemia, which is a very common cardiovascular disease. In a side-by-side comparison with the existing medical imaging modalities such as NIR-I fluorescence imaging, X-ray CT, ultrasound and laser Doppler, NIR-II fluorescence imaging using SWNTs has proved superior with minimum invasiveness, higher spatial resolution and broader dynamic range of blood velocity measurement.11 Chapter 6 applied SWNTs and NIR-II fluorescence imaging in the context of another global medical problem, stroke, which is a representative example of cerebrovascular diseases. By rationally selecting a sub-region of NIR-II in the 1.3-1.4 µm range (termed NIR-IIa window) and chemically enriching SWNTs with fluorescence in this window, we performed vascular imaging inside the mouse brain through intact scalp and skull with a sub-10 µm spatial resolution capable of resolving cerebral capillaries at an imaging depth of up to ~3 mm. Hemodynamic imaging of the cortical blood flow with NIR-II fluorescence also allowed us to pinpoint the site of stroke in real time and real space. In these two chapters, we have demonstrated the unique strengths of in vivo NIR-II fluorescence imaging, which are not attainable by other imaging techniques such as CT (inferior spatial resolution),12 traditional visible and NIR microscopy (inferior penetration depth),13 multi-photon microscopy (invasiveness),14 and laser Doppler blood spectroscopy (invasiveness and poor imaging contrast due to speckles).15,16
The work described in **Chapters 7-8** demonstrated two new NIR-II fluorescent materials other than SWNTs for both *in vitro* and *in vivo* imaging. In **Chapter 7**, the Ag$_2$S QDs were synthesized with strong NIR-II fluorescence emission centered at ~1200 nm, and surface-functionalized with targeting ligands and PEG to afford selective molecular imaging of cancer cells *in vitro* and non-specific tumor imaging *in vivo*. In Chapter 8, a toolbox of low-bandgap conjugated copolymers (termed ‘pDA polymers’) were synthesized with tunable NIR-II fluorescence emission ranging from 1050 nm to 1350 nm and solubilized in water by surfactant coating. The pDA formed a strongly held supramolecular complex with the PEGylated surfactant, affording selective cell imaging through conjugation to the specific targeting ligands. The exceptionally bright NIR-II fluorescence of the pDA polymer allowed for high-speed, video-rate NIR-II imaging of the fast arterial blood flow with an unprecedented temporal resolution of >25 frame/s, with which the periodic oscillation of cardiac cycles was clearly resolved in blood velocity and NIR-II fluorescence intensity. The new NIR-II fluorescent materials expanded the ‘palette’ of NIR-II ‘pigments’ to fit the various needs of NIR-II fluorescence imaging in biological and medical researches.

Although this work has opened up the numerous possibilities of biomedical imaging in the NIR-II window with new therapeutic avenues, there is still a long way to go before NIR-II fluorescence imaging can one day become one of the FDA approved imaging modalities and thus becomes available to doctors and patients in most hospitals. A major roadblock in that aspect remains the potential toxicity and the retarded clearance of the intravenously administered nanomaterials used as the NIR-II contrast agent.$^{17,18}$ Among thousands of available fluorophores with emission
wavelengths in the visible and NIR-I windows, so far there are only three fluorophores, indocyanine green (ICG), methylene blue and fluorescein isothiocyanate (FITC), that have been approved by the FDA to use clinically for fluorescence imaging in the traditional windows. It is noteworthy all of the three FDA-approved fluorophores are small molecules with hydrodynamic size significantly smaller than the reported renal cutoff size of ~5.5 nm, making them readily cleared out from the body within only a few hours after administration, leaving only minimum adverse effects to the patient. Therefore, an important future direction of NIR-II fluorescence imaging is the development of small molecule based NIR-II fluorophores below the cutoff size of renal filtration and with fast body clearance, and biodegradable NIR-II active materials that can be broken down into less toxic and more easily excreted small pieces inside the body.

Another major limitation of current NIR-II fluorescence imaging remains the insufficient penetration depth. Although a penetration depth of up to ~3 mm shown in this work can already provide sufficient depth information for basic science research and preclinical studies on mouse models, this imaging depth is still far from sufficient for humans and some large experiment animals such as pigs and monkeys. The limited penetration depth of NIR-II fluorescence imaging comes from the lingering scattering of photons even at this longer wavelength, and the absorption of fluorescence by water molecules and other biological molecules that are prevalent in any living organism. To overcome this limitation, more efforts can be invested in the following two directions: optimization of NIR-II fluorescent materials, and development of new imaging systems.
To afford the maximum penetration depth for *in vivo* NIR-II fluorescence imaging with the best image sharpness and signal-to-noise ratio (SNR), the optimum NIR-II fluorescent material needs to have even longer emission wavelengths (preferably in the 1.5-1.7 μm region) and higher fluorescence quantum yield (QY). However, the strong overtone absorption peaks at 1.45 μm and beyond 1.8 μm of water\textsuperscript{24} as well as the increasing absorption of other biological molecules in the short-wavelength infrared window (SWIR window, 1.7-3 μm) put a tradeoff between the scattering and absorption of photons, which limits our efforts of synthesizing new fluorophores with even longer wavelengths for NIR-II imaging with deeper penetration. On the other hand, the sub-optimal fluorescence QY of all current NIR-II fluorophores (mostly 0.1-2%) exacerbates the problem of tissue absorption at long wavelengths. Therefore, making new NIR-II fluorophores with higher fluorescence QY should receive more attention, especially when optimizing the emission wavelengths to reduce scattering.

New imaging systems can help achieve deeper imaging penetration by detecting fluorescence in different ways. First of all, it is always desired to detect NIR-II photons more efficiently than the current InGaAs camera, presumably through an avalanche photodiode (APD) or a photomultiplier tube (PMT) with higher sensitivity in the NIR-II window. Second, by rejecting the out-of-focus foreground and scattered signals in a volume sample using a confocal microscope that detects fluorescence in the NIR-II, we are hoping to spatially resolve signals from different depths by optical sectioning of the volume sample (e.g., a live animal or an *ex vivo* organ) to an even greater depth. Third, through further rejection of the scattered photons via non-linear
optics or reshaping the scattered wavefronts by wavefront engineering, deep imaging through a complex media can be substantially improved on a two-photon NIR-II imaging setup or a spatial light modulator (SLM) facilitated NIR-II imaging setup. Fourth, NIR-II fluorescence mediated tomography (FMT) can help visualize deep features from different viewing angles, enabling more accurate localization of deep features by circumventing the strong scattering associated with imaging from one single perspective.

Despite the aforementioned limitations, NIR-II fluorescence imaging holds the promise of advancing the understanding of important biological processes and providing an early-stage diagnosis tool for various diseases. With the development of better NIR-II fluorophores and NIR-II imaging systems, we envisage NIR-II fluorescence to be applied to imaging of the heart and coronary vessels in the context of myocardial infarction, as well as tracking of circulating tumor cells (CTCs) during early-stage cancer development. NIR-II fluorescence can also be combined with super resolution imaging techniques to provide deep-tissue and super resolution imaging of biomarkers and sub-cellular structures in live animals.
References


Imaging Hindlimb Vessel Regeneration with Dynamic Tissue Perfusion Measurement. 


Appendix. A User Manual for Selected Optical Setups

This appendix includes the optical diagram, operation procedure and alignment tips for a few selected imaging and spectroscopy setups I have designed and assembled during my graduate study. The purpose of this appendix is to provide the new users a quick guide on how to assemble, align and use these setups, which are listed as follows,

1) A Whole Body Imaging Setup with Adjustable Magnification in the Second Near-Infrared (NIR-II, 1000-1700 nm) Window;
2) A Whole Body Imaging Setup with Adjustable Magnification in the Visible (400-750 nm) and First Near-Infrared (NIR-I, 750-900 nm) Windows;
3) A Fast Scanning Confocal Microscope Operating in All Windows;
4) A Hyperspectral Microscopic Imaging Setup Operating in All Windows.

For the other existing setups in our lab, including the wide-field microscope setup and the photoluminescence-versus-excitation (PLE) setup, since they were built by previous members in our lab, the detailed alignment tips can be found in the Appendix of Dr. Kevin Welsher’s dissertation and thus are not included here.
A.1 A Whole Body Imaging Setup with Adjustable Magnification in the NIR-II Window

The optical diagram of the NIR-II whole body imaging setup with adjustable magnification is shown in Fig. A.1 as follows,

**Figure A.1 | Optical Diagram of the NIR-II Whole Body Setup.** Optical diagrams are shown for the NIR-II whole body imaging setup with three representative magnifications, 1× (top), 2.5× (middle) and 7× (bottom), where 1× denotes the magnification that covers the entire mouse body within a 60 mm × 48 mm field of view.
view, the 2.5× covers a 25 mm × 20 mm field of view, and the 7× covers a 8 mm × 6.4 mm field of view.

The optical components used in this setup are listed as follows,

<table>
<thead>
<tr>
<th>Part Abbreviation</th>
<th>Full Name</th>
<th>Company</th>
<th>Catalog number</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>collimator</td>
<td>Thorlabs</td>
<td>F230SMA-B</td>
<td>SMA connection</td>
</tr>
<tr>
<td>DT</td>
<td>detector</td>
<td>Princeton Instruments</td>
<td>2D OMA:V</td>
<td>Detector is the 2D InGaAs arrays with sufficient sensitivity in the NIR-II (1000-1700 nm)</td>
</tr>
<tr>
<td>F1</td>
<td>Excitation filter set comprised of an 850-nm short-pass filter and a 1000-nm short-pass filter</td>
<td>Thorlabs</td>
<td>FES0850 FES1000</td>
<td>To clean the laser line centered at 808 nm</td>
</tr>
<tr>
<td>F2</td>
<td>Emission filter set comprised of one or more long-pass and/or band-pass filters for user-defined sub-regions of NIR</td>
<td>/</td>
<td>/</td>
<td>To collect fluorescence in the NIR-II region. Can be customized for different NIR sub-regions.</td>
</tr>
<tr>
<td>L1</td>
<td>200 mm lens, 2” diameter</td>
<td>Thorlabs</td>
<td>AC508-200-C</td>
<td>Anti-reflection coating in the 1050-1620 nm range</td>
</tr>
<tr>
<td>L2</td>
<td>75 mm lens, 2” diameter</td>
<td>Thorlabs</td>
<td>AC508-075-C</td>
<td>Anti-reflection coating in the 1050-1620 nm range</td>
</tr>
<tr>
<td>L3</td>
<td>150 mm lens, 2” diameter</td>
<td>Thorlabs</td>
<td>AC508-150-B</td>
<td>Anti-reflection coating in the 650-1050 nm range. Ideally a C coated lens should be used but the AC508-150-C is too thick to fit into the setup.</td>
</tr>
<tr>
<td>LS</td>
<td>Laser</td>
<td>RMPC laser</td>
<td>808 nm</td>
<td>SMA connection</td>
</tr>
<tr>
<td>M</td>
<td>Mirror</td>
<td>Thorlabs</td>
<td>PF10-03-P01</td>
<td>Protected silver</td>
</tr>
</tbody>
</table>
Assembly and alignment of this setup are detailed in the following step-by-step procedure:

1. Fix the animal stage close to the edge of the optical table. This allows the nose cone that delivers the anesthetic gas to be easily mounted on the stage.

2. Place the 2D InGaAs camera (DT) at a distance of roughly 350 mm from the animal stage with its active area facing the stage. The camera is mounted on a 2D translation stage allowing for fine position adjustment later during the experiment. Therefore, in order to afford the maximum adjustable range, rotate the knobs to the middle of the range before starting the experiments.

3. Assemble the lens set.

   For the 1× magnification, place the 75-mm lens (L2) to the very bottom of a 2” lens tube, and then stack the 200-mm lens (L1) on top of the 75-mm lens. Note that the distance between these two lenses, $d_1$, needs to be as small as possible to afford the largest field of view (i.e., lowest magnification). Therefore these two achromat lenses were allowed to touch each other with the convex surface of both lenses facing the front of the 2” lens tube. Screw in a 2” retaining ring to hold the two lenses in place.

   For the 2.5× magnification, first screw in a 2” retaining ring to an empty 2” lens tube until the ring is approximately 1 cm from the end of the inner thread of the tube. Then place 75-mm lens (L2) inside the tube and it should stop at
the retaining ring. Screw in another 2” retaining ring to hold the 75-mm lens in place. Screw in another 2” retaining ring by a few revolutions in the thread to prevent the 2”-to-1” adaptor (to be mounted later) from going too deep into the tube. Place the 200-mm lens (L1) in a lens holder, which is then fixed immediately in front of the animal stage on the table. This would give a $d_2$ distance between the two lenses of approximately 200 mm. A magnification between 1× and 2.5× can be achieved by adjusting the distance between L1 and L2 in the range of 0 mm (two lenses touching each other) to ~200 mm.

For the 7× magnification, leave the 2” lens tube empty, but do screw in a 2” retaining ring by a few revolutions in the thread. Place the 200-mm lens (L1) in a lens holder, which is then fixed immediately in front of where the 2” lens tube will be located on the table. Place the 150-mm lens (L3) in a lens holder, which is fixed immediately in front of the animal stage. This would give a $d_3$ distance between the two lenses of approximately 150 mm.

4. Screw in the 2”-to-1” adaptor to the 2” lens tube and it should stop at the first retaining ring. Place the desired emission filters (F2) in one or more 1” lens tube(s) and mount the 1” lens tube(s) onto the 2”-to-1” adaptor.

5. Place the lens set with the filter set on a kinematic base held by a pair of magnets. Adjust the height of the lens/filter tubes to be roughly the same as the active area of the camera. Adjust the orientation of the filter tube(s) to facing the animal stage briefly.

6. Put on the blackout cloth to cover the camera and the lens/filter tubes. The 1” filter tube(s) should go through a hole made on the blackout fabric while all
other components should be completely covered by the cloth to prevent any leakage of the ambient light.

7. Adjust the mirror (M) roughly at a 45° angle to the optical table. The height of the mirror should be briefly adjusted to be the same as the active area of the camera. The vertical projection of the mirror should fall onto the animal stage.

8. Cut a new piece of non-woven wiper paper to the size of the animal stage and fix it on the stage by taping its four corners. Run the “2D OMA-V Background Subtraction” VI on the computer to see what part of the paper is being imaged by the camera. Very likely the paper is not in sharp focus of the camera. If this is the case, for the 1× and 2.5× magnification setups where there is at least one lens in the 2” lens tube, unscrew the post holder for the 2” lens tube from the kinematic base and move it back and forth to reach the sharpest focus (one should be able to see the line textures on the paper). For the 7× magnification setups where there is no lens in the 2” lens tube, move the animal stage up and down until a sharp focus is achieved. Note: DO NOT use the knob of the 2D translation stage where the camera is mounted for focusing in this step, since we want to save the maximum adjustable range for later use during imaging.

9. Once a sharp focus is achieved on the paper, it should be possible to determine the four borders of the field of view, which should be parallel to the sides of the optical table (adjust the mirror angle if not). Depending on where the animal will be placed on the stage (need to take into account where to put the nose cone) and what part of the animal will be imaged, the field of view on the paper can be repositioned by adjusting the angle of the mirror and the
height/angle of the lenses. Note that adjusting the azimuth angle might result in an inclined field, evidenced by part of the paper in focus while the rest out of focus (assuming the paper was taped down on a perfectly horizontal surface of the stage). To eliminate the inclined field, the azimuth angles of the lenses need to be close to 0° with respect to the detector array. This step completes the alignment of the emission path from the stage to the camera.

10. Next we need to align the excitation path. Mark the four borders of the finalized field of view on the paper. Use a fiber-coupled 633-nm He/Ne laser for excitation beam alignment through the 4.5-mm fiber collimator (CL), and adjust the position and angle of the collimator so that the excitation beam is centered in the marked field of view. Fix a filter holder underneath the collimator and make sure the beam pass through the center of the empty filter holder. Then put the two excitation filters (F1) into the filter holder.

11. Switch the fiber back to the 808-nm laser (LS). This completes the alignment of the excitation path. To perform imaging with this setup, turn on the 808-nm laser while using an IR card to view the beam location. Use the NIR-II portion in the spectrum of the room light to take white light optical images under a short exposure time of 20-100 ms. Turn off the room light and perform NIR-II fluorescence imaging with the 808-nm laser excitation. Note that the sample or the animal might not be in the same focal plane as the paper, so adjust the knob at the back of the InGaAs camera to change the focus until the sharpest image is reached.
A.2  A Whole Body Imaging Setup with Adjustable Magnification in the Visible and NIR-I Windows

The optical diagram of the whole body imaging setup with adjustable magnification using the Si camera with a sensitivity ranging from 350 to 900 nm is shown in Fig. A.2 as follows,

The Si camera is significantly smaller in size than the InGaAs camera owing to the thermoelectric cooling unit rather than a dewar flask for storage of liquid nitrogen. As a result the Si camera can be easily moved and repositioned on the optical table. The Si camera also has a standard C mount that allows for direct mounting of a 75-mm camera lens with external C-mount threading. These benefits have allowed the Si camera to reach a much larger range of adjustable magnifications by inserting empty, spacer tubes between the Si camera and camera lens.

The optical diagram of the NIR-II whole body imaging setup with adjustable magnification is shown in Fig. A.2 as follows,
**Figure A.2 | Optical Diagram of the Si-Camera Based Whole Body Setup.** This optical diagram shows the general setup for whole body animal imaging using the Si camera with tunable magnifications offered by changing the length \( d \) of the spacer lens tube (LT).

The optical components used in this setup are listed as follows,

<table>
<thead>
<tr>
<th>Part Abbreviation</th>
<th>Full Name</th>
<th>Company</th>
<th>Catalog number</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>collimator</td>
<td>Thorlabs</td>
<td>F230SMA-B</td>
<td>SMA connection</td>
</tr>
<tr>
<td>DT</td>
<td>detector</td>
<td>Hamamatsu</td>
<td>ORCA-03G</td>
<td>Detector is the 2D Si camera with sufficient sensitivity in the visible and NIR-I windows (350-900 nm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C8484-03G02</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>Excitation filter set</td>
<td>/</td>
<td>/</td>
<td>To clean the excitation source. Can be customized for different excitation</td>
</tr>
<tr>
<td></td>
<td>comprised of one or more</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td>short-pass and/or band-pass filters for specific</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Component</td>
<td>Description</td>
<td>Manufacturer</td>
<td>Model</td>
<td>Notes</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>--------------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>F2</td>
<td>Emission filter set comprised of one or more long-pass and/or band-pass filters for user-defined detection ranges</td>
<td>/</td>
<td>/</td>
<td>To reject excitation light and collect fluorescence only. Can be customized for different user-defined detection ranges.</td>
</tr>
<tr>
<td>L</td>
<td>75-mm camera lens</td>
<td>Thorlabs</td>
<td>MVL75L</td>
<td>A camera lens with external C-mount threading and manual focus</td>
</tr>
<tr>
<td>LT</td>
<td>Spacer lens tube</td>
<td>Thorlabs</td>
<td>SM1LXX</td>
<td>A series of stacked empty lens tubes that act as a spacer between the camera and the camera lens to increase magnification</td>
</tr>
<tr>
<td>LS</td>
<td>Laser</td>
<td>/</td>
<td>/</td>
<td>Can be any laser coupled to the other end of the optical fiber</td>
</tr>
<tr>
<td>M</td>
<td>Mirror</td>
<td>Thorlabs</td>
<td>PF10-03-P01</td>
<td>Protected silver mirror with broad reflection band covering the NIR-II</td>
</tr>
</tbody>
</table>

Table A.2 | A List of Components in the Si-Camera Based Whole Body Setup.

Assembly and alignment of this setup are detailed in the following step-by-step procedure:

1. Fix the animal stage close to the edge of the optical table in a similar way to the NIR-II whole body setup.
2. Mount none or more empty lens tubes (LT) to the Si camera (DT) through an SM1/C adaptor. Place all filters (F2) into these lens tubes if there is enough space. Then mount the camera lens (L) to the other end of the lens tube stack through a C/SM1 adaptor. Place an additional lens tube with filters (F2) if necessary on the camera lens (L).

Note: the total length of the stacked lens tubes \( d \) determines the magnification and thus the field of view of this imaging system. The longer the total length \( d \), the higher the magnification and the smaller the field of view. Empirically the following relationship can be followed:

<table>
<thead>
<tr>
<th>Tube length ( d )</th>
<th>Working distance (the distance from L to sample stage)</th>
<th>Field of View</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mm</td>
<td>559 mm</td>
<td>60 mm × 46 mm (‘1×’)</td>
</tr>
<tr>
<td>12.7 mm</td>
<td>241 mm</td>
<td>20 mm × 15 mm (‘3×’)</td>
</tr>
<tr>
<td>25.4 mm</td>
<td>190 mm</td>
<td>15 mm × 11 mm (‘4×’)</td>
</tr>
<tr>
<td>50.8 mm</td>
<td>142 mm</td>
<td>10 mm × 8 mm (‘6×’)</td>
</tr>
<tr>
<td>101.6 mm</td>
<td>114 mm</td>
<td>5 mm × 4 mm (‘12×’)</td>
</tr>
</tbody>
</table>

Table A.3 | Empirical Magnifications Using the 75-mm Camera Lens and the Spacer Tubes.

3. Place the Si camera with the attached tubes and camera lens at a distance from the sample stage where the sharpest image of the non-woven wiper paper taped on the animal stage is achieved (similar to the NIR-II whole body setup). The
camera can be moved freely on the optical table before being fixed down to find the best distance and azimuth angle to the animal stage. A general rule is, the higher the magnification (i.e., the longer the total length of the stacked lens tubes), the closer the focal distance from the animal stage to the camera lens.

4. Mark the edge of the field of view on the wiper paper and then follow steps 9~11 of the NIR-II whole body setup.
A.3 A Fast Scanning Confocal Microscope Operating in All Windows

The optical diagram of the fast scanning confocal microscope is shown in Fig. A.3 as follows,

Figure A.3 | Optical Diagram of the Fast Scanning Confocal Microscope. This optical setup allows for confocal microscopy in the visible, NIR-I and NIR-II windows with an entire wavelength range of 350-1700 nm, using user-customized excitation (LS & F1), emission filter set (F2) and different detectors (DT) to cover desired ranges to collect fluorescence.

A digital camera photo of the fast scanning confocal microscope is shown in Fig. A.4 as follows,
Figure A.4 | A Digital Camera Photo of the Fast Scanning Confocal Microscope.

An excitation source of the 658-nm laser diode and a detector of InGaAs camera are shown in this setup. The excitation beam is shown in red and the emission path is shown in purple.

The optical components used in this setup are listed as follows,

<table>
<thead>
<tr>
<th>Part Abbreviation</th>
<th>Full Name</th>
<th>Company</th>
<th>Catalog number</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>computer</td>
<td>/</td>
<td>/</td>
<td>Synchronizes TR and DT for fast confocal scanning and simultaneous</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Manufacturer</td>
<td>Model/Code</td>
<td>Notes</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------------------------------------------------</td>
<td>--------------</td>
<td>------------</td>
<td>------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>DC</td>
<td>Dichroic filter, 850 nm</td>
<td>Edmund Optics</td>
<td>#69-895</td>
<td>Can be customized to a different one based on the excitation and emission wavelengths.</td>
</tr>
<tr>
<td>DT</td>
<td>Detector</td>
<td></td>
<td>/</td>
<td>Detector can be single-chip photodiode, APD, PMT or 2D array detectors. Si based detectors should be used in the visible and NIR-I ranges (350-900 nm) and InGaAs based detectors should be used in the NIR-II window (1000-1700 nm).</td>
</tr>
<tr>
<td>F1</td>
<td>Excitation filter set comprised of one or more short-pass and/or band-pass filters for specific excitation source</td>
<td></td>
<td>/</td>
<td>To clean the excitation source. Can be customized for different excitation wavelengths.</td>
</tr>
<tr>
<td>F2</td>
<td>Emission filter set comprised of one or more long-pass and/or band-pass filters for user-defined detection ranges</td>
<td></td>
<td>/</td>
<td>To reject excitation light and collect fluorescence only. Can be customized for different user-defined detection ranges.</td>
</tr>
<tr>
<td>I1-I2</td>
<td>Iris diaphragms on SM1 threaded cage plate</td>
<td>Thorlabs</td>
<td>SM1D12D, CP02T</td>
<td>To aid alignment in the cage system</td>
</tr>
<tr>
<td>L1</td>
<td>200 mm lens on a z-axis translation mount</td>
<td>Thorlabs</td>
<td>AC254-200-C, SM1Z</td>
<td>Anti-reflection coating in the 1050-1620 nm range</td>
</tr>
<tr>
<td>L2</td>
<td>30 mm lens in a fixed lens tube</td>
<td>Thorlabs</td>
<td>AC254-030-C, SM1L05</td>
<td>Anti-reflection coating in the 1050-1620 nm range</td>
</tr>
<tr>
<td>LS</td>
<td>Laser</td>
<td></td>
<td>/</td>
<td>Can be any laser</td>
</tr>
</tbody>
</table>
with sufficient power output. A laser beam with small divergent angle is preferred. An excitation pinhole can be used in front of the laser to confine the divergent angle too.

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
<th>Supplier</th>
<th>Model</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1-M5</td>
<td>Mirrors</td>
<td>Thorlabs</td>
<td>PF10-03-P01</td>
<td>Protected silver mirror with broad reflection band covering the NIR-II</td>
</tr>
<tr>
<td>M6</td>
<td>Mirror on a cage mirror mount</td>
<td>Thorlabs</td>
<td>PF10-03-P01, KCB1</td>
<td>The beginning of the cage system</td>
</tr>
<tr>
<td>MM1-MM2</td>
<td>Magnetic mounts for cage system</td>
<td>Thorlabs</td>
<td>CP90F</td>
<td>For easy attachment and detachment of the pinhole and the emission filter(s)</td>
</tr>
<tr>
<td>OB</td>
<td>Objective, 100x</td>
<td>Olympus</td>
<td>ULWD MS Plan 100 IR</td>
<td>Depending on the desired resolution and scanned area, objectives with other magnifications can be used</td>
</tr>
<tr>
<td>P</td>
<td>Pinhole, 150 μm</td>
<td>Thorlabs</td>
<td>P150S</td>
<td>Size of the pinhole is determined by Airy disk size × magnification</td>
</tr>
<tr>
<td>S</td>
<td>Manual shutter</td>
<td>Thorlabs</td>
<td>LB1, FM90</td>
<td>A beam block mounted on a flip mount</td>
</tr>
<tr>
<td>TR</td>
<td>3D translation stage</td>
<td>Newport</td>
<td>VP-25XA-XYZR</td>
<td>This translation stage performs the fast raster scanning</td>
</tr>
</tbody>
</table>

Table A.4 | A List of Components in the Fast Scanning Confocal Microscope Setup.
Assembly and alignment of this setup are detailed in the following step-by-step procedure:

1. Assemble the setup according to Fig. A.3 coarsely. A rule of thumb for good alignment is that the height of every optical component, such as the filter, the mirror and the lens, should be adjusted to be roughly the same as the detector (DT). Make sure the excitation laser beam hits the center of F1, M1, S, M2, DC and M3. This helps maintain the beam path parallel to the optical table.

2. Since the cage system starting from M6 will be mounted onto the camera, and the camera lens L2 will be fixed inside the cage system, the first-time user is recommended to jump to step 11 to determine where to put L2 inside the cage system, and how long a lens tube is needed between MM1 and DT.

3. (Optional) If wide-field microscopic imaging capability is desired with an easy switch in the confocal microscopy setup, place a pair of lenses with focal lengths of 10 mm and 50 mm between LS and F1. This lens pair makes a beam collimator with expanded beam size. These two lenses should be placed on a flip mount to allow for easy switch between the confocal and wide-field modes. The second lens (50-mm lens) should also be mounted on a linear translation stage to allow for adjustment of the expanded beam size. Then adjust the position and angle of these two lenses to make sure the center of the enlarged beam still hits the center of F1, M1, S and M2 roughly. If so this beam should also hit the center of DC. Now place another lens with a focal length of 100 mm (usually referred to as the ‘offset lens’) also on a flip mount between M2 and DC. Adjust the position and angle of this 100-mm lens so that the beam
after passing this lens hit the center of DC and M3. Through this coarse adjustment, the wide-field illumination can be roughly aligned to share the same path as the confocal, point-like illumination. Nonetheless, fine adjustment is needed below. After this step is complete, put all these three additional lenses down via the flip mount and allow the excitation to return to the confocal mode.

4. Align the excitation beam to be perfectly vertical after bouncing off the mirror M3. Do this with an additional iris diaphragm connected to lens tubes with different lengths. Move the iris diaphragm along the vertical axis while adjusting the angles of DC and M3 to walk the beam and make sure the excitation beam always hits the center of the iris.

5. Screw in the objective (OB) with desired magnification on M3 through an RMS/SM1 adaptor. If the alignment in step 4 was well done now the beam should be tightly focused by the objective with maximum throughput power. Because of this, avoid directly looking into the objective to protect your eyes.

6. Use a highly reflective sample coated with NIR-II fluorophores for alignment of the reflected light. Such a sample can be made by spin-coating water-soluble, surfactant-stabilized single-walled carbon nanotubes (SWNTs) onto a thick gold coated glass substrate (thick Au/Au substrate), as aforementioned in Chapter 2.¹ Adjust the height of this sample via the 3D translation stage (TR) and watch for the maximum intensity of the reflected laser beam between DC and M4. Do fine adjustment of TR to reach the smallest beam size.

¹ Chapter 2
7. Relay the reflected excitation beam from M4 to M6 by making sure the beam hits roughly the center of each mirror.

8. Walk the reflected beam by adjusting the angles of M5 and M6 so that the reflected beam inside the cage system is parallel to the cage axis. Make sure the reflected beam always hits the center of the iris I1 as it moves along the rail of the cage system.

9. Open the diaphragm of I1 all the way and move L1 as close as possible to I1. Watch for the focused excitation beam after L1, and perform fine beam walking with I2 by adjusting the angles of M5 and M6 and making sure the focused beam always hits the center of the I2 as it moves along the rail of the cage system.

10. Attach F2 to MM2 while leaving MM1 unloaded. Move I2 with a closed diaphragm as close to MM1 as possible to mimic a pinhole (I2 with closed diaphragm would make a pinhole of ~500 μm). Adjust the position of L1 on the rail of the cage system to make sure $d_2$ and $d_1$ satisfy the following relationship:

$$
\frac{d_2}{d_1} = \frac{f_{L_1}}{f_{OB}}
$$

where $f_{L_1}$ and $f_{OB}$ are the focal lengths of L1 and OB, respectively.

11. Place L2 and DT with respect to MM1 such that $d_3$ and $d_4$ satisfy the following relationship:

$$
\frac{1}{d_3} + \frac{1}{d_4} = \frac{1}{f_{L_2}}
$$
where \( f_{L_2} \) is the focal lengths of L2. It is usually desired to have a large \( \frac{d_4}{d_3} \) ratio since this would give a relatively bigger field of view for the wide-field imaging mode in the same setup.

12. Run the “2D OMA-V Background Subtraction” VI on the computer. Use the white light from a halogen lamp to illuminate a transparent sample and image the sample under a short exposure time. First open the pinhole-mimicking iris \( I_2 \) all the way to find the edges of the field of view, and then close \( I_2 \) to find the edges of the iris. Adjust M6 to make sure the field of view (which should be a circle) and the iris (which should be a regular polygon) are concentric in the image.

13. Open \( I_2 \) all the way and switch to the fluorescence mode by turning off the halogen lamp and turning on the laser. Replace the more transparent sample with the aforementioned highly reflective Au/Au sample coated with SWNTs. It is recommended to switch to the wide-field mode by putting up all the three lenses described in step 3. A fluorescence image of the sample should be seen on the screen after focusing. Fine adjustment of the position and angle of these three lenses might be needed to reach a uniform, flat illumination of the field of view. Note that no other optical components than these three lenses should be touched or moved since it would affect the confocal alignment. Move the sample around by the 3D translation stage TR to find a relatively bright spot and move that spot to the center of the circular field of view. Switch back to the confocal mode (we might need to reduce the exposure time in the
meantime), and close I2 all the way. Now I2 only leave a tiny aperture in the middle of the field of view with a bright dot corresponding to the spot found before the iris was closed.

14. Maximize the fluorescence signal of the bright dot on the detector (read out from the VI) by fine adjustment of the angles of mirrors M5 and M6. Open and close the diaphragm of I2 and make sure the center of the diaphragm is concentric with the laser illumination profile (which should be a diffraction-limited 2D Gaussian if a point source is used as the excitation).

15. Attach the pinhole P to MM1 and then open the diaphragm of I2 all the way. The desired pinhole size of P is given by the following formula:

\[ \text{pinhole size} = \frac{1.22\lambda}{NA} \times M \]

where \( \lambda \) is the average wavelength of the detected fluorescence, \( NA \) is the numerical aperture of the objective and \( M \) is the magnification of the objective given that the right tube lens (L1) with the same focal length as rated by the manufacturer of that specific objective is used.

If the fluorescence intensity has been maximized in step 14 through a closed diaphragm, now the DT should still pick up sufficient signal from the sample, showing up as a tiny bright dot on the detector array. If a 2D detector array is used, zoom into the few pixels where the intensity is the highest by selecting a sub-array of the display and fixing the x and y scales in the VI. Fix the z scale as well and then maximize the intensity of this tiny bright dot (which should take 2 × 2 to 3 × 3 pixels) by fine adjustment of mirrors M5 and M6, as well as
the position and angle of the pinhole P on MM1. It has been found that how the pinhole P was drawn onto MM1 affected the coupling efficiency of fluorescence through the tiny pinhole.

16. Replace the standard Au/Au sample with a real sample for confocal imaging. Adjust M5, M6 and P to reach the maximum intensity. For easy alignment, TR can be moved too to find a bright spot in the sample to start with. If finding a bright spot in the sample becomes a problem, it is recommended to switch to the wide-field mode again, according to step 13. Note that the pinhole P needs to be taken off from MM1 when switching to wide-field.

17. When the intensity is maximized for the real sample in the confocal mode, record the coordinates of the brightest 4 pixels (2 × 2) from the “2D OMA-V Background Subtraction” VI. Type these coordinates into the block diagram of the “2D OMA-V Fast Confocal” VI. Reset the 3D coordinates of the translation stage to (0,0,0). Set the scan parameters according to the brightness and the size of the sample and make all parameters default. Always remember to save the VI after any new parameter is made default. One good rule of thumb to determine the step size of the scan is based on the full width at half maximum (FWHM) of the axial response function of the system (i.e., the axial resolution or z resolution of the system), which can be measured by performing z scan of a sample with thickness < diffraction limit. In our lab, the Au/Au film coated with SWNTs would be a good sample where its effective thickness of the sample is determined by the very sensitive sub-10 nm distance dependence.
of fluorescence enhancement. One can also estimate the \( z \) resolution based on the theoretical depth of focus as follows,

\[
R_z = \frac{\lambda n}{NA^2}
\]

where the refractive index \( n \) is usually 1 for air-immersed objective lenses. Once the \( z \) resolution is determined, the scan step size in the \( z \) direction is set to the \( z \) resolution to avoid unnecessary oversampling, and the scan step size in the \( x \) and \( y \) directions is also set to the same as the \( z \) step size to avoid streak artifact due to imbalanced pixel size.

18. CLOSE ALL VIS AND OTHER RUNNING PROGRAMS. This step is crucial since the “2D OMA-V Fast Confocal” VI is very memory-intense and any other programs running in background could cause the confocal VI to misregister the fast axis or even crash.

19. Relaunch the “2D OMA-V Monitor Temperature” VI and run it. Relaunch the “Newport ESP300 Position Control Panel” VI, run it, reset all coordinates of the stage to (0,0,0) and stop running it. Relaunch the “2D OMA-V Fast Confocal” VI and run it. The order of performing all these operations is very important, and if one operation was not done properly, go back to step 18 and start over. Once again, the “2D OMA-V Fast Confocal” VI needs clean memory to function properly.

20. Once the confocal scan starts, the stage TR will start raster scanning at a high speed while the detector will be collecting signals in a continuous manner. Note that any operation on the computer should be absolutely prohibited to
avoid misregistration of the fast axis (which would generate streak artifacts along the fast axis) or crash of the program.

21. The same operation procedure applies for confocal microscopy using the Si camera, except that a different VI called “Hamamatsu FAST CONFOCAL” is used to synchronize the Hamamatsu Si camera with the 3D translation stage. All VIs for the InGaAs camera run under LabVIEW 8.0, while all VIs for the Si camera run under LabVIEW 2009.

Note: For the InGaAs camera, the overhead time per acquisition is ~1.7 ms, limiting the scanning frequency to $\frac{1}{1.7 \text{ ms}} \approx 588 \text{ Hz}$. For the Si camera, the overhead time per acquisition is ~16 ms, limiting the scanning frequency to $\frac{1}{16 \text{ ms}} \approx 62 \text{ Hz}$. As a result, a significantly longer time is needed for taking confocal images in the visible and NIR-I windows using the Si camera than in the NIR-II window using the InGaAs camera, given roughly the same intensity levels are obtained on both cameras.
A.4 A Hyperspectral Microscopic Imaging Setup Operating in All Windows

The optical diagram of the hyperspectral microscopic imaging setup operating in a broad spectral range of 500-2100 nm is shown in Fig. A.5 as follows,

**Figure A.5 | Optical Diagram of the Hyperspectral Microscopic Imaging Setup.**

This optical setup allows for hyperspectral microscopic imaging in the visible, NIR-I and NIR-II windows with an entire wavelength range of 500-2100 nm, based on the aforementioned confocal setup.

A digital camera photo of the hyperspectral microscopic imaging setup is shown in Fig. A.6 as follows,
Figure A.6 | A Digital Camera Photo of the Hyperspectral Microscopic Imaging Setup. A fiber-coupled 808-nm laser, a 2D array InGaAs detector and a 1D array InGaAs detector are shown in this setup. The excitation beam is shown in red and the emission path is shown in purple.

Many of the optical components used in this setup are the same as in the confocal setup shown in Table A.4 and labeled with the same part abbreviations. The other optical components that are not used in the confocal setup are listed as follows,
<table>
<thead>
<tr>
<th>Part Abbreviation</th>
<th>Full Name</th>
<th>Company</th>
<th>Catalog number</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>One-dimensional InGaAs array detector</td>
<td>Princeton Instruments</td>
<td>OMA:V</td>
<td>This 1D array detector has sufficient detection sensitivity in the 500-2100 nm range</td>
</tr>
<tr>
<td>BD</td>
<td>Beam deflector, comprised of a mirror, a magnetic mirror holder and a magnetic cube</td>
<td>Thorlabs</td>
<td>PF10-03-P01, DFM</td>
<td>The kinematic mirror mount with a pair of magnets allows for accurate and reproducible switch between the imaging mode and the spectroscopy mode.</td>
</tr>
<tr>
<td>L2</td>
<td>60 mm lens in a fixed lens tube</td>
<td>Thorlabs</td>
<td>AC254-060-C, SM1L05</td>
<td>Anti-reflection coating in the 1050-1620 nm range. Note that this L2 has a different focal length from the L2 in the confocal setup.</td>
</tr>
<tr>
<td>L3</td>
<td>200 mm lens, 2” diameter</td>
<td>Thorlabs</td>
<td>AC508-200-C</td>
<td>A relay lens with anti-reflection coating in the 1050-1620 nm range</td>
</tr>
<tr>
<td>L4</td>
<td>50 mm lens, 1” diameter</td>
<td>Thorlabs</td>
<td>AC254-050-C</td>
<td>Anti-reflection coating in the 1050-1620 nm range</td>
</tr>
<tr>
<td>M7-M10</td>
<td>Mirrors</td>
<td>Thorlabs</td>
<td>PF10-03-P01</td>
<td>Protected silver mirror with broad reflection band covering the NIR-II. M7 and M8 make a vertical periscope to correct for the height difference of DT and 1D, while M9 and M10 make a horizontal periscope to correct for the position difference</td>
</tr>
</tbody>
</table>
The shutter allows for baseline subtraction of each acquisition for the spectrum of BD and SP.

A triple turret spectrometer with a built-in grating to spatially resolve different wavelengths for spectroscopy.

Provides white light illumination in the transmission mode for bright-field optical imaging and microscopic absorption measurement.

**Table A.5 | A List of Components in the Hyperspectral Microscopic Imaging**

**Setup.**

Assembly and alignment of this setup are detailed in the following step-by-step procedure:

1. Follow steps 1-14 of the confocal microscope setup described in **Section A.3** to align the right half of the setup shown in **Fig. A.5** for the imaging mode. Note a slightly different focal length of lens L2 \( f = 60 \text{ mm} \) is used here due to the increased distance from the pinhole P to the lens L2 after insertion of the beam deflector (BD) between P and L2.

2. Put the mirror in BD to deflect the beam to the side of the spectrometer (SP). Place a relay lens (L3) to confine the size of the beam. Use a vertical periscope made of two mirrors (M7 & M8) to lower the height of the beam to that of SP.
Then use a horizontal periscope made of an additional two mirrors (M9 & M10) to direct the beam roughly perpendicular to the aperture of SP. A mechanical shutter (MS) needs to be placed between the two periscopes (i.e., between M8 and M9) to chop the beam and take baseline subtraction during spectral measurement. Place the focusing lens (L4) in front of the aperture of SP. Close the aperture to ~0 μm and adjust the position and angle of L4 to direct the beam to the center of the aperture and minimize the beam size at the aperture too.

3. Run the “SP2300i with OMA-V and Shutter” VI and use the excitation laser light reflected from the standard Au/Au sample surface for alignment. Maximize the intensity by adjusting the angle and position of M9, M10 and L4. Once the alignment is optimized, stop the VI and open the aperture to ~350 μm.

4. Place an emission filter between M10 and L4 (it can be a 1000-nm or an 1100-nm long-pass filter to reject the reflected excitation laser light), and run the “SP2300i with OMA-V and Shutter” VI again. This time maximize the fluorescence signal from the standard Au/Au sample by fine adjustment of M9, M10 and L4. Once the fluorescence intensity is maximized too, the alignment of the left half of the setup is complete.

5. To carry out microscopic absorption hyperspectral mapping, a white light halogen lamp (WL) is used as the light source. The fiber head of the lamp needs to be fixed securely on the optical table, allowing for reproducible uniform illumination through the sample and to the objective (OB). The dichroic mirror (DC) needs to be put down and no filter should be used
between M10 and L4. To locate the sample for microscopic absorption measurement, take out the mirror in BD and open the iris I2 all the way without the pinhole P. Find the sharpest focus of the sample, and then confine the area of absorption measurement in the sample by closing the I2 to a desired size or adding in the pinhole P. Once the size of I2 has been decided it should not be changed in the following experiment (same for the pinhole, once P is decided to be used it should be on for all spectral measurements later). Remove the sample from the 3D translation stage (TR) and keep the lamp on. Put the mirror back in BD and then take a spectrum of the lamp light without any sample attenuation as the reference spectrum $S_r(\lambda)$. In this case, and the cases to follow, a VI named “SP2300i with OMA-V and Shutter Get Full Spectra” is used to take the spectrum. Put the sample back on the stage and under the illumination of the lamp, take out the mirror from BD and find the region of interest under the imaging mode. Then put the mirror back in BD and take a spectrum of the lamp light with the attenuation of the sample as the sample spectrum $S_s(\lambda)$. According to the definition of absorbance, the absorption spectrum of the sample at a specific location over a given area determined by the iris/pinhole size can be derived as follows,

$$A(\lambda) = -\log_{10} \frac{S_s(\lambda)}{S_r(\lambda)}$$

Since absorbance measurement is performed with the reference spectrum, no correction for the detector sensitivity profile is needed (i.e., it should cancel out from both numerator and denominator inside the log). A 2D mapping of the
sample can be achieved by taking the spectrum point-by-point along with raster scan. This gives a hyperspectral image with each pixel corresponding to the absorption spectrum of that specific location in the sample.

6. To carry out microscopic fluorescence hyperspectral mapping, WL needs to be turned off. The dichroic mirror (DC) needs to be put up in place and a proper long-pass filter should be added between M10 and L4. If the cutoff wavelength of that long-pass filter is \( \lambda \), then the measurable range for the fluorescence spectrum is \( \lambda - \min(2\lambda, 2100 \text{ nm}) \). The excitation laser should be turned on, and the mirror needs to be taken out from BD for locating the sample and determining the area of fluorescence hyperspectral mapping. The size of \( I_2 \) and \( P \) needs to be determined and fixed according to step 5. Fluorescence is an absolute measurement so no reference is needed as for the absorption measurement. Put the mirror back in BD and then take a fluorescence spectrum of the sample at a specific location over a given area under laser excitation. The spectrum needs to be corrected for the detector sensitivity profile and the extinction profile of the emission filter, which is placed between M10 and L4. A 2D mapping of the sample can be achieved by taking the fluorescence spectrum point-by-point along with raster scan. This gives a hyperspectral image with each pixel corresponding to the fluorescence emission spectrum of that specific location in the sample.
References