Reduction of excitation light leakage to improve near-infrared fluorescence imaging for tissue surface and deep tissue imaging

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Purpose: Fluorescence-enhanced optical imaging using near-infrared (NIR) light developed for *in vivo* molecular targeting and reporting of various diseases provides promising opportunities for diagnostic imaging. However, the measurement sensitivity of NIR fluorescence (NIRF) optical imaging systems is limited by the leakage of the strong backscattered excitation light through rejection filters. In this article, the authors present a systematic method for improving sensitivity and validating the NIRF optical imager currently used for clinical imaging of human lymphatic function.

Methods: The proposed systemic method consists of an appropriate filter combination and a collimation optics adapted to an NIRF optical imager. The spectral contributions were first assessed due to the excitation light backscattered from the tissue and from non-normal-incidence of the excitation light on the optical filters used in the authors' NIRF clinical imaging system. Then two tests were conducted to assess the system with and without the components of appropriate filters combination and collimation optics using: (1) a phantom to evaluate excitation light leakage as a function of target depth and (2) deployment in an actual human study.

Results: The phantom studies demonstrate as much as two to three orders of magnitude reduction in the transmission ratio, indicating that the excitation light leakage can be reduced upon using the appropriate filter combination and collimation optics while an *in vivo* investigatory human study confirms improved imaging.

Conclusions: The method for reducing the excitation light leakage is presented for validating collected signals for fluorescence imaging. © 2010 American Association of Physicists in Medicine. [DOI: [10.1118/1.3497153](https://doi.org/10.1118/1.3497153)]

Key words: optical imaging, optical filter, molecular imaging, fluorescence, noise floor

I. INTRODUCTION

Currently, the “gold-standard” for molecular imaging resides in nuclear medicine whereby upon decay, radiolabeled targeting moieties provide high energy photon events that have minimal tissue absorbance and scatter to provide planar (gamma scintigraphy) or tomographic (single photon emission computed tomography or positron emission tomography) imaging with exquisite sensitivities that can detect femtomolar to picomolar tissues concentrations of contrast agent. The opportunity for near-infrared fluorescence (NIRF) imaging to become a newly accepted method for noninvasive, clinical molecular imaging depends critically on achieving maximum sensitivity to likewise detect tissue concentrations of fluorescent contrast agents that can delineate disease markers on the tissue surface as well as deep within tissues. NIRF imaging utilizes tissue-penetrating excitation light (above >750 nm) to repeatedly and selectively excite exogenous, fluorescently labeled compounds that emit low-energy photons (with Stoke's shift of >20 nm) that experience minimal absorbance but significant scatter. At excitation wavelengths above 750 nm, contributions from endogenous fluorophores are small or nonexistent, providing the lowest background for emission measurement.^{1,2} The signal emanating from tissues is comprised of backscattered excitation and

generated fluorescence light (see Fig. 1). While there are a plethora of studies published in the literature using red and near-infrared (NIR) excited fluorophores in small animals,^{3,4} the extension of fluorescent imaging into large animals and humans has been limited. As recently reviewed by Marshall *et al.*,⁵ NIRF imaging in humans has been limited to the use of indocyanine green (ICG), a dim NIR excitable fluorophore that has no conjugatable group for attaching to targeting moieties, such as antibodies, antibody fragments, proteins, and peptides. For the most part, these studies employ milligram dose of ICG for fluorescent imaging collection at surface and subsurface conditions. Before new “first-in-humans” NIRF imaging agents can be employed at far lower quantities, imaging instrument sensitivity needs to be improved.

One method to improve sensitivity is to reduce the “noise floor” of the instrument by improving the efficiency of passing the weak NIRF signal while rejecting the overwhelmingly large component of scattered excitation light.^{2,5-7} For example, in the case of micromolar concentration of ICG, the re-emitted emission fluence is almost three orders of magnitude less than the backscattered excitation fluence that propagates to the tissue surface, requiring rejection filters with performance optical densities (ODs) greater than 3 at the excitation wavelength. If nanomolar quantities of NIRF dye,

FIG. 1. Schematic of the principle of fluorescence-enhanced optical imaging in a tissue medium.

then the re-emitted fluence is almost five orders of magnitude less than the backscattered excitation fluence, requiring filters with performance ODs greater than 5 (see the Appendix for computations). The physics of NIRF excitation and emission from tissue-like scattering media call into question the plethora of high impact literature reports of small animal imaging of femtomolar tissue concentrations of red and NIR excitable molecular imaging agents with rejection filters of OD 3. Yet as the depth of fluorescent targets increases or the tissue concentration decreases, the requirements for excitation light rejection becomes even more stringent. Few groups determine the contribution of excitation light leakage in collected signals or discriminate between fluorescence and backscattered excitation light in their measurements.

False NIRF signals arise from one or more of the following: (i) Spectral contribution from the excitation source, i.e., lamp, light emitting diode (LED), or laser diode; (ii) loss of interference filter performance arising when collected light is not normally incident;⁸ and (iii) limited holographic rejection filter performance at the excitation wavelength(s). Several investigators attempted to maximize sensitivity and minimize the noise floor with approaches such as (i) employing custom holographic rejection filters designed for laser or laser diode illumination with a small spectral bandwidth,² (ii) optimizing filter combination,^{6,9,10} and (iii) collimating incident images onto the optical filters using collimation optics.⁷ Herein, we present a systematic method for improving sensitivity and validating NIRF imaging instrumentation currently used for clinical imaging of human lymphatic function with microdose (defined as less than 1/100th of the dose calculated to yield a pharmacological effect of the test substance based on primary pharmacodynamic data obtained *in vitro* and *in vivo*, $\leq 100 \mu\text{g}$ or, in the case of a protein-based substance, $< 30 \text{ nM}$) administration of ICG. In the following, we describe the approach and methods used for (i) assessing spectral contributions of excitation light backscattered from the tissue and from non-normal-incidence of the excitation light on the optical filters used in our NIRF clinical imaging system and (ii) validating instrument excitation light rejection performance on a phantom model as well as

on one subject in the clinic. The concepts, approach, and results presented herein are translatable to both preclinical and clinical fluorescence imaging systems under development in other laboratories.

II. APPROACH AND METHODS

In the following, the concepts for accounting for spectral contributions due to incident illumination and filter excitation light leakage through the filters are first described and demonstrated and then the methods for validating improved performance and reduced noise floor are presented. In our preclinical and clinical studies, ICG was used as the fluorophore and was excited at 785 nm with the fluorescent signal collected at 830 nm.

II.A. Spectral contributions from incident illumination

Within small animal and emerging clinical systems reported in the literature,⁵ a variety of illumination sources are employed, including broad spectral lamps, LEDs, laser diodes, and lasers. Since the noise floor is determined by effective rejection of backscattered incident illumination and selective passage of the comparatively weak fluorescence signals, it is preferable to choose an excitation illumination source of smallest bandwidth. For NIRF excitation of ICG, a 785 nm, 500 mW laser diode light source (Intense, Inc., North Brunswick, NJ) driven by a combined current and temperature controller (Thorlabs, Inc., Newton, NJ) was used in both the phantom and human clinical case study described below. Typically, laser diode output is considered monochromatic and its spectra described by a prominent line corresponding to a single output wavelength. However, there often exist side-band components or second peaks due to lower level transitions, plasma, and “glows,” all of which can create background noise when there is spectral overlap with the fluorophore employed. For example, Fig. 2(a) shows the spectra of a 785 nm laser diode whose output was filtered with an 800 nm long pass filter (FF01–800/LP Semrock, Inc., Rochester, NY) and measured with a spectrometer (USB4000-VIS-NIR, Ocean Optics, Inc., Dunedin, FL) at different laser diode driving currents. With the long pass filter used to improve dynamic range of output at wavelengths $> 800 \text{ nm}$, a second peak located at the emission wavelength of the NIR dye was found. Upon introducing a 785 nm laser diode “clean-up” filter with 10 nm full width at half maximum (LD01–785/10, optical density > 5 : 705–765 nm and 803–885 nm, Semrock, Inc.), the spectral contribution in the dye’s emission band was removed [Fig. 2(b)]. In the above measurements, we varied the integration time to have a maximum photon count greater than 50 000 counts. It is noteworthy that the spectral contributions of white light and LED sources may also contribute unwanted signals that may be more difficult to eliminate due to their larger spectral bandwidths.

FIG. 2. Spectra intensity of 785 nm laser diode filtered with (a) an 800 nm long pass filter and (b) a 785 nm band pass and an 800 nm long pass filter combination.

II.B. Spectral contribution of filtering at point of light collection

NIR fluorescence optical imaging requires applying the appropriate interference filters with sufficient optical density for separating weak fluorescent signals from strongly backscattered excitation light. However, interference filters are designed to filter collimated light at a small tolerance from the angle of normal incidence. An increase in the incident angle causes a shift in maximal spectral performance toward shorter wavelengths.⁸ For example, the 800 nm long pass filter (FF01–800/LP-25, Semrock, Inc.) becomes a 725 nm long pass filter at a 45° angle of incidence, as computed from the data provided by Semrock Inc. and as shown in Fig. 3. Yet when tissue is illuminated by excitation light of 785 nm, the collected backscattered light is dispersed in all directions and impinges on to the filter at various incident angles thereby reducing its excitation light rejection capabilities. Consequently, collimation optics is critical to reducing excitation light leakage. As shown in Fig. 3, the Semrock 800 nm long pass can acceptably block 785 nm backscattered excitation light with a spectral discrimination width of <10 nm at 15° angle of incidence ($OD > 5$). To account for non-normal-incidence, a ray tracing software (ZEMAX software, Zemax, Bellevue, WA) was used to design collimation optics, shown in Fig. 4(a), which were integrated into the NIRF imaging system [Fig. 4(b)]. Figure 4(a) illustrates the impact collimation on two points on the image plane from the Nikon

FIG. 3. Optical density of 800 nm long pass filter as a function of incident angle, where AOI represents the angle of incidence.

focus lens (AF NIKKOR 28 mm f/2.8D, Nikon, NY, USA). The ray tracing demonstrates that the maximum angle of incidence of backscattered excitation light passing through the first lens has been reduced to 14° using the collimation optics. Such approaches can be used to design and optimize NIRF tissue imaging systems. As evidence of the approach, in the following we describe the instruments and the methods used to evaluate that the additional components minimized spectral contributions to the noise floor.

II.C. Instrumentation integrating components minimizing spectral contributions to noise floor

Figures 4(b) illustrates the gain modulatable intensified charge-coupled device (ICCD) NIRF system (details of the instrumentation can be found in Ref. 11) before integrating the appropriate filtering and collimation scheme. A schematic of the gain modulatable ICCD NIRF system after integration of the filtering and collimation schemes is illustrated in Fig. 4(c). First, the laser diode with the 785 nm band pass clean-up filter was expanded to illuminate the phantom or tissue surface uniformly through an optical diffuser and a convex lens. Fluorescent signals along with backscattered excitation light were incident upon a 830 nm band pass filter with 10.0 nm full width at half maximum (830FS10, Andover, Salem, NH) placed in front of the Nikon lens [Fig. 4(c)]. The collimation optical system shown in Fig. 4(a) was positioned between the Nikon lens and the intensifier (FS9910C Gen III model, IIT Night Vision, Roanoke, VA), collimating the entering light into a maximum critical angle of 14° and directing it to the 800 nm long pass filter and then to a second 830 nm band pass filter which was used to further reject the excitation light. Finally, the fluorescence signals were magnified by the intensifier and imaged with a 16 bit customized CCD camera (Photometrics, Tucson, AZ). The whole imaging process was implemented under LABVIEW based interface (National Instruments, Austin, TX).

II.D. Test of imaging systems with and without components

Two tests were conducted to assess the system with and without the components of appropriate filters combination

FIG. 4. Schematic of the collimation optics (a) and the ICCD NIRF imaging system (b) before and (c) after integration of filtering and collimation schemes, where “f” represents the focus length of lens.

and collimation optics. First, a phantom was used to evaluate excitation light leakage as a function of target depth (or collection of weaker fluorescent signals), and then the optics were used in human case study of lymphatic imaging to determine if they significantly reduced excitation light leakage in images of fluorescent human lymphatics.

II.D.1. Phantom experiments

II.D.1.a. Surface and subsurface fluorescent targets. While NIRF imaging promises depth penetration for imaging subsurface tissues, NIRF imaging also has the potential for assessing tissue surfaces during intraoperative procedures, endoscopic evaluation, etc. We first sought to demonstrate

the improvement at zero target depth or surface and subsurface detection of NIRF. A simple phantom was made consisting of two Eppendorf tubes filled with 1% (by volume) Liposyn, which was made by volumetric dilution of a 20% stock solution (Hospira, Inc., Lake Forest, IL) to mimic the scattering properties of tissue. ICG was dissolved in de-ionized ultrafiltered water and was then added to one of the tubes to formulate a 10 nM ICG solution. The tubes were illuminated using the 785 nm laser diode with an incident power of 0.86 mW/cm² (comparable to the incident laser power used in the clinical trials described below) and images were obtained with and without the appropriate filter combination and collimation optics components. During image acquisitions, the intensifier and camera gain were constant and the maximum intensity observed with the CCD camera was limited to approximately 40 000 counts by the varying integration time. The quantitative analysis of target and background signals was performed using IMAGEJ software by plotting the fluorescent intensity profile of a line drawn across the portion of the images which contained the tubes.

II.D.1.b. Transmission ratio for excitation light leakage. In order to quantify excitation light leakage, a cylindrical phantom was made containing 1.0% Liposyn scattering solution. Excitation light leakage was defined as the signal $S(\lambda_x)$ or average pixel intensity values associated with the image of the scattering surface taken without ICG in the solution. The fluorescence signal $S(\lambda_m)$ was likewise averaged from the pixel intensity values associated with the images taken after adding ICG to formulate the 1.0 nM solution. Measurement settings such as the intensifier gain and the maximum count of CCD camera were held constant for collection of signals $S(\lambda_x)$ and $S(\lambda_m)$ by varying the CCD integration time under different laser diode illumination powers. The transmission ratio R was then calculated using the following equation:^{6,7}

$$R = \frac{S(\lambda_x)}{S(\lambda_m) - S(\lambda_x)},$$

where $S(\lambda_x)$ signals can be regarded as the “off-band” transmission signals, whereas the differences, signal $S(\lambda_m) - S(\lambda_x)$, represent the “in-band” transmission signals. The smaller the value of the transmission ratio R , the better performance for rejecting excitation and passing fluorescence light and the lower the noise floor.

II.D.1.c. Depth of penetration experiments. A third set of experiments was performed to quantitate excitation light leakage as a function of fluorescent intensity by constructing a phantom in which the depth of the fluorescent target could be varied from 1.0 to 4.0 cm (to mimic fluorescent deep targets such as tumor or lymph nodes). The fluorescent target consisted of a transparent plastic tube of 1.5 mm inner diameter containing a 200 nM of ICG in 1.0% Liposyn solution. The target was submerged in a phantom containing 1.0% Liposyn solution. The fluorescent target depth was increased from 1.0 to 4.0 cm in increments of 1.0 cm. In the first phase of this experiment, a target tube containing 1.0% Liposyn but no ICG contrast was inserted into the phantom and images

acquired at each depth level. Subsequently, a tube containing ICG was inserted and images were again acquired at each depth. In both scenarios, the laser power was adjusted to 1.9 mW/cm² on the surface to mimic the maximum permissible incidence levels established by our Food and Drug Administration (FDA) approved clinical applications (see below). To improve the image quality, we subtracted the corresponding absence image taken under identical measurement conditions (intensifier gain and integration time) and then averaged the subtracted images 25 times to further reduce noise.

II.D.2. Human subject test

The NIRF optical imager was tested in a single case study performed under the auspices of a feasibility study approved by the United States FDA under combinational investigational new drug application 102 827 for the off-label use of ICG as a NIR fluorescent contrast agent. The HIPPA-compliant studies were approved by the Institutional Review Board at the University of Texas Health Science Center and the Memorial Hermann Hospital similarly to studies previously reported.^{5,12} Following informed consent, the single subject was recruited as part of a larger study and received multiple 0.1 ml intradermal injections of 25 mg ICG given in both legs. For the purposes of this test, imaging was performed at a single location using the NIRF optical imager with and without the components at our standard camera integration time of 200 ms. Maximum photon counts of around 40 000 counts were achieved by varying intensifier gain.

III. RESULTS

III.A. Phantom experiments

III.A.1. Surface and subsurface fluorescent targets

Figure 5 shows the comparison of images with and without the components of appropriate filter combination and collimation optics in the NIRF optical imager. Without the components, the outline of the empty Eppendorf tube is visible and the noise floor is relatively high, as shown in Figs. 5(a) and 5(c), respectively. With the added components, the empty Eppendorf tube is indistinguishable from background and the noise floor is reduced to a lower level, as shown in Figs. 5(b) and 5(d). These results demonstrate that the appropriate filter combination and addition of collimation optics results in less excitation light leakage relative to the amount of fluorescence collected as shown by the higher signals not associated with the fluorophore. These results also indicate that excitation light leakage makes a considerable contribution to the noise floor and can be reduced through the appropriate filter combination and collimation optics.

III.A.2. Transmission ratio for excitation light leakage

Table I presents the calculated transmission ratio as a function of laser illumination power for systems with and without the appropriate filter combination and collimation

FIG. 5. Qualitative and quantitative demonstration of excitation light leakage in the measurements (a) without and (b) with the components of appropriate filter combination and collimation optics. The lines in (a) and (b) illustrate the line for which the fluorescent intensity is plotted in (c) and (d). CCD integration times were 55 ms for (a) and 650 ms for (b).

optics. As shown, we observe as much as two to three orders of magnitude reduction in the ratio of off-band to in-band transmission, indicating that the excitation light leakage has been reduced upon using the appropriate filter combination and collimation optics described herein. In these studies, the gain modulatable ICCD NIRF optical imager was operated with the modulation frequency ω set to zero and additional improvements may be expected with frequency-domain operation.⁶

III.A.3. Depth of penetration

Figures 6 and 7 illustrate the NIRF images of the phantom surface containing the ICG target at varying depths. Without the appropriate filter combination and collimation optics, the fluorescence signal is weak at an image depth of 1.0 cm and

is undetectable at a depth 2.0 cm, as shown in Fig. 6. Increased integration times did not improve detection, presumably because the contribution due to excitation light leakage is higher than that from the fluorescent inclusion. In contrast, the NIRF optical imager with the appropriate filter combination and collimation optics can detect the fluorescence signals at depths as much as 4.0 cm by increasing the integration time of CCD camera, as illustrated by Fig. 7. These results clearly indicate that the reduction of the excitation light leakage lowers the noise floor enabling lengthening of camera integration time to detect smaller amount of fluorophore at greater tissue depths.

III.B. Lymphatic imaging

To translate the developed NIRF optical imager with the components of appropriate filter combination and collimation optics to the clinical applications, we imaged a small section of the lymphatic system in a human subject following intradermal injection of ICG. Figures 8(a) and 8(b) shows the lymphatic structure with and without the components of appropriate filter combination and collimation optics, respectively, and their corresponding 3D surface plots are illustrated in Figs. 8(c) and 8(d), respectively. The results show that the noise floor is reduced by the appropriate filter combination and collimation optics. While we do not know the depth of the lymphatic vessels noninvasively imaged, the results nonetheless demonstrate the opportunity for imaging deeper fluorescent tissues and/or tissues contrasted by

TABLE I. Transmission ratio (R) as a function of laser source illumination power for the measurement with and without the components of appropriate filter combination and collimation optics on the NIRF optical imager.

Laser power (mW/cm ²)	R without the collimation and appropriate filter combination	R with the collimation and appropriate filter combination
0.24	12.66 ± 0.10	0.004 31 ± 0.000 20
0.47	5.40 ± 0.02	0.003 62 ± 0.000 09
0.84	4.05 ± 0.02	0.003 49 ± 0.000 07
1.16	3.80 ± 0.01	0.003 46 ± 0.000 06
1.47	3.18 ± 0.01	0.003 37 ± 0.000 05

FIG. 6. Contrasting fluorescent images of a target suspended at different depth in a 1.0% Liposyn solution without the components of appropriate filter combination and collimation optics. The target depths and CCD integration times were (a) 10 mm and 70 ms, and (b) 20 mm and 70 ms.

smaller amounts of NIR contrast agents. Finally, the reduction of the noise floor bodes well for tomographic imaging where instrument response deteriorates with addition of time-dependent measurements.

IV. DISCUSSION

The core of the NIR fluoresce optical imager is the ICCD camera by placing an image intensifier in front of the CCD chip. The image intensifier was made of three main components, namely, a photocathode, a microchannel plate (MCP), and a phosphor screen. At the photocathode, the diffuse light signal is converted into electrical energy (or electrons), which are then amplified at the MCP, and finally the amplified signal is converted back to a light signal at the phosphor screen ready for the CCD to detect. The detection limit of the current fluorescence imaging system is determined by the accumulation of signal noise which originates from sources such as the inherent shot noise, dark current noise, and readout noise which are generated in the intensifier and camera and by the leakage of ambient and excitation light through optical filters. In contrast to the shot noise, the readout and

dark current noises are negligible. However, shot noise is a fundamental property of the quantum nature of light, which arises from statistical fluctuations in the number of photons emitted from the object. This noise source is unavoidable and always present in imaging systems.¹³

Hence, the excitation light leakage currently represents the limiting noise floor for NIRF imaging. In practice, it is nearly impossible to perfectly reject all the backscattered excitation light (as well as ambient light) to selectively collect only the comparatively weaker fluorescence signals. If the noise floor created by excitation light leakage is higher than the fluorescent signal emitted from the NIR fluorescent contrast agent, then one needs to increase the amount of fluorophore to collect an image. The “holy grail” of using molecularly targeted NIR fluorescence agents to guide surgical resection or noninvasively detect diseased tissues on the basis of a disease marker requires the ability to detect low concentrations of agent within tissues that is validated *not* to arise from backscattered excitation light that leaks through rejection filters. Since the intensity of emanating fluorescent signal attenuates with originating depth, increased filter light leakage reduces the depth of penetration. In addition, strong excitation light leakage may in turn contaminate reconstructed fluorescence images with artifacts in fluorescence tomography studies.⁷ It is imperative that both planar and tomographic fluorescence imaging require validation of the collected signals as being from an exogenous fluorophore rather than from excitation light leakage.

There are many factors resulting in excitation light leakage including contributions from illumination sources, limited optical density of interference filters, and the deterioration in filter performance as incident light deviates from normal direction of filter surface. In this contribution, with the appropriate filter combination and collimation optics, we show that the noise floor in the NIRF optical imager can be dramatically reduced and the camera integration time can be lengthened for detecting targets located at deeper depths and lower fluorophore concentrations. The parameter of transmission ratio R can be used as a quantifiable metric for efficient excitation light rejection over improved fluorescence collection and should be used for instrument qualification and performance documentation. Finally, this validation and qualification approach to reduce and document excitation light leakage should be extended to all fluorescence-enhanced optical imaging systems which can potentially suffer from strong excitation light leakage.

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APPENDIX: CALCULATION OF OPTICAL DENSITY

In a continuous wave based measurement approach, the incident excitation energy from a constant intensity (or kHz modulated) source is constant over timescale of milliseconds

FIG. 7. Contrasting fluorescent images of a target suspended at different depth in a 1.0% Liposyn solution with the components of appropriate filter combination and collimation optics. The target depths and CCD integration times were (a) 10.0 mm and 900 ms, (b) 20.0 mm and 41 s, (c) 30.0 mm, 52 s, and (d) 40.0 mm and 58 s.

and the re-emitted fluorescence energy from exogenous agents is likewise constant. As the excitation light travels through the absorption and scattering medium, it is exponentially attenuated with respect to the incident light. The amount of fluorescence generated from a fluorophore within the tissue is proportional to the product of the fluorophore concentration, quantum efficiency, and the local excitation fluence.¹ The propagation of NIR light through tissue is well described by the diffusion equation derived from the radiative transport equation.^{14,15} The coupled diffusion equations predicting the fluorescence light generation and propagation in tissue over three-dimensional domain Ω are

$$\begin{cases} -\nabla \cdot (D_x \nabla \Phi_x) + (\mu_{axi} + \mu_{axf})\Phi_x = S_x \\ -\nabla \cdot (D_m \nabla \Phi_m) + (\mu_{ami} + \mu_{amf})\Phi_m = \phi \mu_{axf}\Phi_x \end{cases} \quad \text{on } \Omega \quad (\text{A1})$$

subject to the Robin boundary conditions on the domain boundary of

$$\begin{cases} \vec{n} \cdot (D_x \nabla \Phi_x) + b_x \Phi_x = 0 \\ \vec{n} \cdot (D_m \nabla \Phi_m) + b_m \Phi_m = 0 \end{cases} \quad \text{on } \delta\Omega. \quad (\text{A2})$$

In the above equation, Φ represents the fluence; μ_a is the absorption coefficient (cm^{-1}), where the subscripts x and m correspond to excitation and emission wavelength, respectively, and where the subscripts i and f denotes the chromophores (i.e., the endogenous chromophores in tissues) and fluorophores, respectively; S_x is the excitation photon source; and ϕ denotes the quantum efficiency of the fluorophore. The optical diffusion coefficient at the excitation wavelength D_x and emission wavelength D_m are given by $D_{x,m} = 1/3(\mu_{ax,m} + \mu'_{sx,m})$, where μ'_s is the reduced optical scattering coefficient (cm^{-1}) and the Robin boundary coefficients are governed by the reflection coefficients (R_x, R_m), $b_{x,m} = (1 - R_{x,m})/2(1 + R_{x,m})$.

Numerical experiments were performed on a $10 \times 10 \times 6.5 \text{ cm}^3$ synthetic domain using area illumination and col-

FIG. 8. Images of lymphatic vessels (a) without and (b) with the components of appropriate filters and collimation optics on the clinical NIRF optical imager and their 3D surface plots shown in (c) and (d), respectively.

lection measurement geometry employed in our NIRF optical imager in order to compare the backscattered excitation fluence and the re-emitted fluence on the tissue surface. The optical properties of this domain were chosen to model tissue mimicking phantom with ICG as the fluorescent agent, with excitation at $\lambda=785$ nm and collected emission at $\lambda=830$ nm. Background optical properties were modeled as homogenous with the following values: $\mu_{\text{axf}}=0.3$ cm⁻¹ and 0.0003 cm⁻¹ (corresponding to 1 μ M and 1 nM concentration of ICG, respectively), $\mu_{\text{axi}}=0.025$ cm⁻¹, $\mu_{\text{axm}}=0.032$ cm⁻¹, $\mu'_{\text{sx}}=10.88$ cm⁻¹, $\mu'_{\text{sm}}=9.82$ cm⁻¹, $\phi=0.011$, $R_{\text{x,m}}=0.431$ on the top, and $R_{\text{x,m}}=0.0282$ on the other five sides. We use the Galerkin finite element method to solve the above second-order coupled diffusion equations. The specular reflectance formula $R_{\text{sp}}=(n_1-n_2)^2/(n_1+n_2)^2$, where n_1 and n_2 are the refractive indices of the outside medium and tissue, respectively, is used to calculate the direct reflections of normally incident excitation fluence from the surface of the tissue mimicking phantom ($n_1=1.0$ and $n_2=1.33$). The total backscattered excitation and emission fluences are 56.6 and 1.44×10^{-1} (mW/cm²), respectively, for 1 μ M concentration of ICG, and 72.9 and 4.42×10^{-4} (mW/cm²), respectively, for 1 nM concentration of ICG in tissue mimicking phantom. Finally, the required OD to detect 1 μ M and 1 nM concentration of ICG in tissue

mimicking phantom are 2.6 and 5.2, respectively. If the excitation light is not normally incident, then specular reflections may contribute a higher contribution to the collected light and a higher OD may be required to detect fluorescent light.

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