Fluorescence depth estimation from wide-field optical imaging data for guiding brain tumor resection: a multi-inclusion phantom study

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Abstract: Studies have shown that fluorescent agents demarcate tumor from surrounding brain tissue and offer intraoperative guidance during resection. However, visualization of fluorescence signal from tumor below the surgical surface or through the appearance of blood in the surgical field is challenging. We have previously described red light imaging techniques for estimating fluorescent depths in turbid media. In this study, we evaluate these methods over a broader range of fluorophore concentrations, and investigate the ability to resolve multiple fluorescent emissions in the same plane or at different depths along the axis of imaging. A tungsten halogen lamp is used as a broadband white light source for reflectance imaging. Fluorescence from Alexa Fluor 647 is excited with a 635 nm diode laser. Reflectance and fluorescence spectral data are gathered between 670 and 720 nm with the use of a liquid crystal tunable filter and recorded on a sCMOS camera. Results show that two fluorescent emissions can be resolved within 2 mm if they are in the same plane or within 3 mm if they are at different depths along the axis of imaging up to 6 mm below the surface.

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OCIS codes: (110.0110) Imaging systems; (170.2520) Fluorescence microscopy.

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1. Introduction

Brain cancers are challenging to treat and come with a poor prognosis. Primary tumors, even low-grade cancers, often progress rapidly. Studies show that extent of resection corresponds with increased survival and better quality of life [1], though even small amounts of residual disease can negatively impact the efficacy of adjuvant therapies [2,3]. The current standard of care for guiding brain tumor resection is co-registration of preoperative magnetic resonance imaging (MRI). However, once surgery begins, brain shift of up to 20 mm causes deviation of the surgical field from the registered MRI [4]. Further, glial tumor tissue is often not visually distinct from normal tissue under normal operative conditions. Providing updated high-resolution guidance during resection procedures would, therefore, be very valuable.

Development of updated MRI through co-registration of intraoperative imaging techniques including ultrasound and stereovision has previously been reported [5–8], but these methods are yet to be widely adopted. Optical techniques including confocal [9,10] and two-photon fluorescence microscopy [11] produce images with resolution and contrast comparable to histopathology. However, imaging depths are limited to a few hundred microns, and the relatively small fields of view (<1mm) limit applicability to surgical guidance. To effectively guide resection in practice, some depth detection over a wider field of view would be desirable, especially for ALA-induced PpIX fluorescence which has gained some clinical acceptance in neurosurgery [12,13].

In previous work, the depth of a single fluorescent source was accurately estimated when located up to 6mm below the surface based on linear relationships between fluorescence
intensity ratio and fluorophore depth derived from analytical expressions [14]. However, the performance of this depth estimation strategy when multiple fluorescent sources are present in various sizes, separations and depths has not been investigated or characterized. The approach is very attractive because of its data acquisition and computational simplicity, but its viability in practice, for example, during surgery depends on the performance that can be attained under these more complex conditions.

In this paper, we investigate the performance of the depth estimation algorithm (and several simple perturbations), when detecting fluorescence emissions from multiple sources. The experiments build on fluorescence excitation with red light that is not strongly absorbed by blood to enable deeper penetration into the tissue. Previous studies have shown that 5-aminolevulinic acid (ALA) induced protoporphyrin-IX (PpIX) fluorescence guided surgery has been successful in demarcation of tumor [15,16]. In the studies described here, we used Alexa Fluor 647 (AF647) since its emission spectrum closely matches that of PpIX when excited with 635 nm light; yet, it is biochemically more stable than PpIX, allowing experiments to occur more efficiently over a much larger set of optical properties and inclusion/phantom parameter variations that impact the accuracy of the depth estimations.

Tissue phantoms, with and without fluorescent inclusions, mimicking the range of optical properties found in the brain were fabricated. Reflectance and fluorescence images of the phantoms were acquired. Image data were analyzed to estimate locations of the fluorescence inclusions, and outcomes were compared to the known orientations. Results demonstrate the depth detection capabilities of the method in the presence of multiple fluorescent sources of varying sizes, separations and depths over a range of biologically relevant optical property backgrounds.

2. Materials and methods

2.1 Imaging system

A commercially available surgical microscope (Carl Zeiss Meditec) previously adapted for PpIX fluorescence imaging was used [14]. A variable-intensity bright-field tungsten-halogen lamp served as a broadband white light source for reflectance measurements. For excitation of AF647 fluorescence, a 635 nm diode laser (Intense Co) was coupled into the system, producing a beam with a peak intensity of 20 mW/cm². To improve the quality of the illumination, two plano convex lenses (Thorlabs, LA1253-A and LA1252-A) created a Gaussian beam with a diameter of 3 cm and peak intensity of 8 mW/cm². Figure 1 shows a schematic of the experimental setup. The microscope optics allow for zoom and field-of-view adjustment. Light returning from the image plane was filtered with a 650 nm long pass filter (Chroma) to eliminate excitation light from the diode laser and a liquid crystal tunable filter (LCTF, Verispec CRI) for select wavelengths, before being registered on a sCMOS camera (Edge, PCO).
2.2 Tissue simulating phantoms

A phantom preparation method previously described [14] was optimized for the current study. Two identical inclusions of 1 cm diameter and 1 mm wall thickness were prepared. To hold the bulk background liquid, a container was designed to be 7 cm in diameter to minimize the amount of media required during the experiments while maintaining adequate optical diffusion distance (> 3 times the penetration depth).

For the bulk media, concentrations of metHb (Sigma Aldrich) and Intralipid (Intralipid 20%, Patterson Veterinary Supply) were diluted to produce absorption coefficient and reduced scattering coefficient values that span the range of optical properties found in brain [17]. AF647 (Sigma Aldrich) was mixed with the bulk media to produce various dilutions for the fluorescent inclusions. The wavelength-dependent absorption features of metHb differ from oxy-and deoxy-hemoglobin contained in whole blood (see Fig. 2). However, similarly to AF647, metHb is biochemically more stable, which was important for this study for practical reasons (because of the long experiments involving a large number of phantom property perturbations that were conducted consecutively). The use of AF647 versus PpIX and metHb versus oxy- and deoxy-hemoglobin is included in section 4.
Fig. 2. Comparison of absorption spectra of oxygenated blood and metHb. Since experiments involved a homogeneous phantom, vessel size was not considered when calculating the absorption coefficient of blood. The concentrations of metHb and blood were 3.66 mg/mL.

2.3 Image acquisition

Bright white light illumination for reflectance and red excitation for fluorescence were performed in succession for each case. Acquisitions were recorded every 5 nm between 650 nm and 720 nm for a total of 14 reflectance and 14 fluorescence images. Total acquisition time for one set of 28 measurements was less than 10 seconds. One reflectance measurement, acquired over the same sampled wavelength range, was recorded per set of optical properties without any fluorescent inclusion. To normalize these reflectance images, a spectralon standard (LabSphere) was measured to eliminate any bias in the instrument.

2.4 Image analysis

In previous work, we reported that depth estimation relies on the ability to acquire zero-depth data on a fluorescent inclusion, and calculation of the slope, $m$ [14], of the linear relationship between the dual-wave fluorescence intensity ratio (DWFR), and the depth of the fluorescence emission [19]. A summary of the techniques and expressions for finding $m$ are summarized in Table 1 [14]. For our tests, zero-depth data was measured with no intervening bulk media between the imaging system and each of the different AF647 inclusions used. By measuring a diffuse reflectance standard (Labsphere), the wavelength-dependent diffuse reflectance, $R_\lambda$, of the medium being imaged was calibrated. From calculation of $R_\lambda$, values of $m$ were estimated by one of three techniques. Technique 1 relied on knowledge of optical properties $\mu_a$ and $\mu_s'$ of the medium to calculate the wavelength-dependent penetration depth, $\delta_\lambda$ at the two wavelengths used for the DWFR. Wavelength-dependent penetration depth can be calculated as described in Table 1. From penetration depth, $m$ can be directly calculated. Technique 2 relied on knowledge of $\mu_s'$ and required diffuse reflectance images acquired at the two DWFR wavelengths, $R_{\lambda_1}$ and $R_{\lambda_2}$. These diffuse reflectance images were used to estimate the attenuation in the tissue due to absorption. For the third technique, $R_{\lambda_1}^b$ and $R_{\lambda_2}^b$ were empirically combined to represent the total light attenuation in the tissue, and did not require previous knowledge of $\mu_a$ and $\mu_s'$ for the range of optical properties typically found in the brain.
Table 1. Summary of three techniques to calculate $m$ for recovering depth information [12].

| Technique                  | Description                                      | Input Parameters                                      | Limitations                                      | $m (mm^{-1})$ |
|----------------------------|--------------------------------------------------|-------------------------------------------------------|-------------------------------------------------|---------------|
| 1                          | Diffusion equation solution                      | $\mu_a, \mu_s$ infinite medium boundary conditions   | Absorption and reduced scattering needed         | $m = \frac{1}{\delta^1} \frac{1}{\delta^2}$ |
| 2                          | Empirical scattering correction                  | $\mu_s$                                               | Reduced scattering needed                        | $m = \ln\left(\frac{R_1}{R_2}\right) \left(\frac{\mu_a(700nm)}{\mu_s}\right)^{3.28}$ |
| 3                          | Diffuse reflectance correction                   | None                                                  | Not accurate for all sets of optical properties  | $m = \ln\left(\frac{R_1}{R_2}\right)$ |

$\delta^1 = \sqrt{\frac{D}{\mu_s}}$, $D = \sqrt[3]{3(\mu_a + \mu_s)}$

To calculate DWFR, the two wavelengths, $\lambda_1$ and $\lambda_2$, were chosen to be 670 nm and 700 nm since they correspond to greater change in absorption of metHb, while still having contributions from the peak AF647 fluorescence (see Fig. 3). From the fluorescence images acquired at these wavelengths, the ratio of fluorescence intensities was computed.

![Emission spectra of AF647 and extinction coefficient of metHb, 670 nm and 700 nm were the wavelengths chosen to calculate the DWFR.](image)

Fig. 3. Emission spectra of AF647 and extinction coefficient of metHb, 670 nm and 700 nm were the wavelengths chosen to calculate the DWFR.

2.5 Experimental setup

The experimental setups for three separate inclusion configurations evaluated in this study are shown in Fig. 4. In the first experiment, we determined the relationship between concentration of AF647 and maximal depth of detection, shown in Fig. 4(A). Here, the concentration of AF647, $C_i$, had values of 0.01, 0.1, 1.0, and 10.0 $\mu$g/mL, and depth, $d$, ranged from 0 to 10 mm in 1 mm increments. For the bulk media, we tested a range of concentrations of metHb, resulting in values of $\mu_a$ equal to 0.001, 0.005, 0.01, 0.03, and 0.05 mm$^{-1}$, while the range of concentrations of intralipid resulted in values of $\mu_s'$ equal to 0.5, 1.0, 1.5, 2.0, and 2.5 mm$^{-1}$. These values represented the range expected in vivo in the brain. For each depth, $d$, a total of 25 combinations of optical properties for the 5 different AF647 concentrations were evaluated in this experimental configuration yielding 125 data sets that were analyzed.

The second experiment, illustrated in Fig. 4(B), tested the ability to differentiate two fluorescent inclusions of increasing radius and separation within the same plane at different depths. The two fluorescent inclusions had AF647 concentrations of 10.0 $\mu$g/mL. The radii and separation of the inclusions, $r$ and $s$, were varied from 1 to 5 mm and 2 to 5 mm, respectively. Depth of the inclusions was varied from 0 to 10 mm. For the bulk media, three sets of optical properties were considered. For the first set, $\mu_a = 0.001$ mm$^{-1}$ and $\mu_s' = 1.0$
mm$^{-1}$, corresponding to low scattering and low absorption, relative to values expected in brain. The next set had optical properties of $\mu_a = 0.01$ mm$^{-1}$ and $\mu_s' = 1.0$ mm$^{-1}$ corresponding to high absorption and low scattering, relative to values expected in brain. The last set incorporated optical properties of $\mu_a = 0.001$ mm$^{-1}$ and $\mu_s' = 2.0$ mm$^{-1}$, corresponding to low absorption and high scattering relative to values expected in brain. For this experimental configuration, the number of combinations of optical properties was reduced to 3 for one concentration of AF647 at each depth. A total of 60 data sets were evaluated in the Experiment 2 setup.

The third experiment, shown schematically in Fig. 4(C), evaluated two fluorescent inclusions with fixed lateral separation and relative depth at increasing depths below the phantom surface. Two 10 mm diameter inclusions of 1 $\mu$g/mL AF647, separated laterally by 15 mm and vertically by 3 mm were placed at depths, $d_1$ and $d_2$. For this experiment, $d_1$ and $d_2$ were varied between 0 and 5 mm, and 3 mm and 8 mm, respectively below the phantom surface. This experiment was designed to determine if algorithms could differentiate between multiple inclusions at different depths along the axis of imaging. Only one set of optical properties of the background medium and one concentration of AF647 was tested for this experimental setup. A total of 6 data sets were evaluated in the Experiment 3 geometry.

Fig. 4. Diagram of phantom experiments evaluated. (A) Experiment 1: varying AF647 concentration, $C_i$, and depth, $d$, of the inclusion. (B) Experiment 2: varying radius, $r$, and spacing, $s$, of inclusions at increasing depth, $d$. (C) Experiment 3: varying depth ($d_1$ and $d_2$) of inclusions separated by 15 mm laterally and 3 mm in relative depth ($d_2 - d_1$).
3. Results

3.1 Experiment 1: variation in fluorophore concentration

Maximum depth at which fluorescence could be detected was determined by having a signal to background ratio (SBR) greater than 2 where the signal was measured at the center of the inclusion and background was measured at the center of the null inclusion (0.0 μg/ml AF647). Figure 5 reports the maximum depths of detection versus absorption coefficient for low (Fig. 5(A)) and high (Fig. 5(B)) scattering. As expected, higher concentrations of AF647 were detected at deeper locations, but these depths decreased as metHb and intralipid concentrations were increased which corresponded to increasing absorption and scattering, respectively. For the case of higher scattering, the SBR for the highest concentration of 10 μg/ml of AF647 falls below 2 when absorption is increased to 0.03 mm$^{-1}$. This is likely due to the AF647 contributing to the overall absorption, decreasing the depth from which signal can be returned to the detector. While the lowest concentration of AF647 (0.01 μg/mL) was difficult to detect in higher scattering and absorbing phantoms, in lower scattering and absorbing media it could be resolved up to 6 mm below the surface.

![Fig. 5. Maximum depth detection for different concentrations of AF647 over the range of absorption coefficients tested. (A) corresponds to the lowest reduced scattering coefficient tested ($\mu_s' = 0.5 \text{ mm}^{-1}$) and (B) corresponds to the highest scattering coefficient tested ($\mu_s' = 2.5 \text{ mm}^{-1}$).](image)

Topographic maps were created to compare errors in the depth estimates from the three estimation methods. Figure 6 shows findings for the bulk optical properties with the largest depth detection (absorption coefficient of $\mu_a = 0.001 \text{ mm}^{-1}$, and reduced scattering coefficient of $\mu_s' = 0.5 \text{ mm}^{-1}$). Results are reported for the four concentrations of AF647 tested – 0.01 μg/mL in Fig. 6(A), 0.1 μg/mL in Fig. 6(B), 1.0 μg/mL in Fig. 6(C), and 10 μg/mL in Fig. 6(D) – and are color-coded as accurate (within 1 mm of the true value), under-predicted or over-predicted.
Fig. 6. Tomographic depth map for $\mu_s' = 0.5 \text{ mm}^{-1}$ and $\mu_a = 0.001 \text{ mm}^{-1}$. Panels (consisting of 4 individual columns) show detection limits and estimation accuracies for four AF647 concentrations: (A) 0.01 $\mu$g/mL (B) 0.1 $\mu$g/mL (C) 1.0 $\mu$g/mL (D) 10 $\mu$g/mL. Each column within a panel summarizes the accuracies of the 3 estimation techniques and shows the raw fluorescence image at 700 nm. Depth increases with each row. Color indicates whether the depth is accurately predicted (green), under-predicted (blue) or over-predicted (red). Below each panel is a plot of the estimated depth at the center of the inclusion as a function of the true depth for each estimation technique.

Below each map is a comparison of estimated versus true depth of the inclusion for the three estimation algorithms. Looking at Case C, with AF647 concentration of 1.0 $\mu$g/ml, technique 3 (using an empirical diffuse reflectance correction) over predicts depth estimation over the inclusion area by over 1mm for depths beyond 2mm. Using the reduced scattering coefficient in Techniques 1 and 2 improve the depth estimation with 50% of inclusion returning estimated depth within 1mm of true depth up to depths of 8 mm. For Cases A and B, lower AF647 concentration cases indicate that using the absorption coefficient in Technique 1 improves the depth estimates with 50% of inclusion returning estimated depths within 1 mm of true depth up to 9 mm vs. only 3 mm for Technique 2. In Case D, at higher AF647 concentrations, Technique 1 overestimates depth with 50% of inclusion returning estimated depth within 1 mm of true depth only up to depths of 2 mm, compared to depths up to 9 mm for technique 2 in which absorption is not taken into account. Uncorrected reabsorption of the fluorescence at the concentrations of AF647 higher than 1.0 $\mu$g/ml may be contributing to the overall absorption thereby negatively affecting Technique 1 when $\mu_a$ is used.

3.2 Experiment 2: variation in inclusion size and lateral separation

In these experiments, detection of two inclusions located in the same plane was evaluated as a function of inclusion size and lateral separation distance. Here, a watershed transform [20] was applied to determine when the two inclusions were distinguishable. This transform evaluates image gradients and identifies areas that flow to the same region or watershed of the
image. If two inclusions are found to be in the same watershed, they are considered to be indistinguishable within the image. Figure 7 illustrates application of the watershed transform to data from Experiment 2. The first case (Fig. 7(A)) results in unique watersheds (Fig. 7(C)), and therefore the inclusions are deemed to be distinguishable, whereas the second case (Fig. 7(B)) results in a single watershed as shown in Fig. 7(D), and the inclusions are scored as indistinguishable.

Fig. 7. Sample images of phantoms with inclusions that are 2mm in diameter spaced 4mm apart. The inclusions are at depths, d = 6mm in (A) and d = 7mm in (B). After applying the watershed transform, the two inclusions from A result in unique watershed regions, (C). The two inclusions in (B) result in the same watershed, (D). The lines in (C) and (D) indicate the locations of the centers of the two inclusions.

Figures 8(A), 8(B), and 8(C) report the maximum distinguishable depth of the two inclusions according to the watershed transform as a function of separation distance and size of inclusions for the three sets of optical properties tested. In this experiment, a high AF647 concentration (10 μg/ml) was used, and depth estimates shown were calculated from Technique 2 as the better option given results in Fig. 6. Depth estimation errors, along with raw fluorescence intensity at 700 nm appear in Fig. 8(D) for inclusions with a radius of 5mm having four separation distances. Errors begin to mount beyond a depth of 2 mm and are greater at the smaller separations.
3.3 Experiment 3: variation in inclusion axial separation

Inclusions in this experiment were 10mm in diameter and separated by 15mm laterally and 3mm vertically (i.e., in the depth direction). Initial measurements were acquired with the shallow inclusion positioned at the phantom surface (i.e., $d_1 = 0$ mm) and the deeper inclusion placed at a 3mm depth ($d_2 = 3$ mm), and the depth of the inclusion pair was increased in 1 mm increments until the shallower inclusion was located at depth, $d_1 = 5$ mm, and the deeper inclusion was held at a depth, $d_2 = 8$ mm. The concentration of AF647 concentration was 1 μg/ml, and results in Fig. 9 used Technique 2 for estimating inclusion depth. These data suggest that two inclusions separated by 3mm along the imaging axis can be resolved when located within the first 5mm below the surface.
Fig. 9. Depth estimations from Technique 2 for deeper and shallower inclusions in Experiment 3. Bulk media optical properties were $\mu_s' = 1.0 \text{ mm}^{-1}$ and $\mu_a = 0.001 \text{ mm}^{-1}$ and the concentration of AF647 used in the inclusions was 1.0 $\mu$g/ml.

3.4 Error in estimation technique

To further compare the three techniques for depth estimation, regions of interest corresponding to the true size of the inclusion were selected in the depth estimation maps as shown in Fig. 10. The region of interest is determined by the size of the inclusion and then overlayed over the image, and average error is calculated over this region. The error in estimation versus actual depth was then averaged over the regions of interest for the three experimental setups; variation in fluorophore concentration, variation in inclusion size and lateral separation, and variation in inclusion axial separation. A summary of the depth estimation error for each technique is provided in Table 2. Techniques 1 and 2 averaged 1.86 mm and 1.93 mm error (i.e. the inclusion was deeper than the techniques estimated), respectively. The third technique, relying only on the diffuse reflectance images can provide depth estimation under certain optical properties; however, when averaged over a larger set of bulk media properties and orientations of fluorescent inclusions, the error averaged to over 2 mm deeper than the actual depth of inclusion.
Table 2. Absolute error in depth estimation measured in mm for the three depth experiments.

| Technique | Experiment 1 | Experiment 2 | Experiment 3 | Total |
|-----------|--------------|--------------|--------------|-------|
| 1         | 1.92         | 1.87         | 1.82         | 1.86  |
| 2         | 1.94         | 1.98         | 1.84         | 1.93  |
| 3         | 2.40         | 3.40         | 2.50         | 2.70  |

4. Discussion and conclusions

In this study, a modified clinical microscope was used to investigate simple techniques for estimating the depth of localized fluorescence in phantoms. Resolving focal, spatially distinct fluorescence emissions is likely to be important in determining whether a tumor is shallow and contains low concentration of the fluorophore or is deeper and contains higher concentration of the fluorophore.

Depth determination relied on being able to estimate the value of $m$, which represents the slope of the linear relationship between a dual-wave fluorescence intensity ratio (DWFR) and the depth of the fluorescence emission. Experimentally, a fluorescence ratio was recorded for an inclusion placed at the phantom surface. To estimate $m$ during surgery, a fluorescence ratio measurement acquired on cancer located on the surgical surface is needed. Here, the surgeon could rely on preoperative imaging to approximate the location of the tumor, and the depth detection techniques evaluated here could prove valuable later in surgery when preoperative imaging deviates substantially from the surgical field due to the procedural intervention.

This study reported on limits in resolving the fluorophore, Alexa-Fluor 647, at depth when embedded in a background of optical properties containing methemoglobin. For concentrations of AF647 comparable to those expected for PpIX in tissue (0.1-3 μg/mL), the detection limit was between 1 and 5 mm below the surface. Multiple inclusions could be detected up to 5-6 mm deep for separations in the same plane as small as 2 mm, and inclusions were differentiated when placed at multiple depths up to about 5 mm and separated by 3 mm along the imaging axis. In general, for depths greater than 2 mm below the surface, depth estimation errors were greater than 1 mm in many cases. In the clinic, creating topographic maps of subsurface tumor will be challenging as disease may be present in any arrangement. Nonetheless, given the maximum detectable depths observed here, as well as our simple depth estimation techniques, rapid imaging and mapping through 5 mm of tissue is likely to provide additional valuable information on remaining tumor. Assuming a resolution of ~2 mm on pre-operative MRI, the estimation techniques described here would provide surgeons with information sufficient to localize fluorescence signals when the surgical field deviates from the co-registered preoperative scans.

For these methods to be used in mapping of brain tumors, evaluations of PpIX in bulk media containing whole blood are still needed. Differences exist in the absorption spectra of methHb, oxy- and deoxy-hemoglobin which may change these results. Moreover, the quantum yield of AF647 is different from PpIX [21]. To model the relative fluorescence signals from AF647 and PpIX, the diffuse light transport of a point source in infinite medium was used (Eq. (1)) as described elsewhere [22,23].

$$I = \frac{I_o}{4\pi Dr} e^{-\delta}$$  \hspace{1cm} (1)

Here, $I$ is the intensity at a distance $r$ from the source where the source has an initial intensity $I_o$, and $D$ and $\delta$ are the diffusion coefficient and penetration depth respectively at the wavelength $\lambda$. Calculating the fluence to reach a point below the surface of turbid media accounts for the differences in absorption between methemoglobin and blood at 635 nm. The difference in fluorescence yield of AF647 and PpIX can be calculated using Eq. (2):
where $Q_{AF}$ is the quantum yield of AF647 (0.33), $Q_{PpIX}$ is the quantum yield of PpIX (0.06), $\varepsilon_{AF}(\lambda_{ex})$ is the absorption coefficient at the excitation wavelength of AF647 (125 L/g/cm), and $\varepsilon_{PpIX}(\lambda_{ex})$ is the absorption coefficient at the excitation wavelength of PpIX (9.12 L/g/cm). The result is that at the same concentration, AF647 will yield 75 times more fluorescence emission photons. Calculating the photon fluence from the emitting fluorescent source back at the detector, we can estimate the total detectible fluorescence for fluorescence inclusions in bulk media in terms of initial intensity as $I_{em}/I_{O} = I_{ex}/I_{O} \times I_{em}/I_{ex}$ for metHb and for blood it is $I_{em}/I_{O} = I_{ex}/I_{O} \times I_{em}/I_{ex}/75$ for blood.

In Fig. 11 we present the results of our model. For metHb and blood concentrations of 3.66 mg/mL the depths at which inclusions of concentrations 1 mg/mL of PpIX in a bulk media using blood as an absorber (dashed line) and AF647 in a bulk media using methemoglobin as the absorber (dashed line) yield fluence ratios. These ratios describe the amount of fluorescence emitted relative to the initial intensity of the source. Given a certain fluence ratio, we can then determine the difference in depths at which the two different fluorophore-bulk media combinations would provide the same level of fluorescence. Looking at the dotted line in Fig. 11, for equal concentrations, AF647 at a depth, $d_{AF647}$ of 6 mm in metHb will provide the same amount of fluorescence at the detector as PpIX at a depth, $d_{PpIX}$ of about 5.1mm in blood. The depths, $d_{AF647}$ and $d_{PpIX}$ will vary for different concentrations of fluorophores and different bulk media properties, but we feel that this model shows the use of AF647 and metHb provides a suitable substitute for PpIX in whole blood for running large quantities of phantoms.

Our use of 635 nm illumination demonstrated that the technique should allow for imaging beyond the absorption bands of whole blood. Future experiments with these techniques will explore phantoms with inclusions of PpIX to assess these differences.

The depth detection techniques evaluated in these studies have certain advantages over other approaches (e.g. diffuse fluorescence tomography, modulated imaging, time-of-flight...
imaging) in that they are less sensitive to calibration errors and less expensive to compute, yet still yield errors that are typically within 1 mm in phantom experiments at depths of a few millimeters, at least for the two methods that use optical properties in the calculations. These results show the importance of knowing at least the reduced scattering coefficient of the surrounding media to estimate depth of fluorescence signals, which puts more emphasis on developing reliable methods for estimating optical properties very quickly over fields of view relevant to surgery. Thus, improvements in our estimation techniques are likely to occur by incorporating spatial frequency domain imaging (SFDI) to more accurately map optical properties.

**Funding**

This work was supported by US National Institutes of Health (US) under Grants No. R01 NS052274-09 and K25 CA164248-01 (CCK) and NSERC (Canada) Discovery Grant Program.