Impact of the emission wavelengths on in vivo multiphoton imaging of mouse brains

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Abstract: Tissue scattering and absorption impact the excitation and emission light in different ways for multiphoton imaging. The collected fluorescence includes both ballistic photons and scattered photons whereas multiphoton excited signal within the focal volume is mostly generated by ballistic photons. The impact of excitation wavelengths on multiphoton imaging has been extensively investigated before; however, experimental data is lacking to evaluate the impact of emission wavelengths on fluorescence attenuation in deep imaging. Here we perform three-photon imaging of mouse brain vasculature in vivo using green, red, and near-infrared emission fluorophores, and compare quantitatively the attenuation of the fluorescence signal in the mouse brain at the emission wavelengths of 520 nm, 615 nm and 711 nm. Our results show that the emission wavelengths do not significantly influence the fluorescence collection efficiency. For the green, red and near-infrared fluorophores investigated, the difference in fluorescence collection efficiency is less than a factor of 2 at imaging depths between 0.6 and 1 mm. The advantage of long wavelength dyes for multiphoton deep imaging is almost entirely due to the long excitation wavelengths.

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1. Introduction

Multiphoton microscopy (MPM) utilizes nonlinear excitation to confine the fluorescence generation within the focal volume, which allows the use of efficient, large-area detectors for the fluorescence collection to improve the signal-to-noise ratio (SNR) for deep imaging in scattering biological tissue [1–7]. Light attenuation in biological tissues is a combined effect of absorption and scattering. Multiphoton excited signal within the focal volume is mostly generated by ballistic photons that are not scattered along the excitation path. However, because of the wide-field geometry for fluorescence collection in MPM, both ballistic and scattered fluorescence photons contribute to the image formation [8]. MPM relies on efficient fluorescence generation and collection for deep tissue penetration [9]. Understanding the transport of light in biological tissue is important in selecting the optimum excitation and emission wavelengths for MPM. Although the advantage of using long excitation wavelengths for MPM has been extensively explored [10–15], the impact of emission wavelengths on deep imaging has not been systematically investigated before.

In this paper, we performed in vivo three-photon imaging in the same mouse brains using the same excitation wavelengths but with green, red, and near-infrared (NIR) emission dyes, and compared the signal attenuation in the tissue at these emission wavelengths. To ensure that all three fluorescent dyes are three-photon excited and there is no bleed-through of third harmonic generation (THG) signal into the fluorescence collection channels, we did two different sets of imaging on dye-labeled brain vasculature in vivo. One uses 1450 nm to excite fluorescein and Texas Red, and the other uses 1700 nm to excite Texas Red and Alexa Fluor 647. The wavelength of the collected fluorescence is defined by the optical filters centered at 520 nm, 615 nm and 711 nm. Our experimental results show that the impact of the emission wavelengths on multiphoton deep imaging (within ~1 mm depth in the mouse brain) is small (less than a factor of 2) for the green, red and NIR fluorescent dyes investigated, and the
advantage of long wavelength dyes for deep imaging is mostly contributed by the long excitation wavelengths.

2. Characterization of the three-photon imaging setup

The three-photon imaging setup is similar to that described previously [5,6]. The excitation source was a wavelength-tunable optical parametric amplifier (OPA, Opera-F, Coherent) pumped by a Monaco amplifier (Coherent) operating at 330 kHz repetition rate. The excitation spectra at 1450 nm and 1700 nm were measured by an Optical Spectrum Analyzer (OSA, Thorlabs), shown in Fig. 1(a).

The generated fluorescence was detected by photomultiplier tubes (PMTs) with GaAsP photocathodes (H7422-40, Hamamatsu Photonics). Three bandpass filters, 520/15 nm (Semrock, FF01-520/15-25), 615/20 nm (Semrock, FF02-615/20-25) and 711/25 nm (Semrock, FF01-711/25-25), were positioned in front of the PMTs to separate the fluorescence generated by fluorescein, Texas Red, and Alexa Fluor 647, respectively. The spectral response of the emission filters (provided by Semrock) is shown in Fig. 1(b). The transmission at wavelengths >800 nm of these filters has no impact on the experiments due to the spectral response of the GaAsP PMT (cutoff < 750 nm). The emission spectra for most fluorescent dyes are not symmetrical, and usually display long emission tails at the long wavelengths; however, the bleed-through between fluorescence emission spectra does not alter the measurements since the wavelengths of the detected fluorescence were defined by the optical filters rather than the fluorescent dyes. Indeed, a single fluorescent dye would be preferred in this experiment if it could cover a broad emission spectrum from the green to the NIR.

Three-photon excitation was chosen to compare the impact of the emission wavelength in order to suppress the generation of out-of-focus fluorescence background, which would introduce uncertainties in the comparison [16–18]. To ensure that all three dyes are three-photon excited, we measured the dependence of the fluorescence on the excitation power for fluorescein and Texas Red with 1450 nm excitation, and Texas Red and Alexa Fluor 647 with 1700 nm excitation (Fig. 2). A low repetition rate of 330 kHz was used to increase three-photon excitation relative to two-photon excitation for the redder dyes in the comparisons since the ratio of three-photon to two-photon excited fluorescence is inversely proportional to the duty cycle of the laser pulse train [19]. The generated fluorescence was recorded by a photon counter (SR400, Stanford Research Systems). The slopes in the log-log power-dependence plots confirmed three-photon excitation for all the fluorescent dyes used here.

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![Fig. 1.](image) Fig. 1. (a) Measured spectra of the laser source operating at 1450 nm and 1700 nm. (b) Transmission data for the three emission filters used for fluorescein (520/15 nm), Texas Red (615/20 nm) and Alexa Fluor 647 (711/25 nm).

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3. In vivo comparison of the impact of the emission wavelengths on deep imaging

To compare multiple fluorescent dyes excited by the same wavelengths without systematic bias, we did simultaneous imaging of fluorescein and Texas Red excited at 1450 nm, and Texas Red and Alexa Fluor 647 excited at 1700 nm. 5-mm craniotomies were performed on wild-type mice (C57BL/6J). A glass window was placed directly on the intact dura of each mouse for imaging. The mouse was anesthetized using isoflurane (3% in oxygen for induction and 1.5–2% for surgery and imaging to maintain a breathing frequency of 1 Hz). Body temperature was kept at 37.5 °C with a feedback-controlled blanket (Harvard Apparatus), and eye ointment was applied.

The imaging conditions were kept the same for all the imaging sessions. The pulse energy at the focus is similar at each imaging depth (< 2nJ). As shown in Fig. 2, there is no fluorescence saturation at such pulse energy. Three-photon images were acquired in 20 µm depth increment from the brain surface. The images were taken at a frame rate of 0.24 Hz (512 x 512 pixels/frame) with a field-of-view (FOV) of 340 x 340 µm, and 10 frames were averaged at each depth to ensure high signal-to-noise ratio for all the images.

Since the fluorescence was acquired from the two fluorescent dyes at the same time by separating the signal into two detection channels using the emission bandpass filters, the imaging FOV and the imaging depth of each frame is identical for the two fluorophores. Example images are shown in Fig. 3.
For the comparison experiment of fluorescein and Texas Red excited at 1450 nm, dextran-conjugated fluorescein (70kDa, Invitrogen) and Texas Red (70kDa, Invitrogen) were injected retro-orbitally at the same time prior to imaging. Three-photon excited fluorescence was collected by the two detection channels simultaneously. The average value of the brightest 1% of pixels in the x-y image was selected at each depth as the fluorescence signal. To ensure that the results are not dependent on the selection criteria for the brightest pixels, we varied the selection criteria from 0.5% to 2%, and found that this variation did not affect the comparison results significantly.

The fluorescence signal at the depth \( z \) (\( F_z \)) was normalized to that at the surface (\( F_0 \)). This is defined as the normalized fluorescence signal \( F_N \), i.e., \( F_N = F_z/F_0 \). \( F_N \) is calculated at each depth and plotted in Fig. 4(a). We then calculated the ratio of the normalized Texas Red signal (\( F_{N-TR} \)) and the normalized fluorescein signal (\( F_{N-FL} \)) at each depth, i.e., \( F_{N-TR}/F_{N-FL} \). The results are shown in Fig. 4(b).

Similarly, for the comparison of Texas Red and Alexa Fluor 647 excited at 1700 nm, dextran-conjugated Texas Red and Alexa Fluor 647 (10kDa, Invitrogen) were injected simultaneously into the same mouse and three-photon imaging was performed. We plotted the normalized fluorescence signal \( F_N \) as a function of depth in Fig. 5(a) and the ratio of the normalized Alexa Fluor 647 signal and Texas Red signal at each depth (i.e., \( F_{N-AF647}/F_{N-TR} \)) in Fig. 5(b).
We repeated the simultaneous imaging experiments as described above in different mice with age from 8 weeks old to 6 months old (10 mice total for the comparison between fluorescein and Texas Red, and 7 mice total for the comparison between Texas Red and Alexa Fluor 647). For each mouse, we calculated the average ratio of the normalized Texas Red and fluorescein signal at every 100 μm depth interval, e.g., the ratios \( \frac{F_{NR,TR}}{F_{NR,FL}} \) obtained from the images between 0 and 100 μm are averaged, and the value is plotted at the 50 μm depth as the average ratio for the depth between 0 and 100 μm (Fig. 6(a)). Similar analysis was done for the Alexa Fluor 647 and Texas Red signal (Fig. 6(b)). We also averaged over all mice to get an average ratio at each depth interval. The results are summarized in Table 1 and Table 2. Based on the experimental results, with the same signal at the brain surface, the Texas Red signal is about 1.34 times the fluorescein signal at 510-600 μm depth, and 1.43 times at 710-800 μm depth. The Alexa Fluor 647 signal is 1.14 times the Texas Red signal at 710-800 μm depth, and 1.14 times at 910-1000 μm depth.
Fig. 6. (a) Ratio of the normalized fluorescence of Texas Red and fluorescein averaged every 100 μm depth interval in 10 different mice. Each color/marker represents a different mouse. (b) Ratio of the normalized fluorescence of Alexa Fluor 647 and Texas Red averaged every 100 μm depth interval in 7 different mice. Each color/marker represents a different mouse.

Table 1. Ratio of the normalized fluorescence of Texas Red and fluorescein for all 10 mice at 100 μm depth interval.

| Depth Range (μm) | 0-100 | 110-200 | 210-300 | 310-400 | 410-500 | 510-600 | 610-700 | 710-800 |
|------------------|-------|---------|---------|---------|---------|---------|---------|---------|
| Average Ratio    | 1.03  | 1.07    | 1.15    | 1.20    | 1.24    | 1.34    | 1.32    | 1.32    |
| Minimum Ratio    | 0.92  | 0.88    | 0.99    | 1.03    | 1.11    | 1.13    | 1.15    | 1.25    |
| Maximum Ratio    | 1.25  | 1.26    | 1.35    | 1.39    | 1.39    | 1.68    | 1.50    | 1.61    |

Table 2. Ratio of the normalized fluorescence of Alexa Fluor 647 and Texas Red for all 7 mice at 100 μm depth interval.

| Depth Range (μm) | 0-100 | 110-200 | 210-300 | 310-400 | 410-500 | 510-600 | 610-700 | 710-800 | 810-900 | 910-1000 |
|------------------|-------|---------|---------|---------|---------|---------|---------|---------|---------|-----------|
| Average Ratio    | 1.02  | 1.01    | 1.08    | 1.06    | 1.09    | 1.11    | 1.13    | 1.14    | 1.22    | 1.14      |
| Minimum Ratio    | 0.90  | 0.89    | 0.90    | 0.90    | 0.90    | 0.99    | 1.02    | 1.01    | 1.09    | 0.94      |
| Maximum Ratio    | 1.08  | 1.18    | 1.31    | 1.27    | 1.23    | 1.29    | 1.32    | 1.40    | 1.48    | 1.50      |

4. Discussion

Although the comparison experiments were performed by three-photon microscopy to reduce the out-of-focus background, the results also apply to two-photon microscopy. In order to put our results into proper context, we discuss several important considerations below, such as interaction between the dyes and the difference in the detection path between the two collection channels. We will then compare our experimental results with estimations obtained from the photon diffusion approximation.

4.1 Variations between imaging sessions

In order to calibrate the interaction between the dyes and investigate the difference in the detection path between the two collection channels, we must perform multiple imaging sessions on the same mouse. Therefore, we first calibrated the variations between different imaging sessions by performing three-photon imaging of the same dye in the same mouse brain repeatedly. The detection path including the detectors and the collection optics was kept the same for all the fluorescent dyes. For each dye, two imaging sessions were performed on the dye-labeled blood vessels and the normalized fluorescence signal is plotted as a function of depth for fluorescein excited at 1450 nm (Fig. 7(a)), Texas Red excited at 1450 nm (Fig. 7(b)), Texas Red excited at 1700 nm (Fig. 7(c)), and Alexa Fluor 647 excited at 1700 nm (Fig. 7(d)). We then calculated the difference between the two imaging sessions at each
depth. After averaging over all depths, we find a difference of 32%, 35%, 29% and 15% for the data presented in Fig. 7(a), 7(b), 7(c) and 7(d), respectively. The difference is mostly caused by the depth difference between the two imaging sessions since it’s difficult to match the imaging depth exactly in two sequential imaging sessions, especially with the 20 µm depth interval in our experiments. We note that such difficulties do not exist in our comparison experiments where simultaneous imaging of the two dyes was performed. These variations between the imaging sessions will be used as references in the following discussion.

### 4.2 Interaction between the dyes

Interaction between the dyes could be a concern, e.g., the emission of the bluer dye may potentially excite the redder dye. Therefore, we compared the imaging results with and without the presence of the second dye. To test whether the presence of Texas Red alters the signal attenuation of fluorescein in the mouse brain, we first imaged fluorescein. Then Texas Red was injected into the mouse, and fluorescein was imaged again with the presence of Texas Red. The results are shown in Fig. 8(a). We performed similar experiments for the dyes in all possible combinations, and the results are summarized in Fig. 8. With the same signal level at the brain surface (normalized to 1), the average difference for the fluorescein signal with and without the presence of Texas Red is 23% (Fig. 8(a)), which is comparable to the variations between imaging sessions discussed in Section 4.1. Similar results are obtained for all the other dye combinations (Fig. 8), which confirmed that the presence of a second dye in the simultaneous imaging experiments has negligible impact on the imaging of the first dye.
4.3 Difference between the collection channels

To verify that the difference in the collection path for the two channels in simultaneous imaging does not alter the results, we performed experiments by swapping the collection channels. For the comparison of fluorescein and Texas Red excited at 1450 nm, we first performed simultaneous three-photon imaging with channel 1 (ch 1) for fluorescein and channel 2 (ch 2) for Texas Red. We then repeated the imaging experiment by swapping the two channels, with channel 1 for Texas Red and channel 2 for fluorescein. The results are shown in Fig. 9a. We compared the signal level before and after channel swapping, and found that there is an average difference of 9% and 15% for fluorescein and Texas Red, respectively, which is within the variations between imaging sessions. We also performed the same channel-swapping experiments for the comparison of Texas Red and Alexa Fluor 647 excited at 1700 nm (Fig. 9b). The average signal difference before and after channel swapping is 21% for Texas Red and 8% for Alexa Fluor 647, which is again within the variations between imaging sessions. This set of experiments proved that the slight difference in the signal collection path between channel 1 and channel 2 does not introduce significant systematic bias in the comparison results.
4.4 Diffusion theory and Beer’s law predictions

Diffusion theory can be used to estimate the light transport in biological tissues based on the tissue optical properties, such as absorption coefficient ($\mu_a$), scattering coefficient ($\mu_s$), and anisotropy factor ($g$).

For the mouse brains in vivo, blood absorption dominates the absorbance at the wavelengths investigated in this paper. Oxy-hemoglobin (HbO$_2$) and deoxy-hemoglobin (HbR) are the main optical absorbers in biological tissue in the visible spectral range [20]. Based on the reported values for oxygen saturation (sO$_2$) measurements, we assume that all the hemoglobin in the arteries are HbO$_2$ while the veins contain 75% HbO$_2$ and 25% HbR [21–29]. Thus, the absorption coefficient for the veins $\mu_{vein}$ and the arteries $\mu_{artery}$ can be expressed based on the relative concentration of oxy- and deoxy-hemoglobin: $\mu_{vein} = 0.75 \mu_{HbO2} + 0.25 \mu_{HbR}$ and $\mu_{artery} = \mu_{HbO2}$. Assume that there are 70% veins and 30% arteries in the mouse brain vasculature in vivo [30], the effective blood absorption coefficient $\mu_b$ is $\mu_b = 0.7 \mu_{vein} + 0.3 \mu_{artery}$.

Calculated from the 2-dimensional three-photon images we took, we found that there is an average of 5% (standard deviation 0.5%, from all 17 mice) blood volume in the imaged brain regions. However, this likely over-estimates the blood volume concentration because the axial resolution (approximately 4 µm in our experiments [5]) is comparable to the size of the capillary vessels. Nevertheless, the small standard deviation indicates that the blood content for the mice we imaged is overall very consistent. On the other hand, previous measurements using high-resolution 3-dimensional reconstructions showed that the blood volume concentration in the same mouse brain region is approximately 3% [31]. Using 3% as the blood volume concentration, the effective absorption coefficient ($\mu_b$) can be estimated as: $\mu_b = 0.03 \mu_b = 0.03 \times (0.825 \mu_{HbO2} + 0.175 \mu_{HbR})$. The effective absorption length (1/$\mu_a$) is plotted in Fig. 10(a).

To estimate the scattering coefficient $\mu_s$ and the anisotropy factor $g$ in the brain tissue, Mie scattering is used for a tissue-like colloidal solution containing 1-µm diameter beads at a concentration of 5.4x10$^9$ beads/mL [32–34]. The scattering mean free path (MFP, $l_s = 1/\mu_s$) is also plotted in Fig. 10(a).

Radiative transfer equation (RTE) can be used to model the photon migration in biological tissues. The diffusion approximation of RTE provides an analytical solution for cases where the absorption of the tissue is negligible compared to the scattering, and the light sources and detectors are far apart so that the photon trajectories can be treated as diffuse. The transport MFP, defined by $l_t = l_s/(1-g)$, describes the average distance over which a photon loses the...
memory of its initial direction. When the imaging depth \((z)\) is much larger than the transport MFP of the emission photons \((z \gg \ell_t)\), the emitted photons are in the diffusive regime, and the diffusion approximation is well suited to model their propagation [35,36].

Solving the diffusion equation for the simple case of a time-independent point source in an infinite homogeneous medium, we can find the effective attenuation coefficient [37]:

\[
\mu_{\text{eff}} = \sqrt{3\mu_s[\mu_a + \mu_s(1-g)]}
\]

This simple model needs to be modified in order to apply to the visible spectral regions \textit{in vivo} where tissue absorption is not negligible. A modified solution of the diffusion equation with a weighted dependence on the absorption coefficient can be written as:

\[
\mu_{\text{eff}} = \sqrt{3\mu_s[\alpha\mu_a + \mu_s(1-g)]}
\]

Here we use \(\alpha = 0.5\) based on the calculations and experimental validations for a highly anisotropic scattering medium \((g \geq 0.8)\) [38–40]. Using Eq. (2), we plot the effective attenuation length \((l_e = 1/\mu_{\text{eff}})\) in the mouse brain \textit{in vivo} in Fig. 10(a).

The ratio of the normalized fluorescence signal from the same point source at depth \(z\) for two different emission wavelengths \(\lambda_1\) and \(\lambda_2\) is:

\[
\text{Ratio}(z) = e^{-\frac{z}{l_{e_{\lambda_1}}}}/e^{-\frac{z}{l_{e_{\lambda_2}}}} = e^{\frac{z}{l_{e_{\lambda_2}}} - \frac{z}{l_{e_{\lambda_1}}}}
\]

We calculated the transmission spectrum (Fig. 10(b)) of a point emitter at depths of 600 \(\mu\text{m}\), 800 \(\mu\text{m}\), 1 mm, and 1.6 mm in the mouse brain using Eq. (3). We find that the ratio for the emission between 615 nm and 520 nm is 1.72 at \(z = 600 \mu\text{m}\) and 2.07 at \(z = 800 \mu\text{m}\). The ratio for the emission between 711 nm and 615 nm is 1.16 at \(z = 800 \mu\text{m}\) and 1.21 at \(z = 1 \text{ mm}\).

As seen from Fig. 10, absorption by the brain tissue is relatively small at imaging depth < 1 mm for the green, red and NIR wavelength investigated. This is the main reason that the impact of the emission wavelength on multiphoton imaging is small. On the other hand, as we push the imaging depth further, the impact of the emission wavelength will become more important. For example, the difference between red and green emissions at 1.6 mm is \(\sim 4.27\).

The estimation using the diffusion approximation matches well with the experimental data for the red and NIR fluorophores. On the other hand, our experimental results showed that the difference in signal collection between green and red fluorescence is less than a factor of 1.5,
while the diffusion theory predicts the difference to be close to a factor of 2. The small discrepancies between experimental results and diffusion theory could be caused by several factors such as the difference in blood concentration. Table 3 summarizes our experimental data and the estimations using Eq. (3) at various blood volume concentrations. As the blood concentration is lowered, the experimental results and the estimations get closer.

Table 3. Summary of the experimental results and theoretical calculations with different blood concentrations.

| Data                      | ratio 615/520 at 600 μm | ratio 615/520 at 800 μm | ratio 711/615 at 800 μm | ratio 711/615 at 1 mm |
|---------------------------|-------------------------|------------------------|------------------------|-----------------------|
| Experimental results      | 1.34                    | 1.43                   | 1.14                   | 1.14                  |
| Estimation with 2% blood  | 1.53                    | 1.77                   | 1.13                   | 1.16                  |
| Estimation with 2.5% blood| 1.63                    | 1.92                   | 1.15                   | 1.19                  |
| Theory with 3% blood      | 1.72                    | 2.07                   | 1.16                   | 1.21                  |
| Estimation with 3.5% blood| 1.82                    | 2.22                   | 1.18                   | 1.22                  |
| Estimation with 4% blood  | 1.92                    | 2.38                   | 1.19                   | 1.24                  |

In this paper, we used narrow bandwidth filters to define the wavelength detected precisely. In practical fluorescence imaging, where broader bandwidth filters or even long-pass filters are usually used to improve the fluorescence collection efficiency. In order to evaluate the emission transmission efficiency under the practical fluorescence collection settings, we performed calculations by combining the fluorescence emission spectra (provided by Thermo Fisher Scientific) and the calculated tissue transmission spectrum in Fig. 10(b) with commonly used broad bandwidth (40 nm bandwidth) filters centered at 530 nm for fluorescein, 610 nm for Texas Red, and 660 nm for Alexa Fluor 647. We further performed calculations with no filters at all and allowed the entire emission spectrum to be detected. The calculated results are summarized in Table 4. Compared to the results in Table 3, the differences caused by the filter settings are small (< 10%).

Table 4. Summary of the fluorescence transmission ratios for fluorescein (FL), Texas Red (TR) and Alexa Fluor 647 (AF647) with 40-nm bandwidth filters and with no filters at all (full emission spectrum). The blood volume concentration is assumed to be 3%.

| Filter settings          | ratio TR/FL at 600 μm | ratio TR/FL at 800 μm | ratio AF647/TR at 800 μm | ratio AF647/TR at 1 mm |
|--------------------------|-----------------------|-----------------------|--------------------------|------------------------|
| Filters with 40 nm bandwidth | 1.83                  | 2.25                  | 1.02                     | 1.06                   |
| Full spectrum            | 1.85                  | 2.25                  | 1.13                     | 1.16                   |

Beer’s law is a more straightforward and accessible method compared to the diffusion theory in predicting the absorbance of photons especially for the high absorption regimes such as for the green fluorophores. Here we used Beer’s law to calculate the transmission spectra through various thickness of the brain tissue, assuming a uniform concentration of hemoglobin. To be consistent with the diffusion approximation, we used the same oxygen saturation value (82.5%) of the whole blood and the blood concentration (3%). Figure 11 shows the transmission spectra at depths of 600 μm, 800 μm, 1 mm, and 1.6 mm in the mouse brain. The ratio for the emission between 615 nm and 520 nm is 1.25 at z = 600 μm and 1.35 at z = 800 μm. The ratio for the emission between 711 nm and 615 nm is 1.02 at z = 800 μm and 1.03 at z = 1 mm. Figure 11 better predicts the shorter wavelength transmission ratio but
underestimates the measurement results in the longer wavelength regime. On the other hand, Fig. 11 significantly overestimates the absolute transmission through the brain tissue at all wavelengths by not accounting for tissue scattering (i.e., the zig-zagging photon path).

By comparing the fluorescence signals across the entire FOV, our results represent the average signal attenuation at various depths. Because of the heterogenous nature of the brain tissue, however, locally large variations of attenuation could occur. For example, fluorescence emitted directly below a large diameter blood vessel could have a much stronger dependence on the emission wavelength than those measured in this paper. To illustrate this argument, we show the transmission of fluorescence through a 50-μm-diameter vessel using Beer’s law in Fig. 11. The ratio for the transmission between 615 nm and 520 nm is 1.87, which is larger than what we measured at 0.8 mm deep. A possible example of this phenomenon was observed experimentally in a previous publication (Fig. 5 of ref. 11).

![Fig. 11. Beer’s law calculations of emission light transmission from a depth of 600 μm, 800 μm, 1 mm, and 1.6 mm as well as the emission light transmission through a 50-μm-diameter vessel.](image)

5. Conclusion

We systematically compared the emission attenuation of green, red and near-infrared fluorophores by three-photon imaging of the mouse brain in vivo. Based on the experimental results, we found that, within 1 mm depth in the mouse brain, the attenuation of the green fluorescence (~520 nm) is slightly higher than the red fluorescence (~615 nm) fluorescence, with the difference being 1.34-1.43 times for imaging depths of 0.6 to 0.8 mm. The attenuation of the near-infrared fluorescence (~711 nm) is comparable (within ~14%) to the red fluorescence. The reason for the small impact of emission wavelength on fluorescence attenuation with depth is that the collected fluorescence is contributed by both scattered and ballistic photons, whereas the signal generation within the focal volume is mainly contributed by ballistic photons. Our results show that the advantage of long wavelength dyes for multiphoton deep imaging is mainly due to the long excitation wavelengths, and the impact of emission wavelengths on multiphoton imaging depth is negligible for imaging within the superficial 1 mm of the mouse brain.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.

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