Multicolor three-photon fluorescence imaging with single-wavelength excitation deep in mouse brain

Yusaku Hontani1*, Fei Xia1,2, Chris Xu1*

Multiphoton fluorescence microscopy is a powerful technique for deep-tissue observation of living cells. In particular, three-photon microscopy is highly beneficial for deep-tissue imaging because of the long excitation wavelength and the high nonlinear confinement in living tissues. Because of the large spectral separation of fluorophores of different color, multicolor three-photon imaging typically requires multiple excitation wavelengths. Here, we report a new three-photon excitation scheme: excitation to a higher-energy electronic excited state instead of the conventional excitation to the lowest-energy excited state, enabling multicolor three-photon fluorescence imaging with deep-tissue penetration in the living mouse brain using single-wavelength excitation. We further demonstrate that our excitation method results in a 10-fold signal enhancement for some of the common red fluorescent molecules. The multicolor imaging capability and the possibility of enhanced three-photon excitation cross section will open new opportunities for life science applications of three-photon microscopy.

INTRODUCTION

Over the last several decades, multiphoton microscopy such as two-photon microscopy (2PM) and three-photon microscopy (3PM) has been a powerful tool in deep-tissue, live-cell structural and functional imaging (1–9). When compared to 2PM, 3PM has two advantages: (i) longer attenuation length in scattering tissue by using longer-wavelength excitation and (ii) tighter excitation confinement by using higher-order nonlinear excitation. As a result of these advantages, 3PM improves the penetration depth and the image contrast in deep-tissue imaging such as in the hippocampus of the mouse brain (3–8). The 1300- and 1700-nm excitations have been used for three-photon excitation (3PE) of green and red fluorophores, respectively (3, 4, 6, 10–12). While simultaneous deep-tissue observation of labeled cells of different colors is of great practical importance in life science, the large spectral separation for excitation of green and red fluorophores makes multicolor three-photon (3P) imaging challenging. Simultaneous dual-color 3P imaging of green and red fluorophore in the past required two different excitation wavelengths, 1300-nm 3PE for the green fluorophores and 1700-nm 3PE for the red fluorophores (12). However, the dual-wavelength excitation requires a special excitation source and a complex optical setup. For the same amount of fluorescence signal (e.g., green and red), excitation using two separate laser wavelengths will inevitably increase the total excitation power. Hence, multicolor 3P fluorescence microscopy using a single excitation wavelength is highly desirable for expanding 3PM applications in life science. In addition, 3P imaging suffers from the weak signal intensity because of the higher-order nonlinear excitation. With typical 2PE and 3PE cross sections, the pulse energy at the focus required for 3PM is approximately one order of magnitude higher than that in 2PM (13), which is one of the disadvantages of 3PM. Therefore, increasing the 3PE probability is of paramount importance.

Here, we report a new approach for multicolor 3P fluorescence imaging using a single excitation wavelength. For multiphoton microscopy, ~1300 and ~1700 nm are the best spectral windows for deep-tissue penetration due to their long attenuation lengths in scattering tissues (3, 14). In 3PM, the 1300- and 1700-nm spectral windows are typically used for green and red fluorescent molecules, respectively, to excite the molecules to the lowest electronic excited state (14–17). Although it is commonly believed that the 3PE spectrum of a fluorophore will be similar to its one-photon excitation (1PE) spectrum, when scaled to the same transition energy, we show here that the peak of the 3PE spectrum can be blue-shifted relative to the peak of the 1PE spectrum (with the 1PE wavelength scaled by 3×) by hundreds of nanometers, allowing simultaneous excitation of green and red fluorophores using a single wavelength. The blue-shifted excitation for red fluorescent molecules drives the molecules to a higher-energy state via the 3PE process. We report that this excitation method enables simultaneous imaging of commonly used green and red fluorescent molecules using a single wavelength at the 1300-nm spectral window while fully preserving the deep imaging capability of long-wavelength 3PM. Furthermore, we demonstrate that the blue-shifted 3P cross sections in the 1300-nm window are more than 10 times larger than those at the 1700-nm window for some of the widely used red fluorophores, which greatly enhances the signal strength in 3PM.

RESULTS

3P cross sections with excitation to a higher-energy excited state

We first investigated two-photon (2P) and 3P cross sections of red fluorescent molecules: organic dyes Texas Red (or sulforhodamine 101 acid chloride) (18), sulforhodamine 101 (SR 101), and Alexa Fluor 546 (19); fluorescent proteins DsRed (20), tdTomato, and mCherry (21); and a quantum dot, Qdot 605, upon excitation at 1220 to 1360 nm and 1600 to 1700 nm. Figure 1 shows 2P (top panels) and 3P (bottom panels) action cross sections, together with their one-photon (1P) absorption spectra. Figure S1A shows the experimental setup to obtain the 2P and 3P action cross sections of fluorophores in solution, which is similar to that described in a previous publication (15). For Texas Red, a common red fluorescent molecule for labeling various proteins (18) or serving as a fluorescent tracer for blood plasma (22), the 1P absorption peak is at ~590 nm (excitation to the lowest-energy excited state). Some shorter-wavelength absorption bands (excitation to higher-energy excited states) are also present in

1School of Applied and Engineering Physics, Cornell University, Ithaca, NY 14853, USA.
2Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY 14853, USA.
*Corresponding author. Email: yusaku.hontani@cornell.edu (Y.H.); cx10@cornell.edu (C.X.)
Table 1. Slopes in the logarithmic plot of fluorescence signal for Texas Red versus incident intensity at selected wavelengths with low-NA (NA = 0.26) and high-NA (NA ~ 0.8) excitation at the pulse energy indicated in the experiments (fig. S2). All calculations were based on the 2P and 3P cross sections shown in Fig. 1, assuming a pulse duration (full width at half maximum) of 60 fs.

<table>
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<th>Excitation wavelength</th>
<th>1220 nm</th>
<th>1240 nm</th>
<th>1260 nm</th>
<th>1280 nm</th>
<th>1300 nm</th>
<th>1320 nm</th>
<th>1340 nm</th>
<th>1360 nm</th>
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<tr>
<td>Low NA (experiment)</td>
<td>2.02 ± 0.01</td>
<td>2.02 ± 0.01</td>
<td>2.16 ± 0.05</td>
<td>2.43 ± 0.03</td>
<td>2.56 ± 0.02</td>
<td>2.74 ± 0.01</td>
<td>2.85 ± 0.02</td>
<td>3.07 ± 0.08</td>
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<td>Low NA (calculation)</td>
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<td>–</td>
<td>2.16</td>
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<td>2.70</td>
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<tr>
<td>High NA (experiment)</td>
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<td>1.98 ± 0.01</td>
<td>2.52 ± 0.01</td>
<td>2.71 ± 0.01</td>
<td>–</td>
<td>–</td>
<td>2.96 ± 0.02</td>
<td>–</td>
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<tr>
<td>High NA (calculation)</td>
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<td>2.75</td>
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<td>2.98</td>
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Fig. 1. 1P absorption spectra and 2P and 3P action cross sections of Texas Red, SR 101, Alexa Fluor 546, DsRed, tdTomato, mCherry, and Qdot 605 in solution. Top: 2P action cross section (green plots) overlapped with 1P absorbance (black lines). Bottom: 3P action cross section (magenta plots) overlapped with 1P absorbance (black lines). The 1P absorbance is normalized to the absorption peak at 550 to 590 nm. The lines are used to connect the measurement data points. The orange lines in the 3P cross-section panels indicate $1 \times 10^{-82}$ cm$^2$ (s/photons)$^2$. The 1P absorption spectra of DsRed, tdTomato, mCherry, and Qdot 605 are regenerated from http://spectra.arizona.edu/
the 1P absorption spectrum, e.g., at ~430 nm. As seen in the top panels of Fig. 1, the 1220- to 1360-nm band overlaps with the long-wavelength tail of 2PE to the lowest-energy excited state (23), while large 3P cross sections were detected at 1260 to 1360 nm (Fig. 1, bottom panels). At some excitation wavelengths, e.g., at 1220 and 1240 nm for Texas Red, no 3PE contribution was observed under our experimental condition. 3PE at ≤1360 nm is likely the result of excitation to the higher-energy bands indicated in the 1P absorption spectra, e.g., at ~430 nm for Texas Red. On the other hand, 3PE at 1650 and 1700 nm excites the fluorophores to the lowest-energy excited state shown in the 1P absorption spectrum, e.g., at ~430 nm. As seen in the top panels of Fig. 1, the 1220- to 1360-nm band overlaps with the long-wavelength tail of 2PE to the lowest-energy excited state. We studied this transition in detail for Texas Red. For each excitation wavelength, fluorescence signal as a function of excitation power was measured with a low–numerical aperture (NA) objective lens (NA = 0.26) (fig. S2).

Figure S3A shows a logarithmic plot of fluorescence signal of Texas Red dextran (70,000 MW) was injected to a wild-type mouse (C57BL/6J, female, 13 weeks old, 22 g, under anesthesia with 1.0 to 1.5% isoflurane in oxygen) to label the blood vessels. The images were obtained with an FOV of 270 μm × 270 μm (512 pixels × 512 pixels) at (A) 700-μm and (B) 1150-μm depth from the brain surface. The pulse energy at the focus was ~0.4 nJ (1220 nm), ~0.6 nJ (1260 nm), and ~1.0 nJ (1300 to 1650 nm). The repetition rate was 330 kHz. For the images obtained with 1220- to 1340-nm and 1650-nm excitation, 10 and 50 frames were averaged, respectively (with a frame rate of 1.0 Hz and a pixel dwell time of 3.1 μs). (C) and (D) are fluorescence intensity profiles along the yellow lines in (A) and (B), respectively. (E) SBR at 1150-μm depth as a function of excitation wavelength. Background is calculated by averaging the pixel values between 0 and 15 μm and between 35 and 50 μm in the line profile plots in (D). For the total fluorescence, the maximum pixel values of the line profiles were used. The SBRs were calculated as (total fluorescence – background)/background. The line is used to connect the measurement data points. Scale bars, 50 μm.
Red versus excitation power obtained with 1300-nm excitation and fitted with power functions. In the low-power region, the slope of the power-law fit was 2.03 ± 0.05, while in the high-power region, the slope was 2.56 ± 0.02. This observation indicates that 2PE is dominant in the lower-power region, while 2P and 3P fluorescence signals are mixed when excited in the higher-power region. At the short wavelengths of 1220 and 1240 nm for Texas Red, pure 2P excited fluorescence is observed even with high-NA excitation (NA ~ 0.8) used in the 3PM setup (fig. S4); at the long wavelength of 1340 nm, on the other hand, 3P excited fluorescence dominates under the experimental conditions typically used for 3PM. More details of the 2PE-to-3PE transition are illustrated in Table 1.

Comparison of mouse brain imaging at different wavelengths

We compared the in vivo imaging performance with 2PE at 1220 nm, mixed 2PE and 3PE at 1260 and 1300 nm, blue-shifted 3PE at 1340 nm, and conventional 3PE at 1650 nm by imaging Texas Red–labeled blood vessels in the same mouse brain and within the same field of view (FOV). Figure 2 shows blood vessel images at 700 and 1150 µm beneath the dura of the mouse brain using the various excitation wavelengths. At 700-µm depth, which is above the white matter of the brain, images of all excitation wavelengths showed a high signal-to-background ratio (SBR). In contrast, at 1150-µm depth, which is in the hippocampus below the white matter, large difference in SBR

![Graphs and images showing absorption, emission, and transmittance spectra for GCaMP6s and Texas Red](https://searchlight.semrock.com/)

Fig. 3. Multicolor 3P fluorescence imaging of GCaMP6s-labeled neurons (green), Texas Red–labeled blood vessels (red), and THG (blue) upon 1340-nm excitation. (A) 1P absorption spectra of GCaMP6s and Texas Red, and the excitation laser spectrum centered at 1340 nm. (B) Emission spectra of GCaMP6s and Texas Red, and THG spectrum calculated from the excitation laser spectrum, and transmittance of bandpass filters used for detection. The absorption, emission, and transmittance spectra are obtained from https://searchlight.semrock.com/. (C) Z-stack images down to 1200 µm from the brain surface with an FOV of 270 µm × 270 µm (512 pixels × 512 pixels per frame). The images were taken with 4-µm z-steps, and the GCaMP6s signals are shown down to 1048 µm. Above 844 µm, the laser repetition rate was set at 2 MHz, and 20 images (1.0-Hz frame rate) were averaged for each depth. Between 848 and 1200 µm, the laser repetition rate was set at 330 kHz, and 40 images (1.0-Hz frame rate) were averaged for each depth. The pixel dwell time was 3.1 µs. The laser power was varied according to the imaging depths to maintain ~1.5-nJ pulse energy at the focus. Maximum average power under the objective was 70 mW. (D) Selected 2D images at various imaging depths. (E) Activity recording site at 750 µm beneath the dura with an FOV of 270 µm × 270 µm (256 pixels × 256 pixels). (F) Spontaneous brain activity traces recorded in an awake mouse from the labeled neurons indicated in (E). The entire recording session is shown in movie S2. Scale bars, 50 µm.
was seen at different excitation wavelengths. The measured SBR at 1150-μm depth was 0.77, 1.4, and 12 for excitation at 1220, 1260, and 1300 nm, respectively. Upon excitation at 1340 and 1650 nm, the SBR at 1150-μm depth was 47 and 16, respectively. This result shows that 1340-nm 3PE provides ~60× larger SBR than 1220-nm 2PE at 1150-μm depth. The SBR at 1340 nm is comparable to previously reported SBR of 3PM of Texas Red–labeled mouse brain vasculature with ~1700-nm excitation at a similar depth (3). The smaller SBR at 1150-μm depth observed here with 1650-nm excitation is due to the small signal intensity, as indicated by the nearly identical baseline levels at 700- and 1150-μm depths (Fig. 2, C and D, black lines). The large SBR difference for the images obtained at the various wavelengths (Fig. 2) is due to the different 2PE contribution at these excitation wavelengths as shown in Fig. 1 and Table 1.

Figure S5 shows image comparison at 50-μm depth upon 1340- and 1650-nm excitation with the same excitation power (~1.0-nJ pulse energy at the focus), demonstrating that 1340-nm excitation gives ~14-fold stronger signal than 1650 nm in vivo, which is in a close agreement to the small signal intensity, as indicated by the nearly identical baseline levels at 700- and 1150-μm depths (Fig. 2, C and D, black lines). The large SBR difference for the images obtained at the various wavelengths (Fig. 2) is due to the different 2PE contribution at these excitation wavelengths as shown in Fig. 1 and Table 1.

**Fig. 4. Multicolor 3P fluorescence images of fluorescent proteins in a PrismPlus transgenic mouse upon 1340-nm excitation.** (A) 1P absorption spectra of the four fluorescent proteins and the excitation laser spectrum centered at 1340 nm. (B) Emission spectra of the four fluorescent proteins, a THG spectrum calculated from the excitation laser spectrum, and transmittance of bandpass filters used for detection. The absorption, emission, and transmittance are obtained from https://searchlight.semrock.com/. (C) Z-stack images down to 1100 μm from the brain surface with an FOV of 260 μm × 260 μm (512 pixels × 512 pixels per frame). Blue, THG; cyan, Cerulean; yellow, Cerulean; EGFP, and YFP; red, DsRed-Max. The excitation wavelength is 1340 nm, and the excitation power was varied according to the imaging depth to maintain ~2.5-nJ pulse energy at the focus. The maximum laser power was ~100 mW under the objective lens. The images were taken with 4-μm z-steps. Between 0 and 800 μm, the laser repetition rate was 2 MHz, and 25 images (1.0-Hz frame rate) were averaged for each depth. Between 800 and 1100 μm, the laser repetition rate was 330 kHz, and 50 images (1.0-Hz frame rate) were averaged for each depth. The pixel dwell time was 3.1 μs. (D) Separate z-stack images of (A) for the four detection channels. (E) Selected images at different depths. Scale bars, 50 μm.
with the result measured in solution (Fig. 1). These observations indicate that 1340-nm excitation of Texas Red provides enhanced 3P fluorescence signal while preserving the high SBR for deep imaging of the mouse brain. Similar data were obtained in two additional mice (i.e., n = 3 in total).

To further investigate the wavelength-dependent SBR, we calculated the SBRs on the basis of the 2P and 3P cross sections in Fig. 1 (see note S1 for details of the SBR calculation). Figure S6A shows the calculated ratios of the SBR (SBRtotal) for a mixture of 2P and 3P fluorescence to the SBR of pure 2PE (SBR2P) when imaging Texas Red with a mixture of 2P and 3P fluorescence, assuming that the signals from the focal volume are generated by the ballistic excitation photons. SBRtotal/SBR2P shows the SBR improvement resulting from the 3PE contribution. Because the background for 3PE is negligible at these imaging depths (3, 25), the background contribution is entirely from 2PE when a mixture of 2PE and 3PE exists. The calculated SBR upon 1340-nm excitation is 4.6×, 15×, and 82× larger than that at 1300, 1260, and 1220 nm (pure 2PE), respectively, because of the smaller contribution from 2PE at 1340 nm (fig. S6B). In the experiment at 1150-μm beneath the dura (Fig. 2, B, D, and E), the SBR upon 1340-nm excitation was 3.9×, 34×, and 61× larger than that at 1300, 1260, and 1220 nm, respectively, showing a trend similar to the calculation (fig. S6B). In fig. S7, SBRtotal/SBR2P is plotted for the other red fluorophores. For all the red fluorophores investigated, our calculations show that 3P imaging in the 1300-nm window provides >40× SBR improvement over 2P imaging at 1220 nm (figs. S6 and S7), indicating that the advantage of the high SBR for 3PM is largely preserved when exciting these red fluorophores within the 1300-nm window.

Multicolor fluorescence imaging and activity recording in the mouse brain with single-wavelength excitation
Combining 3PE to a higher-energy band for red fluorophores with 3PE to the lowest-energy band for green fluorophores, we demonstrate simultaneous multicolor 3PM using a single-wavelength excitation at 1340 nm.

Figure 3 shows multicolor mouse brain images of GCaMP6s-labeled neurons (26), Texas Red–labeled blood vessels, and third-harmonic generation (THG) obtained using a single excitation wavelength at 1340 nm. THG is mainly generated from the red blood cells and myelin (27, 28). The corresponding three-dimensional (3D) images are shown in fig. S8. As seen in Fig. 3 (C and D), high-contrast images of GCaMP6s, Texas Red, and THG are simultaneously captured up to 1200 μm deep in the mouse brain (z-stack images are shown in movie S1). Figure 3 (E and F) shows Ca2+ activity traces at 750-μm depth (the entire recording session is shown in movie S2).

Multicolor 3PM can also be applied to other combinations of fluorescent molecules upon 1340-nm excitation, even for fluorescent molecules that do not exhibit large cross-section enhancement. Figure 4 shows multicolor brain imaging of a PrismPlus mouse (29) that expresses cyan fluorescent protein (CFP; Cerulean) in oligodendrocytes, enhanced green fluorescent protein (EGFP) in microglia, yellow fluorescent protein (YFP) in neurons, and DsRed-Max in astrocytes, using a single excitation wavelength at 1340 nm. DsRed-Max is a less cytotoxic variant of DsRed (30). Figure 4 (C and D) shows 3D images down to 1100 μm beneath the dura. Selected images at different depths are shown in Fig. 4E. For the multicolor detection, we used four photomultiplier tube (PMT) channels: 445-nm channel for THG; 485-nm channel for Cerulean fluorescence; 525-nm channel for the mixed fluorescence from Cerulean, EGFP, and YFP; and 617-nm channel for DsRed-Max. The mixed fluorescence signal in the 525-nm channel is due to the large spectral overlap of fluorescence emission of Cerulean, EGFP, and YFP (see the emission spectra of the various fluorescent proteins in Fig. 4B).

In Fig. S9A, selected multicolor images of GCaMP6s-labeled neurons, SR 101–labeled astrocytes, and THG are shown. Ca2+ activity and astrocyte images at 762-μm depth beneath the dura are shown in fig. S9 (B and C) and movie S3. In fig. S10A, multicolor images of fluorescence-labeled blood vessels, SR 101–labeled astrocytes (23), and THG are shown. Figure S10B shows fluorescence intensity line profiles of SR 101 and fluorescence-labeled blood vessels at 250- and 800-μm depths. In the SR 101 channel, large variations in the “baselines” outside the astrocytes are seen. In particular, the lowest pixel values of the SR 101 channel are the same as those in the fluorescent channel, which shows that the nonstained areas remain background free. In addition, this elevated “baseline” (i.e., the hazy appearance) in the SR 101 channel appears to be the same regardless of the imaging depth (e.g., at 800 μm and at 250 μm), which is inconsistent with the behavior of out-of-focus fluorescence excitation (e.g., the SBR decreases with imaging depth). Therefore, we conclude that the elevated background outside the astrocytes in the SR 101 images is caused by nonspecific staining rather than out-of-focus fluorescence excitation.

With all the labeling combinations, high-contrast, multicolor 3P images were successfully obtained over a large depth using single-wavelength excitation at 1340 nm. These results indicate that deep-tissue, multicolor 3PM of multiple targets, such as neurons, blood vessels, astrocytes, and myelin, can be obtained simultaneously using a single excitation wavelength without any elaborate optical setup.

Photobleaching comparison of SR 101 in the mouse brain with 1340- and 1650-nm excitation
We performed photobleaching measurements of SR 101 (labeling astrocytes) at ~50 to 60 μm beneath the dura in vivo in the mouse brains to compare the photobleaching properties with 1340- and 1650-nm excitation (Fig. 5 and fig. S11). Because the area of the focus is proportional to the excitation wavelength squared, we used 1.5× higher pulse energy at 1650 nm to maintain approximately the same excitation intensity at the focus. Fig. 5 (B and C) shows time traces of SR 101 fluorescence in five different astrocytes with 1340- and 1650-nm excitation. The fluorescence time traces were fitted with a single exponential function. To compare the photostability of SR 101 with 1340- and 1650-nm excitation, relative photobleaching quantum yields were calculated from the experimental data (Fig. 5D; see note S2 for the analysis). As seen in Fig. 5D, the photobleaching quantum yield of SR 101 at 1340 nm is less than that at 1650 nm in all the astrocytes measured. Figure S11 shows another set of measurements of the photobleaching properties at 1340 and 1650 nm. Although the photobleaching data differ somewhat in different cells, SR 101 is noticeably more photo-stable with 1340-nm excitation than with 1650-nm excitation in all datasets. There are a number of possible pathways for photobleaching (31), and photobleaching properties are likely different for different fluorophores (32). While our data show that the blue-shifted 3PE of SR 101 at the 1300-nm window is more photo-stable than 3PE at 1650 nm, further investigation is needed for other fluorophores and fluorescent proteins to compare their photobleaching properties at these two spectral windows. Data collected in these photobleaching measurements also allowed us to compare the fluorescence signal intensity in vivo for
SR 101. Similar to fig. S5, fig. S12 shows pixel intensity histograms in the images shown in fig. S11A at time zero (i.e., before any photobleaching) upon 1340-nm (1.3 nJ) and 1650-nm (2.0 nJ) excitation. 3PE at 1340 nm gives ~3.7-fold stronger signal than 3PE at 1650 nm in vivo, which is in close agreement with the result measured in solution (Fig. 1) when the differences in excitation power, pulse durations, and tissue attenuation lengths are taken into account (see the figure caption of fig. S12 for details).

**DISCUSSION**

We demonstrated large 3P cross sections of red fluorophores upon excitation to a higher-energy excited state (3PE at ~1300 nm) instead of the conventional 3PE to the lowest-energy excited state (3PE at ~1700 nm) (Fig. 1). Using Texas Red, SR 101, and DsRed-Max, together with cyan (CFP) and green fluorophores (EGFP, GCaMP6s, and fluorescein), we showed high-contrast multicolor 3P images in deep brain using 1340-nm excitation (Figs. 3 and 4 and figs. S8 to
Because the 3PE wavelengths reside within the 1300-nm spectral window, the deep-tissue penetration capability of long-wavelength 3PM is preserved. Enhanced 3P cross sections (>10×) were observed at the 1300-nm window for some of the red fluorophores when compared to excitation at the 1700-nm window. The 3P action cross section of mCherry at 1340 nm is ~1.9 × 10^{-48} cm^6/(s/photons)^2, which is the smallest among the red fluorophores studied here. However, the value of the 3P action cross section of mCherry is still larger than that of fluorescein (15) at 1300 nm, and it is two times smaller than that of DsRed at 1340 nm and three times smaller than that of Texas Red at ~1700 nm (Fig. 1). Both fluorescein and Texas Red have been used routinely in the past for 3PM (3–5), and we showed high-contrast deep-tissue 3PM of DsRed-labeled cells at 1340 nm (Fig. 4). Therefore, all the red fluorophores studied here have sufficiently large 3P cross sections at ~1300 nm for 3PM.

A sharp focus in 3D space is essential for high-contrast imaging in deep tissue. Long-wavelength 3PM is advantageous compared to conventional 2PE for deep-tissue imaging because the longer wavelength excitation reduces the effects of tissue scattering and the higher-order nonlinear excitation increases the spatial confinement of the excitation. An important metric for quantifying the contrast of the images in deep tissue is the SBR. As shown in note S1, a mixture of 2PE and 3PE fluorescence improves the SBR when compared to pure 2PE. The SBR improvement is directly proportional to the ratio of 3PE to 2PE signal strength, which is dependent on the 2PE and 3PE cross sections and the peak intensity of the excitation beam (eq. S8 in note S1). One can increase or decrease the relative contributions of 2PE and 3PE by choosing the excitation wavelength, which determines the values of the 2PE and 3PE cross sections, and by varying the pulse width, repetition rate, and focal spot size (i.e., NA of the focusing beam). To increase the 3PE contribution to maximize the SBR, longer wavelength and higher peak intensity are desirable.

For the selection of the optimum imaging wavelength, the signal intensity, which depends on the 2P and 3P cross sections, should be considered in addition to the SBR. For Ca^{2+} indicators, consideration of ΔF/F is also important. For example, the ΔF/F of GCaMP6s under 3PE at 1350 nm is nearly 5× that at 1250 nm (33). Therefore, excitation at 1340 nm as shown here is a good choice for dual-color 3P imaging of GCaMP6s and Texas Red to achieve the combination of high signal, SBR, and ΔF/F.

Our results show that the 1P absorption spectra cannot reliably predict the 3P cross sections of fluorophores. For Texas Red, SR 101, Alexa Fluor 546, and DsRed, the 3P cross sections in the 1300-nm window are larger than those in the 1700-nm window, although their 1P absorptions at ~430 nm are much smaller than those at ~550 to 590 nm (Fig. 1). These enhanced 3P cross sections at the shorter wavelengths likely result from the resonance enhancement effect. As seen in Fig. 1, 3PE at 1260 to 1360 nm overlaps with the long-wavelength tails of 2PE of the red fluorophores. When the combined energy of two excitation photons (2ħω) is close to the energy difference (ΔE) between the lowest excited state and the ground state of the fluorophore, i.e., ΔE ~ 2ħω, the quantum perturbation theory shows that the 3P cross sections are possibly enhanced (i.e., resonance enhancement) (34). This resonance-enhanced 3PE is analogous to the reported resonance-enhanced 2PE (35–38). On the other hand, large enhancements of 3P cross sections were only observed in some of the red fluorophores that we measured. While the precise mechanism requires further investigation, the results of this paper suggest using the following two guidelines for finding fluorophores with large 3P cross sections: (i) using excitation wavelengths that are close to the long-wavelength edge of conventional 2PE and (ii) using fluorophores with a local 1P absorption maximum at about one-third of the excitation wavelength. The first condition is necessary for achieving 2P resonance enhancement. The second one is based on the empirical observations of the experimental data. Fluorophores such as Texas Red and SR 101 have a local 1P absorption maximum at 420 to 450 nm, which may indicate an energy level that is excitable by 3P at 1260 to 1360 nm. These fluorophores showed large, enhanced 3P cross sections at 1260 to 1360 nm. On the other hand, mCherry, which does not show large 3P cross sections at 1220 to 1360 nm, has a local 1P absorption minimum at 420 to 450 nm. These observations may suggest that both resonance enhancement and the local 1P absorption maximum at 420 to 450 nm are features in the red fluorophores to attain large 3P cross sections at ~1300 nm. Similar situations were observed for blue-shifted 2PE in the past (39). To fully explore the opportunities presented by the blue-shifted 3PE, systematic studies of 3PE cross sections of a large number of fluorophores and over a large spectral range must be performed.

The method presented here opens up the opportunity of multicolor 3PM with a single excitation wavelength, which will enable real-time observation of molecular and cellular interactions in the brain and other organs over a large depth in vivo. The new excitation scheme does not need any modification of the 3PM setup. Therefore, multicolor 3P imaging can be immediately applied in any laboratory that has a 3PM setup. Furthermore, large enhancement in 3PE cross sections was found in some of the red fluorescent molecules when excited within the wavelength window of ~1300 nm. While efforts in the last two to three decades have shown that it is extremely challenging to create new fluorophores with extraordinarily large 3PE cross sections for in vivo imaging, our discovery may indicate a fruitful path for finding large 3PE cross sections from existing fluorophores that are commonly used for imaging living tissues. Weak fluorescence signal strength is the greatest difficulty for deep-tissue imaging. Large 3P cross sections will enhance the signal for 3PM and will immediately enable 3PMs to image faster and deeper in scattering tissues.

**MATERIALS AND METHODS**

**Sample preparation for 2P and 3P cross-section measurements**

Fluorescent dyes and proteins were prepared in phosphate-buffered saline (pH 7.4) with the final concentrations of 42.2 μM for Texas Red (sulforhodamine 101 acid chloride, 82354-19-6, Sigma-Aldrich), 50.0 μM for sulforhodamine 101 (SR 101) (60311-02-6, Sigma-Aldrich), 108 μM for Alexa Fluor 546 NHS Ester (A20002, Thermo Fisher Scientific), 44.6 μM for DsRed (rDsRed-monomer protein, 632503, Takara Bio, USA), 11.4 μM for tdTomato (tdTomato-19, Creative BioMart), and 44.8 μM for mCherry (4993, BioVision). The concentrations were calculated from the absorbance measured using an ultraviolet-visible spectrometer (Cary 300 Bio, Agilent) and the known extinction coefficients of the dyes and proteins. For quantum dot Qdot 605 (Q21701MP, Thermo Fisher Scientific), 1.0 μM solution in decane was used. For Texas Red, SR 101, Alexa Fluor 546, and Qdot 605, the sample solution filled the 600- to 800-μm-thick cavity of a glass slide (MS15C1 Thorlabs), which was then
sealed by a cover glass using nail polish. For DsRed, tdTomato, and mCherry, a 120-μm sample spacer was used to hold the solution on a glass slide.

**Laser source**
A noncollinear optical parametric amplifier (Spirit NOPA, Spectra Physics) pumped by a hybrid fiber–solid-state laser (Spirit 1030-70, Spectra Physics) was used as the excitation source. The repetition rate was set at 2 MHz or 330 kHz. The wavelength was varied in the range of 1220 to 1360 and 1600 to 1700 nm (see fig. S13 for the spectra of the laser output). After passing through a prism-pair compressor (for 1220 to 1360 nm) or a 3-mm-thick silicon wafer at Brewster’s angle (for 1600 to 1700 nm) for dispersion precompensation, the laser was focused on the sample with an objective lens. To control the excitation power, a half wave plate and a polarization beamsplitter were used.

**Action cross-section experiment**
Figure S1A shows the setup for the action cross-section measurement, which is similar to that described in a previous publication (15). The laser beam size was expanded to ~40 mm (1/e²) to overfill the back aperture of the objective lens (UPLFLN 0.3 NA/10×, Olympus). Therefore, approximately diffraction-limited focus was achieved in the sample. The repetition rate of the laser was set at 2 MHz, and the excitation wavelengths were varied in the range of 1220 to 1360 nm and 1600 to 1700 nm. The excitation beam was focused at ~50 μm from the sample surface. The NA of the objective lens was 0.26, measured using the knife-edge method at 1300 nm. This value was comparable to that reported previously (15). After a 757-nm longpass dichroic mirror (FF757-Di01, Semrock) and a set of proper emission filters (FESH0700, FESH0800, and FESH0900, Thorlabs; FF02-617/73 or FF01-607/70, Semrock), the generated fluorescence was detected with a PMT (HC125-2, Hamamatsu) and recorded with a photon counter (SR400, Stanford Research Systems). We used an integration time of 30 s for each measurement. The excitation power was recorded with a power meter (S122C and PM100D, Thorlabs) after the sample and without the sample. The effective excitation power at the focus was calculated considering the light reflections at the various interfaces and the absorption by water. For each excitation wavelength, the excitation power was varied to study the dependence of the fluorescence intensity on the time-averaged excitation photon flux $〈 P(t) 〉$ (photons/s). Time-averaged fluorescence photon fluxes under 2PE and 3PE, $S_{2P}$ and $S_{3P}$, respectively, are described as below (24, 39)

$$S_{2P} = \frac{1}{2} \frac{g^{(2)}_F}{\tau} \phi \sigma_n c n_0 \frac{a_2(\lambda)}{8 \pi \lambda} 〈 P(t) 〉^2$$

$$S_{3P} = \frac{1}{3} \frac{g^{(3)}_F}{\tau} \phi \sigma_n c n_0 \frac{a_3(\lambda)}{8 \pi \lambda} 〈 P(t) 〉^3$$

where $\tau$ is the laser pulse duration, $\phi$ is the system collection efficiency, $\eta$ is the fluorescence quantum efficiency, $c$ is the sample concentration, $n_0$ is the refractive index of the sample medium (i.e., water or decane), $\lambda$ is the excitation wavelength in vacuum, NA is the numerical aperture of the objective lens, and $a_2 = 64, a_3 = 28.1$. $g^{(n)}_F$ is the $n$th-order temporal coherence of the excitation source, and we used $g^{(2)}_F = 0.587$ and $g^{(3)}_F = 0.413$, assuming hyperbolic-secant-squared pulse intensity profile. The collection efficiency was calculated considering the reflection by the cover glass, the fluorescence collection by the objective lens, transmittance of the objective lens, reflectivity of the dichroic mirror, the transmittance of the filters, and the quantum efficiency of the PMT. The measured fluorescence signals at various excitation power levels were fitted by a polynomial function, $A_2 〈 P(t) 〉^2 + A_3 〈 P(t) 〉^3$. Using the known and calculated parameters in Eqs. 1 and 2, $\sigma_2$ and $\sigma_3$, which are 2P and 3P action cross sections, respectively, were calculated from the fitting parameters $A_2$ and $A_3$. In the cross-section measurements, the uncertainty is estimated to be about 30% of the mean value mainly due to the uncertainty in the determination of the collection efficiency of the system. The uncertainty is similar to that reported in previous cross-section work (15, 39, 40).

**3PM setup**
The multiphoton images were acquired with a commercially available microscope (Bergamo II, Thorlabs) that has four detection channels with GaAsP PMTs (PMT2101, Thorlabs) (fig. S1B). A high-NA objective lens (XPLN25XWMP2, Olympus, NA = 1.05) was used with D₂O as the immersion medium. The diameters of the beams at the objective lens back aperture (aperture size, ~15 mm) were ~13 mm (1/e²). The back aperture of the objective is underfilled, and the effective NA was estimated to be ~0.8. The signal was epi-collected through the objective lens and then reflected by a 705-nm longpass dichroic beam splitter. For all images except for Fig. 4, the signals were separated into four detection channels by the 562-, 488-, and 635-nm (FF562-DI03, Di02-R488, and Di02-R635, Semrock) dichroic beam splitters, and bandpass filters and a longpass filter were used in front of each PMT. The bandpass filters are centered at 447 nm (FF02-447/60, Semrock), 525 nm (FF03-525/50, Semrock), and 617 nm (FF02-617/73, Semrock), and the cutoff wavelength of the longpass filter is 647 nm (BL01-647R, Semrock). The 447-nm channel was used to detect the THG of 1340-nm excitation; the 525-nm channel was used to detect fluorescence from the GCaMP6s and fluorescein; and the 617-nm channel was used to detect fluorescence from Texas Red and SR 101. For Fig. 5 and fig. S11, the >647-nm channel was used to detect fluorescence from SR 101. For Fig. 4, a different filter set, which consists of three dichroic beam splitters (Di02-R488, FF458-DI02, and FF562-DI03, Semrock) and four bandpass filters (FF01-445/20, FF02-485/20, FF03-525/50, and FF02-617/73, Semrock), was used to detect the THG and fluorescence by four PMT channels. The 445-nm channel detected THG of 1340-nm excitation; the 485-nm channel detected cyan fluorescence from Cerulean; the 525-nm channel detected a mixture of fluorescence from Cerulean, EGFP, and YFP; the 617-nm channel detected fluorescence from DsRed-Max. To prevent thermal damage, we used 100 mW or lower average power at ~1300 nm for all the images (13).

**Animal procedure**
All animal experimentation and housing procedures were conducted in accordance with the Cornell University Institutional Animal Care and Use Committee guidance. Craniotomy was performed before the mouse brain imaging. Procedure for mouse brain craniotomy followed closely to what was described previously (4). The craniotomy was centered at ~2.5 mm lateral and ~2 mm caudal from the bregma point over the somatosensory cortex. For Fig. 3, we crossed Cre-lox transgenic mice [Slc17a7-IRES2-Cre (B6;129S-Slc17a7tm1.1(cre)Hze/J],

transgenic mouse expresses CFP (Cerulean) in oligodendrocytes, of Texas Red or fluorescein [25 mg/ml; dextran conjugate, 70,000 molecular weight (MW), Thermo Fisher Scientific] was injected retro-orbitally before imaging. For Fig. 4, a PrismPlus transgenic mouse [Cx3cr1milItgP;Prism]1989Hz/J, JAX stock number 031478 (29), female, 8 weeks old, 19 g was used. The PrismPlus transgenic mouse expresses CFP (Cerulean) in oligodendrocytes, EGFP in immune cells including microglia, YFP in nucleolus and cytoplasm of neuronal soma, and DsRed-Max in astrocytes (29).

For Fig. 5, a wild-type mouse (C57BL/6); female, 15 weeks old, 19 g, under anesthesia with 1.0% isoflurane in oxygen) with SR 101–labeled astrocytes was used. The images were obtained at ~50 μm beneath the dura with an FOV of 17 μm × 17 μm (512 pixels × 512 pixels per frame), a frame rate of 0.20 Hz, and a pixel dwell time of 183. μs. The laser repetition rate was 2 MHz. The images were obtained continuously for 140 s at each FOV. Each FOV contains a single astrocyte, and the same astrocyte was imaged at 1340 and 1650 nm. For astrocytes 1 to 3, 1340-nm excitation was performed first, followed by 1650-nm excitation, and for astrocytes 4 and 5, the sequence of the excitation was reversed. For SR 101 imaging in Fig. 5 and figs. S9 to S11, SR 101 solution in phosphate-buffered saline was topically applied on the dura after opening the skull for 10 min (43, 44), before attaching the cranial window. Images were obtained after >4 hours from the dye soaking. The concentration of the topically applied SR 101 solution was 300 μM for Fig. 5 and figs. S9 and S11 and 1 mM for fig. S10. During all the surgery, the mice were anesthetized with 1.0 to 1.5% isoflurane

Data processing for 3D structural images

For the z-stack images shown in Figs. 3C and 4 (C and D) and fig. S8, Imaris software (Oxford Instruments) was used, after applying 1-pixel-radius median filter using ImageJ software. For the selected images shown in Fig. 3D and the z-stack movie shown in movie S1, 1-pixel-radius median filter was applied. In Fig. 3C and movie S1, GCaMP6s signals are shown down to 1048 μm. For the depth measurement, the slightly larger index of refraction in brain tissue, relative to water, resulted in a slight underestimate (5 to 10%) of the actual imaging depth within the tissue, because the imaging depths reported here are the raw axial movements of the objective.

Activity recording

For Ca²⁺ activity recording in Fig. 3 (E and F) and fig. S9 (B and C), the frame rate was set at 8.3 Hz with a pixel dwell time of 0.51 μs. The laser repetition rate was 2 MHz, and the average power under the objective lens was 56 and 70 mW for Fig. 3 (E and F) and fig. S9 (B and C), respectively. Each trace was normalized to its baseline and lowpass filtered using a hammering window of 0.72-s time constant. Mechanical drift and mouse motion in the horizontal plane were corrected with Template-Matching plug-in (ImageJ) by using the fluorescence signals from Texas Red (for Fig. 3, E and F) or SR 101 (for fig. S9, B and C) as a template for each frame. Regions of interest corresponding to the neurons were visually identified and selected. Baseline of the traces (F₀) is defined as approximately the lower 20% of each trace during the whole recording time. Intensity traces (F) are normalized according to the formula (F – F₀)/F₀. For the activity videos shown in movies S2 and S3, 16-frame rolling average, Kalman filtering (gain, 0.9), and gamma correction with a correction value of 0.8 were applied for the purpose of visualization.

REFERENCES AND NOTES


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Yusaku Hontani, Fei Xia and Chris Xu

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