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SUPPLEMENTARY INFORMATION

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Supplemental Information and Figures for "*In vivo* three-photon microscopy of subcortical structures within an intact mouse brain," by Nicholas G. Horton, et al.

1) Comparing SBRs in 2PM and 3PM

We derive approximate expressions for SBRs in 2PM and 3PM. Our derivations assume that the imaging depth (z) is much greater than the tissue effective attenuation length (l_e), and l_e is much greater than the focal depth of the imaging system. These conditions are generally valid for high resolution imaging deep into scattering tissues. We further assume that the volume fraction that is labelled is one. For situations where the labelling is non-uniform and only a small fraction of the total volume (V_s/V) is labelled, the actual SBR needs to be scaled by approximately a factor of V/V_s.[1]

The signal generated by a diffraction-limited beam in scattering media for 2PM is approximately[2]

$$< F(t)_{2P} >= 8C_2 n_0 \frac{<P>^2}{\pi\lambda} e^{\frac{-2z}{l_e}}, \quad (1)$$

while that of 3PM is[3]

$$< F(t)_{3P} >= 3.5C_3 n_0 \frac{(NA)^2<P>^3}{\lambda^3} e^{\frac{-3z}{l_e}}, \quad (2)$$

where NA is the numerical aperture of the focusing lens, n_0 is the index of refraction of the imaging medium, $< P >$ is the time-averaged power of the excitation beam, λ is the excitation wavelength, and z is the imaging depth in the tissue. C_2 and C_3 are constants that include the contributions such as the concentration of the dye, absorption cross section, etc.

The background is generated mainly within one effective attenuation length of the sample surface, and is given by,

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$$B_n \propto < I >^n Al_e = \frac{<P>^n}{A^{n-1}} l_e, \quad (3)$$

where n is the photon order, $< I >$ is the time-averaged intensity of the excitation beam, and A is the area of the excitation beam on the tissue surface. The 2PM background is

$$B_2 \approx C_2 < P >^2 \frac{n_0^2 l_e}{\pi z^2 NA^2} \quad (4)$$

and the 3PM background is

$$B_3 \approx C_3 < P >^3 \frac{n_0^4 l_e}{\pi^2 z^4 NA^4}. \quad (5)$$

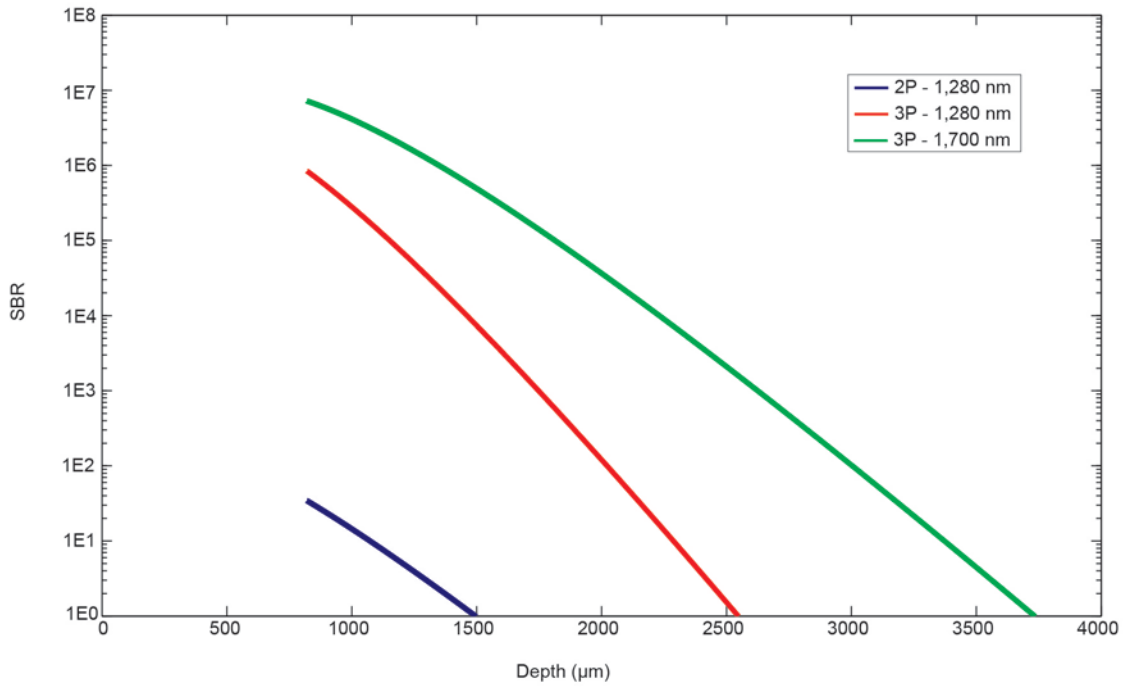
Therefore, the 2PM SBR is

$$SBR_{2P} \approx \frac{6(NA)^2 z^2}{\lambda l_e} e^{-\frac{2z}{l_e}} \quad (6)$$

and the 3PM SBR is

$$SBR_{3P} \approx \frac{14.7 z^4 (NA)^6}{\lambda^3 l_e} e^{-\frac{3z}{l_e}}. \quad (7)$$

Equation (6) is in agreement with previous calculation for SBR in 2PM[1]. Supplementary Fig. 1 compares the 2PM SBR with 1,280 nm excitation (l_e = 285 μm), 3PM SBR with 1,280 nm excitation (l_e = 285 μm), and 3PM SBR with 1,700 nm excitation (l_e = 400 μm). It is clear that 3PM imaging provides a significant improvement in SBR over 2PM, even when equal effective attenuation lengths are considered.



Supplementary Figure 1: Comparing SBRs in 2PM and 3PM.

Calculated SBRs of 2PM and 3PM at $NA = 1$. The effective attenuation lengths are 285 μm and 400 μm for excitation at 1,280 nm and 1,700 nm, respectively.

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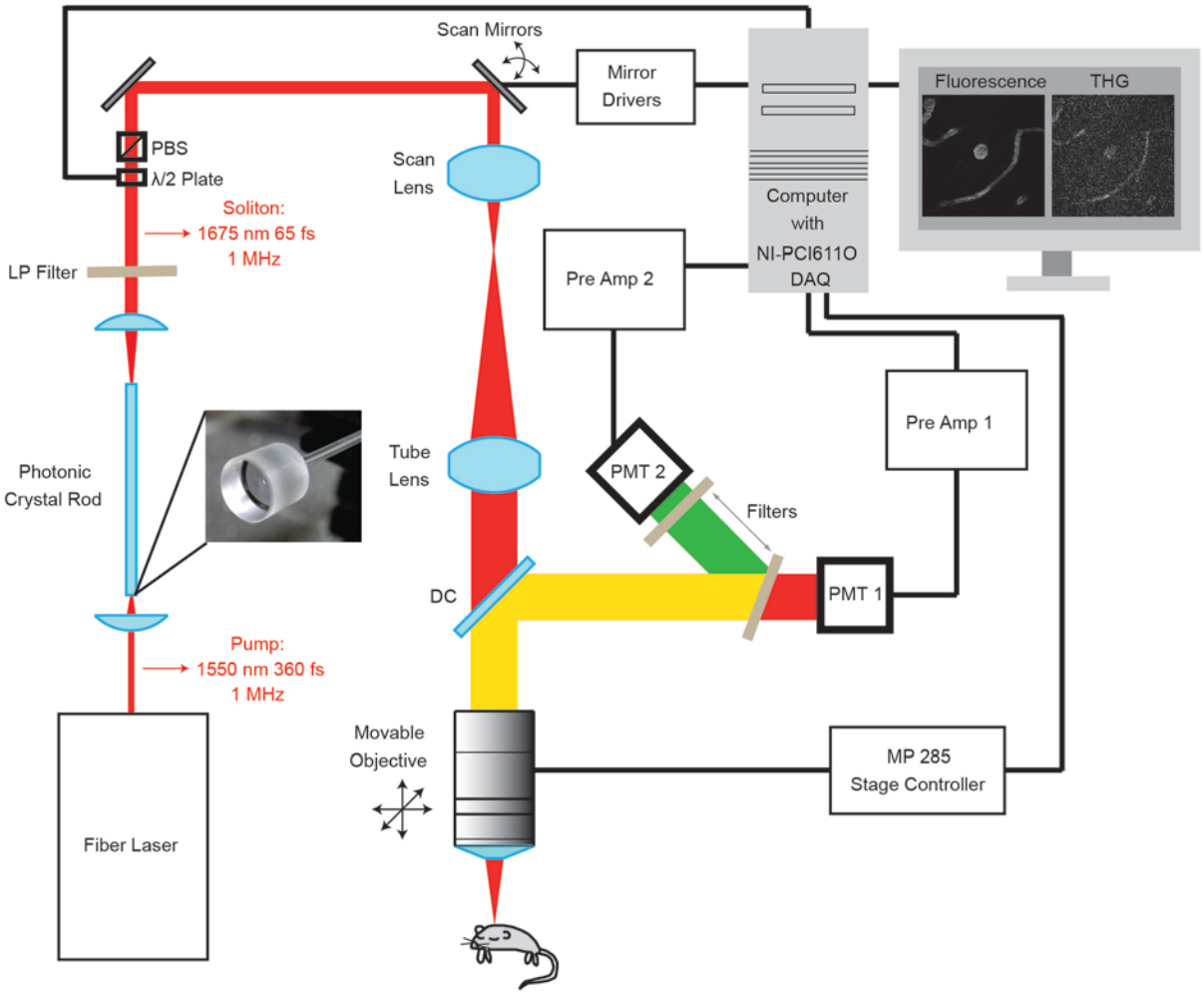
2) 3PM experimental setup



Supplementary Figure 2: 3PM experimental setup.

Schematic of the imaging setup. PBS: polarization beam splitter, DC: dichroic mirror, PMT: photomultiplier tube, $\lambda/2$ Plate: half wave plate, LP Filter: 1,600 nm long-pass filter. The excitation beam is raster-scanned by two galvanometer-driven mirrors (6215H, Cambridge Technology). Scan mirrors are imaged onto the back aperture of the microscope objective with a magnification of 6 times via a scan lens and a tube lens. The scan lens is a C-coated achromat (AC254-030-C-ML, Thorlabs) for high transmission (97%) at 1,675 nm, and the transmission of the tube lens (Sutter Instrument) is 82%. We use a custom high NA water immersion microscope

objective (XLPlan N, Olympus, 25× 1.05 NA), which is specially coated for high transmission (83%) at 1,675nm, to focus the excitation beam into the sample and to epi-collect the fluorescence and third harmonic signals. Epi-collected fluorescence and third harmonic signals are directed to the PMTs by the DC (750DCXXR, Chroma Technology). The power at the sample surface is adjusted by the $\lambda/2$ Plate (AQWP05M-1600, Thorlabs) and the PBS (PBS054, Thorlabs).

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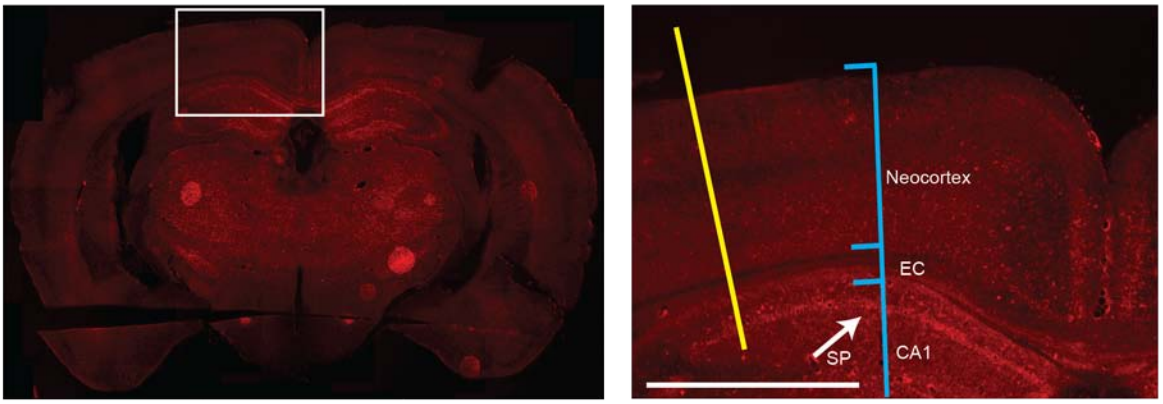
3) Epifluorescence image of the B6.Cg-Tg(Thy1-Brainbow1.0)HLich/J mouse brain



Supplementary Figure 3: Epifluorescence images of the

B6.Cg-Tg(Thy1 Brainbow1.0)HLich/J mouse brain

Left: Coronal section of the mouse brain, 2 mm caudal to bregma. The bright circles in the lower half of this frame are artefacts from the slide mounting process. Right: Zoomed-in region. The yellow line indicates the approximate location where 3PM imaging was performed, and the white arrow indicates the location of the SP. The scale bar is 1 mm.

References

1. Theer, P., Hasan, M.T. & Denk, W. Two-photon imaging to a depth of 1000 µm in living brains by use of a Ti:Al_2O_3 regenerative amplifier. *Opt. Lett.* **28**, 1022-1024 (2003).
2. Xu, C. & Webb, W.W. Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm. *J. Opt. Soc. Am. B* **13**, 481-491 (1996).
3. Xu, C. & Webb, W. W. Multiphoton excitation of molecular fluorophores and nonlinear laser microscopy, *In Topics in fluorescence spectroscopy*, Vol. 5 (Ed, Lakowicz, J. R.), 471-540.