Whole-brain imaging and characterization of *Drosophila* brains based on one-, two-, and three-photon excitations

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1 **Abstract**

2 To study functional connectome, optical microscopy provides the advantages of
3 *in vivo* observation, molecular specificity, high-speed acquisition, and sub-micrometer
4 spatial resolution. Now, the most complete single-neuron-based anatomical
5 connectome is built upon *Drosophila*; thus it will be a milestone to achieve whole-
6 brain observation with sub-cellular resolution in living *Drosophila*. Surprisingly, two-
7 photon microscopy cannot penetrate through the 200- μ m-thick brain, due to the
8 extraordinarily strong aberration/scattering from tracheae. Here we achieve whole-
9 *Drosophila*-brain observation by degassing the brain or by using three-photon
10 microscopy at 1300-nm, while only the latter provides *in vivo* feasibility, reduced
11 aberration/scattering and exceptional optical sectioning capability. Furthermore, by
12 comparing one-photon (488-nm), two-photon (920-nm), and three-photon (1300-nm)
13 excitations in the brain, we not only demonstrate first quantitative reduction of both
14 scattering and aberration in trachea-filled tissues, but unravel that the contribution of
15 aberration exceeds scattering at long wavelengths. Our work paves the way toward
16 constructing functional connectome in a living *Drosophila*.

1 Introduction

2 *Drosophila* is an important model animal to study connectomics since its brain
3 is complex with 10^5 neurons but still small enough to be completely mapped by
4 optical microscopy with single-cell resolution. Compared to other model animals, the
5 genetic tool box is more complete with *Drosophila*, and a connectome map based on
6 *in vitro* structural registration of more than 30,000 cells has been established (Chiang
7 et al., 2011), serving as an invaluable reference for functional connectome study. To
8 study the functional connectome, two-photon fluorescence (2PF) microscopy is now
9 the most popular tool because of its advantages on low photobleaching and
10 phototoxicity, subcellular spatial resolution, and deep penetration depth (Helmchen &
11 Denk, 2005). When observing living mouse or zebrafish brain with 2PF microscopy,
12 the penetration depth approaches 1 mm, which is typically limited to about five
13 scattering lengths (Helmchen & Denk, 2005; Horton et al., 2013). However, even
14 using the same fluorophore and excitation wavelength, the reported imaging depths in
15 a living *Drosophila* brain are much more limited. For example, when imaging
16 GCaMP with excitation wavelength around 920-nm, activities from mushroom bodies
17 (MB) had been recorded at only several tens of micrometers in depth (Honegger,
18 Campbell, & Turner, 2011; Y. L. Wang et al., 2004). Using the same combination of
19 laser and probe, the neuronal activities from antennal lobes (AL) are obtained with
20 imaging depth less than 100 μm (Ignell et al., 2009; Root, Semmelhack, Wong, Flores,
21 & Wang, 2007; Ruta et al., 2010; J. W. Wang, Wong, Flores, Vosshall, & Axel, 2003).
22 Although the thickness of a *Drosophila* brain is only about 200 μm , which is much
23 smaller than the typical imaging depth of 2PF microscopy in other model animals like

1 mouse and zebrafish, to the best of our knowledge, no study has demonstrated *in vivo*
 2 whole-brain imaging in *Drosophila*, nor has characterized the image attenuation of a
 3 living *Drosophila* brain. The whole-brain observation capability is a major milestone
 4 toward establishing functional connectome in this model animal.

5 The underlying difficulty of living *Drosophila* whole-brain imaging is that,
 6 different from mouse and zebrafish, where blood vessels are responsible for oxygen
 7 exchange, air vessels, i.e., tracheae, are in charge of oxygen exchange in *Drosophila*
 8 brains. The micro-tracheae in the brain are a few micrometers in diameter (Beitel &
 9 Krasnow, 2000), comparable to near infrared wavelengths, and thus induce
 10 extraordinarily strong aberration/scattering from the air/tissue interface since the
 11 refractive index (RI) difference between air and tissue is much larger than that
 12 between blood and tissue. This tracheae-induced aberration/scattering impedes deep
 13 tissue observations inside a living *Drosophila* brain. However, the optical properties
 14 of trachea-filled tissues have not been well studied. Therefore, the aim of this work is
 15 to unravel the optical effect of tracheae, and to design a suitable method to increase
 16 imaging depth in trachea-filled tissues.

17 To increase imaging depth in living animals, there are several known
 18 approaches. For example, photo-activatable fluorophores (PAFs) have been used to
 19 suppress out-of-focus fluorescence (Wei, Chen, & Min, 2012), and high-energy lasers
 20 were used to enhance excitation efficiency at deep tissue (Theer, Hasan, & Denk,
 21 2003). However, long converting time is required for PAFs, which is unfavorable to
 22 observe fast neural activities such as calcium dynamics, and high-energy lasers are
 23 potentially harmful due to multiphoton ionization. Adaptive optics (AO) is able to

1 correct tissue-induced aberration, and has demonstrated significant image contrast and
2 depth enhancement (Booth, 2014; Pedrazzani et al., 2016; Tang, Germain, & Cui,
3 2012; C. Wang et al., 2014). Nevertheless, the best depth achieved by AO in a living
4 *Drosophila* brain to date is still less than 100 μm (Pedrazzani et al., 2016), since the
5 aberration inside the insect's brain is much larger than that of vertebrate's brain (RI
6 difference between air and tissue is at least ~ 1 order of magnitude larger than that
7 between blood and tissue). In addition, typically AO does not compensate the
8 scattering effect (Booth, 2014), which limits its impact in improving the imaging
9 depth in *Drosophila*.

10 On the other hand, long excitation wavelength is well known to greatly improve
11 penetration depth by substantially reducing scattering (Chu et al., 2003; Horton et al.,
12 2013; Kobat, Horton, & Xu, 2011; Tao et al., 2017). In addition, the phase error
13 caused by aberration (i.e., wavefront distortion) is inversely proportional to the
14 excitation wavelength. The long wavelength approach is expected to reduce the
15 amount of aberration caused by the RI difference, and thus enhancing imaging depth.
16 Furthermore, high-order optical nonlinear excitations, such as three-photon absorption,
17 are often combined with long wavelength, thus providing better excitation
18 confinement, i.e. better optical sectioning capability, than 2PF, to improve image
19 contrast in deep tissue. Combining these factors together, long wavelength excitation
20 is promising for whole-brain imaging in *Drosophila*.

21 Here, two approaches are adopted to achieve whole-brain observation in a
22 *Drosophila* brain. The first one is to pump our air inside the tracheae, i.e. degassing.
23 Since the tracheae-induced aberration/scattering is largely removed in the degassed

1 brain, 2PF microscopy penetrates through the whole brain. However, the *Drosophila*
 2 is not alive after degassing. To achieve *in vivo* whole-brain observation, the second
 3 approach is to use three-photon imaging based on excitation wavelength at 1300-nm
 4 in a GFP-labeled living *Drosophila* brain. The three-photon fluorescence (3PF)
 5 method provides exceptional excitation confinement and simultaneously reduced
 6 aberration/scattering, thus allows high-contrast and high-resolution image throughout
 7 the whole brain. The accompanying third harmonic generation (THG) modality
 8 provides detailed map of the densely distributed tracheae in the brain, useful for
 9 structure-function studies.

10 Furthermore, the optical attenuations of the *Drosophila* brains at various
 11 wavelengths are characterized, via 1PF (488-nm), 2PF (920-nm) and 3PF (1300-nm)
 12 excitation modalities. For the first time, the attenuations contributed from tracheae are
 13 quantitatively determined, with most contribution from aberration. We show that at
 14 short wavelength, scattering is the dominating attenuation; while at long wavelengths,
 15 the contribution of aberration can exceed that of scattering, and become the main
 16 impeding factor of whole-brain observation in *Drosophila* brains.

17

18 **Results and discussion**

1
2 Figure 1. **Living and degassed *Drosophila* brain images under different imaging modalities.** (A),
3 (C) and (E) are 1PF, 2PF and 3PF images at 30, 60, 130 and 180 μm depths of a living brain. (B) and
4 (D) are 1PF and 2PF images of a degassed brain at the same depths. Although brain-edge structures
5 (arrowheads) are still visible at 60 μm depth in (A), the image loses contrast in brain center (arrow). In
6 the degassed brain, the penetration depth of 1PF is significantly improved in (B), but not approaching
7 the bottom of brain. In (C), 2PF penetrates deeper than 1PF, but becomes blurry at depth beyond 100
8 μm . Whole-brain imaging is achieved in (D) by 2PF in a degassed brain, which nevertheless has no

1 function. (E) shows only 3PF maintains contrast and reasonable resolution throughout the whole-brain
2 *in vivo*. By comparing with structural connectome, LPUs can be identified in (E). MB: mushroom
3 bodies; AL: antenna lobes; VLP: ventrolateral protocerebrum; SOG: subesophageal ganglion, which is
4 the deepest LPU in a *Drosophila* brain. (F) THG images, a complementary contrast with 3PF, in the
5 living brain show clear tracheae distribution. All the images are intensity normalized with individually
6 brightest 1% pixels. Scale bar: 50 μm . Complete depth-images are given in Videos 1 - 3.

7
8 Within a living brain, the 1PF images lose the image contrast at around 40 μm
9 (Video 1), as no structures are visible in the brain center where the white arrow points
10 in the 60- μm panel of Figure 1A. The arrowheads indicate structures that located at
11 the edge of the brain. To verify the effect of trachea, a brain is degassed, i.e. air in
12 tracheae is pumped out. Figure 1B shows that 1PF in the degassed brain provides
13 much better contrast in the center of brain at the same 60 μm depth, but cannot exceed
14 130 μm . The 1PF imaging depth of the degassed brain is comparable with that in
15 mouse brains, which is mainly limited by scattering (Helmchen & Denk, 2005).
16 Comparing the results in Figures 1A and 1B, it is obvious that degassing removes the
17 additional attenuation contributed by tracheae.

18 On the other hand, it is well known that using long excitation wavelengths
19 with 2PF modality efficiently improves imaging depth, approaching 1 mm in mouse
20 brains (Helmchen & Denk, 2005). Using the same excitation wavelength ($\sim 920\text{-nm}$)
21 and contrast agent (GFP families), Figure 1C shows the 2PF imaging depth in a living
22 *Drosophila* brain indeed increases compared to Figure 1A, but reaches only about 100
23 μm , which is not adequate to penetrate the whole brain, mainly due to the tracheae.

24 By combining 2PF modality with a degassed brain, Figure 1D presents the

1 first whole-brain imaging in a *Drosophila*. Reasonable contrast and resolution are
 2 maintained throughout the nearly 200 μm depth, manifesting that the trachea-induced
 3 aberration/scattering is the major restraint for deep-brain imaging in this model
 4 animal. However, please note that the animal is no longer alive after degassing.
 5 Moreover, the degassing process distorts the brain structures, making its association
 6 with the *Drosophila* structural connectome difficult.

7 In order to enable the study of whole-brain functional connectome, 3PF at
 8 excitation wavelength $\sim 1300\text{-nm}$ was adopted. Figure 1E shows that this imaging
 9 modality provides subcellular resolution throughout a living *Drosophila* brain.
 10 Associating the 3PF images with an existing *Drosophila* brain database (*FlyCircuit*,
 11 <http://www.flycircuit.tw>), several local processing units (LPUs) (Chiang et al., 2011)
 12 are identified and marked by the white arrows.

13 During the three-photon excitation process, a complementary contrast, i.e.
 14 THG, is generated by the interfaces with RI differences, and thus is sensitive to the
 15 tissue/air boundaries in tracheae. Figure 1F shows that THG signals reveals the
 16 detailed distribution of tracheae, which are randomly distributed inside the brain and
 17 causing strong aberration/scattering.

Figure 2. 1PF, 2PF, and 3PF signal attenuations in living and degassed brains, and quantitative analysis on contributions from scattering and aberration. (A) and (B) show the semi-logarithmic plot of 1PF and 2PF signal attenuations with depth, inside a living (black) and degassed (green) brains, respectively corresponding to Figure 1A - 1D. (C) 3PF signal attenuation of a living brain in Figure 1E. The inset in (A) shows the area (white box) for signal extraction at different depths. The signals are obtained by averaging the brightest 1 % pixels at different depths inside the white box. In (A) - (C), the corresponding attenuation coefficients (μ_{att}) are given in the bottom. (D) Quantitative comparison of μ_{att} in living brains (black squares) and degassed brains (green diamonds) at each wavelength, together with corresponding attenuations from mouse brains, i.e. neuron scattering (pink circles) and tracheae (red triangles). Scale bar in inset of (A): 50 μm .

To quantify signal attenuation coefficients (μ_{att}) of different imaging modalities,

1 Figures 2A - 2C show the decay of 1PF, 2PF, and 3PF signals, corresponding to
 2 Figures 1A - 1E respectively, within living or degassed brains (see Materials and
 3 Methods and Figure supplement 1 for derivation). Apparently, both degassing and
 4 long wavelength excitation help to reduce μ_{att} significantly. The μ_{att} of 2PF and 3PF
 5 are half and one-third of that of 1PF in living *Drosophila* brains, while the lowest μ_{att}
 6 is provided by 2PF plus degassing. Comparing to mouse brain whose μ_{att} of 2PF and
 7 3PF are less than one-third and one-eighth of 1PF (Horton et al., 2013), the influence
 8 of longer excitation wavelength is much smaller in *Drosophila* brains. To better
 9 understand the results, the μ_{att} has to be decomposed, as we explain in the following.

10 In Figure 2D, the attenuation coefficients of *in vivo* and degassed brains are
 11 plotted in black and green, respectively. It is interesting to note that the attenuations of
 12 degassed brains are comparable to that of a mouse brain (pink circles), which is
 13 mostly contributed from scattering of neurons (Horton et al., 2013). The slight
 14 difference may be due to the effect of residual wax in the tracheae. From the
 15 comparison of *in vivo* *Drosophila* brain (total attenuation, μ_{att}) and mouse brain
 16 (attenuation from neurons, μ_{neu}), the contribution of trachea $\mu_{tra} = \mu_{att} - \mu_{neu}$ is derived
 17 as red triangles in Figure 2D.

18 The results show that both μ_{neu} and μ_{tra} reduce with increasing wavelengths, with
 19 respectively λ^{-2} and $\lambda^{-0.5}$ dependencies (see Figure supplement 2). Since the
 20 attenuation of neuronal tissue μ_{neu} is dominated by scattering, which includes
 21 Rayleigh scattering (λ^{-4} dependence) and Mie scattering (λ^{-1} dependence), the λ^{-2}
 22 dependency should be a natural result of the mixture. On the other hand, the $\lambda^{-0.5}$

1 dependency suggests that μ_{tra} may not be governed by scattering, whose wavelength
2 dependency should be in between λ^{-4} and λ^{-1} , but by aberration from air-tissue
3 interfaces.

4 To further confirm that scattering does not play the key role in the attenuation of
5 the trachea-filled brain, we compare the 2PF μ_{att} of *Drosophila* and mouse in Figure
6 2D, the latter ($\sim 5.55 \text{ mm}^{-1}$) is less than half of the former ($\sim 13.5 \pm 0.5 \text{ mm}^{-1}$).
7 Nevertheless, 2PF imaging in mouse brains typically reaches almost 1 mm, i.e. about
8 five scattering lengths; while in living *Drosophila* brains, only about 100 μm , i.e. less
9 than two attenuation lengths, is achieved. Similar situation occurs with the 3PF
10 modality. The surprisingly limited penetration depth suggests that scattering does not
11 dominate the attenuation, but the trachea-induced aberration dictates, especially in
12 multiphoton imaging cases. The underlying reason of the limited depth is that
13 aberration not only induce excitation intensity loss, but also strongly deteriorates
14 point-spread-function (PSF), especially in the axial direction (Figure supplement 3).
15 As a result, capability of 2PF optical sectioning quickly loses at relatively shallow
16 region, leading to blurred images and considerably limited imaging depth, as
17 manifested in Figure 1C. On the other hand, 3PF imaging modality not only exhibits
18 less aberration (Figure 2D), but also benefits from better optical sectioning (Figure
19 supplement 3), thus provides deeper imaging depth than 2PF. In contrast, the single-
20 photon fluorescence images in Figure 1A (see the arrow) demonstrate loss of image
21 contrast mainly due to scattering.

22 In Figure 2A and 2B, the trachea-contributed aberration is more efficiently
23 removed by degassing in the 2PF modality (2-fold reduction in μ_{att} , Figure 2B) than

1 1PF (~ 30 % reduction, Figure 2A). In addition, from Figure 2D, μ_{tra} for 2PF and 3PF
2 are larger than μ_{neu} , especially in the 3PF case. These two observations further suggest
3 that tracheae-contributed aberration is the main factor to impede multiphoton deep-
4 brain imaging in a living *Drosophila*.

5
6 **Figure 3. Signal-to-background ratio (SBR) and resolution analysis.** (A) Color-coded depth
7 projection image of 3PF throughout the whole-brain, showing different structures at different depths.
8 The white box in the center represents the region of background selection. (B) SBR of 2PF and 3PF
9 signals at each depth. (C) and (D) show the depth images of 3PF and THG at 186 μm, respectively. The
10 lateral resolutions are ~1.0 μm (E) for 3PF and ~1.5 μm (F) for THG signals. The axial resolutions are
11 ~6.5 μm for 3PF (G) and ~8.4 μm for THG (H), respectively. Scale bars: 50 μm in (A), 5 μm in (C).
12

13 As shown in Figure 1, to reaching the milestone of *in vivo* whole-brain imaging
14 in *Drosophila*, 3PF at 1300-nm should be the optimal choice. Here we quantify the
15 depth limit of 3PF by analyzing its signal-to-background ratio (SBR) in Figure 3A and
16 3b, along with comparison between 2PF and 3PF (see Materials and Methods for SBR

calculations). The former reaches unity at $\sim 100 \mu\text{m}$, since no structure is distinguishable beyond this depth (Video 2), while the SBR of the latter significantly outstands the former. From Figure 3B, the imaging depth of 3PF reaches at least $200 \mu\text{m}$. The result once again supports that 3PF is capable to image through a whole *Drosophila* brain.

The spatial resolutions of 3PF and THG in deep-brain region ($186 \mu\text{m}$ depth) are given in Figure 3C - 3H. For 3PF, the minimal full-width-at-half-maximum (FWHM) is $1.0 \mu\text{m}$ laterally (Figure 3E) and $6.5 \mu\text{m}$ axially (Figure 3G), which is sufficient to resolve a single cell in the *Drosophila* brain (Tuthill, 2009). For THG, the FWHM is slightly larger, $1.5 \mu\text{m}$ in lateral (Figure 3F) and $8.4 \mu\text{m}$ in axial directions (Figure 3H). Considering the width of tracheae, the results should be considered as the upper bound of the THG spatial resolution.

In conclusion, we have, for the first time, characterized the optical properties of the *Drosophila* brain, which is filled with air, with single-photon, two-photon, and three-photon modalities. We found that the main limiting factor that impedes *in vivo* whole-brain single-photon imaging is scattering, but for multiphoton imaging, aberration from tracheae structures plays a more dominant role. The aberration affects not only signal attenuation, but also image visibility. Although degassing enables whole-*Drosophila*-brain imaging by reducing trachea aberration, the only way to achieve *in vivo* whole-brain imaging with single cell resolution is 3PF at 1300-nm excitation, which exhibits less scattering, aberration, and better optical sectioning. It is possible to combine with AO to further reduce aberration (Tao et al., 2017), thus allowing functional imaging on the scale extending from a single neuron, a complete

1 brain network, toward a whole-animal connectome (Lo & Chiang, 2016).

2

3 **Materials and Methods**

4 **Microscope setup**

5 **One-photon microscope.**

6 The one-photon imaging was done on a commercial microscope LSM 780
7 (Zeiss, Germany). The built-in laser (488-nm) and photomultiplier tube was used to
8 single-photon excitation and signal detection. A water immersion objective was used
9 (Olympus, XLPlan N, 25× NA 1.05) for its high transmission in both visible and IR
10 wavelength ranges. A pinhole with ~ 60 μm diameter was used to achieve optical
11 sectioning. The image formation was done by the controlling software Zen (Zeiss,
12 Germany).

13 **Multiphoton microscope.**

14 The setup was the same as that in Ouzounov et al (Ouzounov et al., 2017). A
15 home-built laser-scanning microscope that is compatible to long wavelength
16 excitation is constructed. A Ti: sapphire laser at 920-nm with an 80 MHz repetition
17 rate, and an optical parametric amplifier at 1300-nm with a 400 kHz repetition rate,
18 were used as excitation sources of 2PF and 3PF respectively. The same water
19 immersion objective as single-photon microscope was used. The power levels for
20 both lasers after the objective were limited to less than 20 mW for all imaging depths.
21 The fluorescence and THG signals were epi-collected with a dichroic beamsplitter
22 (Semrock, FF705-Di01-25 × 36), and then detected by a GaAsP photomultiplier tube

(Hamamatsu, H7422-40) and a bialkali photomultiplier tube (Hamamatsu, R7600-200) in non-descanned configurations to maximize the collection efficiency. A 488-nm dichroic beamsplitter (Semrock, Di02-R488-25×36) was used to split the fluorescence and THG signals, which were further separated by a 520/60 band-pass filter (BPF, transmission at center 520-nm, FWHM 60 nm) for the fluorescence and a 420/40 BPF for the THG. A living *Drosophila* was fixed and placed onto a motorized stage (M-285, Sutter Instrument). A computer running the ScanImage 3.8 under Matlab (MathWorks) was used to synchronize the stage movement and image acquisition. The signal current from the detectors was converted to voltage, amplified and low-pass filtered by a transimpedance amplifier (Hamamatsu, C9999) and another 1.9 MHz low-pass filter (BLP-1.9+, Minicircuits). Analog-to-digital conversion was performed by a data acquisition card (PCI-6115, National Instruments).

Sample preparations.

All the sample preparation methods followed the protocol of previous publication on *in vivo Drosophila* brain imaging (H.-H. Lin, Chu, Fu, Dickson, & Chiang, 2013). The samples were adult, female *Drosophila* between 5 and 10 days old. GFP was pan-neuronal expressed by genetic drivers (Gal4-elav.L/CyO × UAS-EGFP). The living *Drosophila* was immobilized in a pipette tip with volume 100 µL after anesthetized by ice bathing. A window was cut into the head by using fine tweezers, after placing a drop of Ca²⁺-free saline on the brain to prevent desiccation, and fat bodies above the brain were removed, under a stereomicroscope. The dissection saline was then replaced with a drop of Ca²⁺-containing saline (108 mM NaCl, 5 mM KCl, 2

1 mM CaCl₂, 8.2 mM MgCl₂, 4 mM NaHCO₃, 1 mM NaH₂PO₄, 5 mM trehalose, 10 mM sucrose, and 5 mM HEPES [pH 7.5, 265 mOsm]). No cover glass was placed between the brain and the objective.

4 To check the optical effect caused by the tracheae structure, degassing the *Drosophila* brain was performed by pumping out the air inside tracheae. The degassing protocol followed the previous publication of *in situ Drosophila* brain imaging (C.-W. Lin et al., 2015). The degassing protocol started from immersing the *Drosophila* in 4 % paraformaldehyde and 2 % triton, expelling air in tracheae by using a vacuum chamber that was depressurized to – 72-mmHg for 2.5 minutes, wait for 1.5 minutes, and then releasing to normal pressure for 2 minutes. The degassing process was completed by repeating the above procedure 4 times. After degassing, the same microsurgery preparation was performed, and observed under the same microscope.

15 **Signal analyses.**

16 **Attenuation coefficient calculation.**

17 This section explains how to derive the attenuation coefficients (μ_{att}), which is the inverse value of attenuation length (l_a), i.e., $\mu_{att} = l_a^{-1}$, in the results of Figure 2. The calculation of attenuation length inside a biological tissue has been detailed in an earlier work (Horton et al., 2013). The well-known light attenuation equation is:

$$21 \quad I(d) = I_0 e^{-d/l_a} = I_0 e^{-\mu_{att} \times d} \quad (1),$$

22 where $I(d)$ is the excitation intensity at imaging depth of d , and I_0 is the intensity at

1 tissue surface. With fluorescence excitation processes, the n -th order fluorescence
2 intensity, $F^{(n)}(d)$, is related to the excitation intensity as:

$$3 \quad F^{(n)}(d) \propto (I(d))^n \quad (2).$$

4 For 1PF, 2PF and 3PF, n equals 1, 2 and 3, respectively. Combining eqs. 1 and 2,
5 $F^{(n)}(d)$ is obtain in eq. 3,

$$6 \quad F^{(n)}(d) = a \times e^{-n \times \mu_{att} \times d} \quad (3),$$

7 where a is a proportional constant. $F^{(n)}(d)$ and d are determined experimentally, and
8 μ_{att} can be obtained from their dependencies. More explicitly, by taking the natural
9 logarithmic value of both sides in eq. 3, it becomes,

$$10 \quad \ln(F^{(n)}(d)) = \ln(a) - n \times \mu_{att} \times d \quad (4)$$

11 By plotting the dependence of $\ln(F^{(n)}(d))$ on d , as shown in Figure 2A – 2C
12 and Figure supplement 1B - 1D, the decay slopes of the curves are $-n \times \mu_{att}$.

13 Therefore, μ_{att} is determined by dividing the inverse value of the slopes over n .

14 To obtain the slopes of the curves in Figure 2A - 2C, the data points for linear
15 regression fitting are selected by the same criteria. They are all selected with the
16 starting point of signal decay to the depth limit of the corresponding imaging modality.
17 For example, the depth range for fitting in Figure 2B starts $\sim 20 \mu\text{m}$ and ends ~ 110
18 μm . However, the imaging depth limit of 3PF represent the bottom of the brain,
19 instead of the penetration depth limit.

20

21 **Signal-to-background (SBR) calculation.**

22 The SBR calculation was used in Figure 3 to determine the imaging depth

1 limitation, and the method was similar to our previous publication (Ouzounov et al.,
2 2017). Briefly, the signal value was averaged from the pixels with top 1 % intensities
3 from the white box in inset of Figure 2A, and the background value was the average
4 of all pixel intensities in the white box of Figure 3A, where no brain tissue was found
5 throughout the whole depth range. With this definition, the signals mostly came from
6 the in-focus regions, and the background arose from out-of-imaging-plane
7 contributions. Both SBR results are normalized to the 2PF value at 100 μ m depth,
8 where no distinguishable features are observable.

9

10 **Acknowledgements**

11 The three-photon brain imaging cannot be done with the great help from Chris Xu and
12 Tianyu Wang from School of Applied and Engineering Physics in Cornell University.
13 In addition, we appreciate the generous support of transgenic *Drosophila* from Chun
14 Han and Rushaniya Fazliyeva from Institute of Cell and Molecular Biology in Cornell
15 University. This work was financially supported by the Ministry of Science and
16 Technology (MOST), Taiwan, under grant MOST-107-2321-B-002-009-, MOST-105-
17 2628-M-002-010-MY4 and MOST 104-2218-E-007 -022 -MY2, and by the Brain
18 Research Center from The Featured Areas Research Center Program within the
19 framework of the Higher Education Sprout Project by the Ministry of Education
20 (MOE) and MOST in Taiwan. SWC acknowledge the generous support from the
21 Foundation for the Advancement of Outstanding Scholarship.

22

1 Competing Interests

2 No Competing Financial Interests.

3

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A

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Figure supplement 1

C

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Figure supp 2

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Figure supplement 3

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