Label-free imaging of *Drosophila* larva by multiphoton autofluorescence and second harmonic generation microscopy

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Abstract. The fruit fly *Drosophila melanogaster* is one of the most valuable organisms in studying genetics and developmental biology. To gain insight into *Drosophila* development, we successfully acquired label-free, in vivo images of both developing muscles and internal organs in a stage 2 larva using the minimally invasive imaging modality of multiphoton autofluorescence (MAF) and second harmonic generation (SHG) microscopy. We found that although MAF is useful in identifying structures such as the digestive system, trachea, and intestinal track, it is the SHG signal that allowed the investigation of the muscular architecture within the developing larva. Our results suggest that multiphoton microscopy is a powerful in vivo, label-free imaging technique to examine *Drosophila* physiology and may be used for developmental studies.

2 Materials and Methods

2.1 Sample Preparation

Stage 2 larvae of the *D. melanogaster* strain *w*^1118^ was used in this study. The larvae were anesthetized by exposure to ether fumes for about 4–5 min. The anesthetized second larva was then mounted into a phosphate-buffered saline observation chamber made of coverslips and spacers. After imaging, the anesthetized larva was retrieved from the observation chamber and placed on a grape agar plate with wet nutritional yeast.

2.2 Multiphoton Microscope Setup

A femtosecond, titanium-sapphire (Ti–Sa) laser was used as the excitation source. The Ti–Sa laser (Tsunami, Spectra Physics, Mountain View, California) was tuned to 780 nm with 55–70 mW output at the objective. The Ti–Sa laser has a 80-MHz repetition rate and 150-fs pulse duration. The nonlinear optical imaging system used in this experiment was based on Zeiss Meta LSM510 with a Fluor 40X 1.3NA oil-immersion objective (Zeiss) as the imaging objective. The detection bandwidths of the broadband MAF and narrowband SHG signals are approximately 435–700 and 380–400 nm, respectively.

3 Results and Discussion

Depth-resolved, MAF and SHG images of a stage 2 *Drosophila* larva are shown in Fig. 1. Because both the muscular architecture and internal organs have evolved at this point of...
development, studying a stage 2 *Drosophila* larva is significant. To demonstrate that we can image throughout the thickness of the larva, we conducted multiphoton imaging at different depths in the second larvae stage. Images acquired at the depths of 0, 15, 30, 45, 60, 75, 90, and lateral 15 \( \mu \text{m} \) are shown in Fig. 1(a)–1(h). Shown in Fig. 2 is the SHG image of the larva and the MAF image is shown in Fig. 3. From our results, a number of significant observations can be made. First, the muscular architecture can be imaged by the second harmonic signal. In addition, we found that the trachea system can also be visualized by SHG imaging. Furthermore, MAF can be used to image the outer surface and various internal organs, such as the digestive system, trachea, and intestinal track. Our label-free images are structurally consistent with the known internal architecture of the *Drosophila* larva.\(^\text{12}\) In addition, we did not observe visible structural alteration from the femtosecond laser illumination, suggesting that the photodamage caused by multiphoton imaging is minimal.

In this work, we demonstrated label-free multiphoton in vivo imaging in a stage 2 *Drosophila* larva. Although *Drosophila* is one of the important models in developmental biology, this study shows that the combination of MAF and SHG

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**Fig. 1** (a–h) *In vivo*, depth-resolved multiphoton imaging of the stage 2 larva. The imaging depths are 0, 15, 30, 45, 60, 75, 90, and lateral 15 \( \mu \text{m} \) (Green: autofluorescence, Red: SHG).

**Fig. 2** (A) *Drosophila* stage 2 larva SHG imaging. (B) is the enlarged SHG image of a selected region of interest. Note that both the muscular architecture and the trachea system can be imaged by the SHG signal. Scale bar is 200 \( \mu \text{m} \).

**Fig. 3** Large area and detailed MAF images (A–H) of the stage 2 *Drosophila* larva. Shown in details are (A, D) digestive system, (B) trachea, and (C, E) intestinal track of the developing larva.
microscopy is capable of imaging different organelles of stage 2 Drosophila larva and that this approach may be used for the detailed investigation of developmental and other significant physiological processes in Drosophila in the future.

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References