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 autofuorescence (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.
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1 Introduction

Near-infrared (NIR) multiphoton microscopy has become the preferred tool of choice for microscopic imaging of biological specimens. A number of unique applications, such as biomedical diagnostics, initiation of photochemical reactions, and nanoprocessing within living cells/tissues, can be achieved with NIR illumination.[1] In biomedical imaging, the noninvasive and highly penetrative multiphoton microscopy with spatial resolution of <500 nm has the potential of offering new insight into morphological and developmental studies *in vivo*. Specifically, multiphoton imaging allows submicrometer three-dimensional (3-D) resolution, millimeter-penetration depth, and minimally invasive nature.[2,3]

Drosophila melanogaster is one of the most studied model organisms in biological research, particularly in genetics and developmental biology. Moreover, $\sim$61% of known human disease genes have recognizable orthologs in the *Drosophila* genome, and 50% of fly protein sequences have mammalian analogs.[4] Because of the similarity between human and fly proteome, *Drosophila* is a useful organism for studying mechanisms underlying immunity disorders, diabetes, cancer, and even psychiatry problems, such as drug abuse. In the past, multiphoton microscopy has been successfully applied in the imaging of *Caernorhabditis elegans* and other important biological systems.[5–8] Specifically, the nonlinear imaging modalities of fluorescence, second harmonic generation (SHG), and third harmonic generation (THG) imaging have been used to study physiological processes in *Drosophila*.[9–12] However, to the best of our knowledge, the combination of multiphoton auto fluorescence (MAF) and SHG imaging for the structural exploration of *Drosophila* larval organelles on the whole-body scale has not been demonstrated. In this study, we attempt to achieve label-free imaging of living stage 2 *D. melanogaster* larva, and to test the feasibility of using this imaging modality for investigating the development and other significant physiological phenomena in *Drosophila*.

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 Fluar 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

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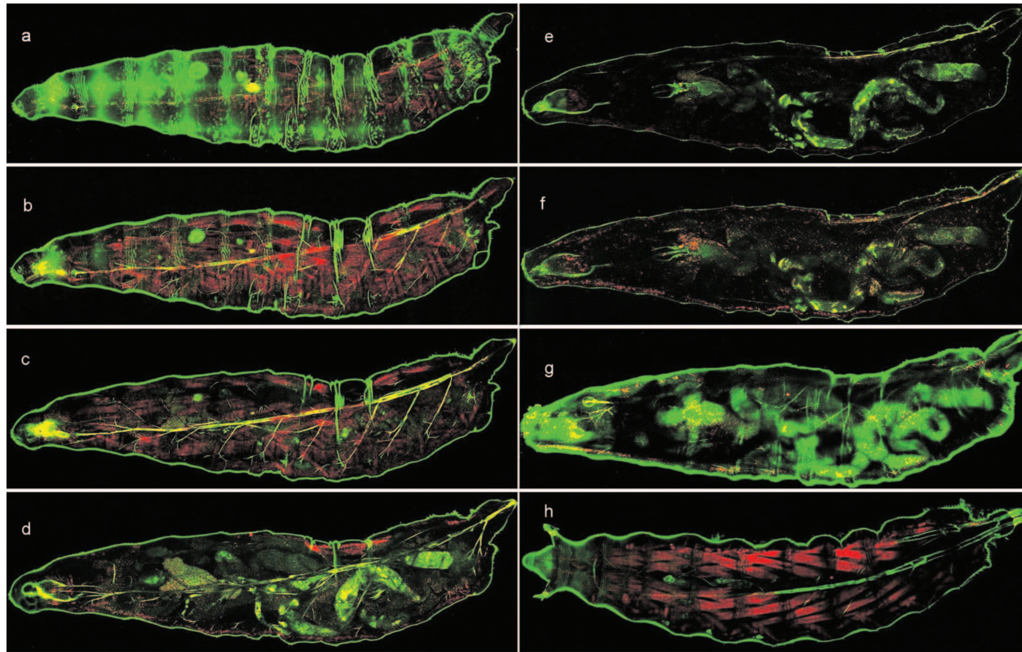

Fig. 1 (a–h) *In vivo*, depth-resolved multiphoton imaging of the stage 2 larva. The imaging depths are 0, 15, 30, 45, 60, 75, 90, and lateral 15 μm (Green: autofluorescence, Red: SHG).

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 μm 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.[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

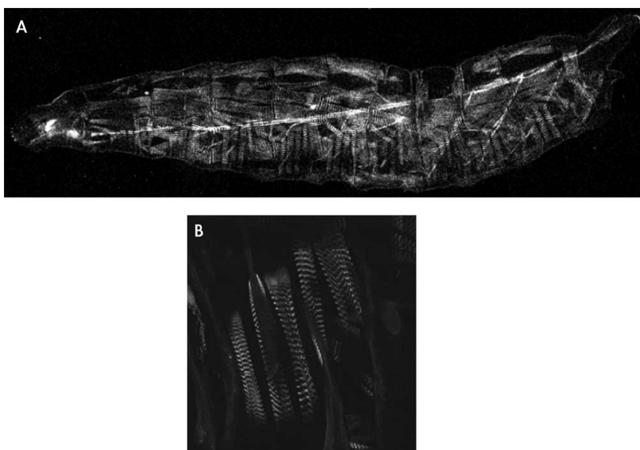

Fig. 2 (A) *Drosophila* stage 2 larva SHG imaging. (B) is the enlarged SHG image of a selected region of interest. Note that both the muscular architecture and the trachea system can be imaged by the SHG signal. Scale bar is 200 μm.

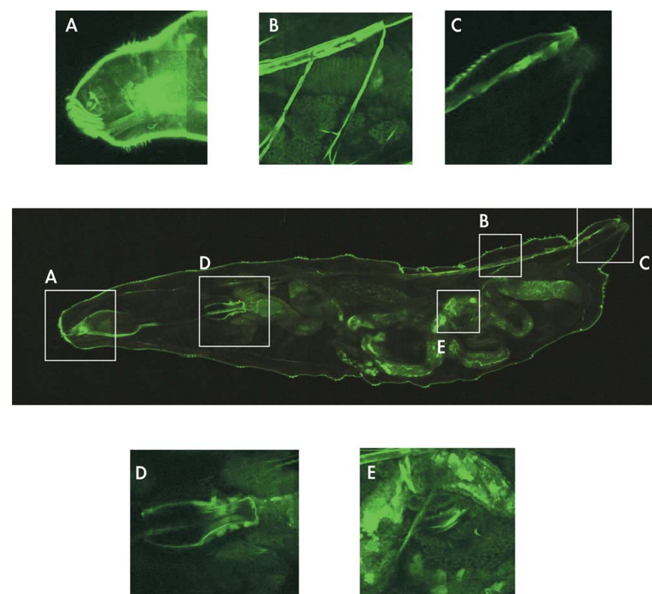

Fig. 3 Large area and detailed MAF images (A–H) of the stage 2 *Drosophila* larva. Shown in details are (A, D) digestive system, (B) trachea, and (C, E) intestinal track of the developing larva.

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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