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Honghai Liu
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Honghai Liu,[a] **Wan Qin,**[a] **Yonghong Shao,**[b] **Zhen Ma,**[a] **Tong Ye,**[c] **Tom Borg,**[d] **and Bruce Z. Gao**[a]
[a]Clemson University, Department of Bioengineering and COMSET, Clemson, South Carolina 29634
[b]Shenzhen University, Institute of Optoelectronics, Shenzhen, Guangdong, 518060, China
[c]University of Alabama at Birmingham, Department of Neurobiology, Birmingham, Alabama 35294
[d]Medical University of South Carolina, Department of Regenerative Medicine and Cell Biology, Charleston, South Carolina 29425

Abstract. We developed a hybrid two-photon excitation fluorescence-second harmonic generation (TPEF-SHG) imaging system with an on-stage incubator for long-term live-cell imaging. Using the imaging system, we observed the addition of new sarcomeres during myofibrillogenesis while a cardiomyocyte was spreading on the substrate. The results suggest that the TPEF-SHG imaging system with an on-stage incubator is an effective tool for investigation of dynamic myofibrillogenesis. © *2011 Society of Photo-Optical Instrumentation Engineers (SPIE)*. [DOI: 10.1117/1.3662457]

Keywords: two-photon excitation fluorescence; second harmonic generation; on-stage incubator; cardiomyocytes; myofibrillogenesis; sarcomere addition.

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The study of myofibrillogenesis in live cardiomyocytes is essential for understanding heart-muscle formation and remodeling.[1,2] In conventional fluorescence technique, this process inside live cells has been studied by labeling, with green fluorescent protein (GFP) technology,[3] specific molecules. The growth of myofibrils and the maturation of premyofibril were investigated by monitoring the distribution of alpha-actinin fused with GFP in live embryonic cardiomyocytes under fluorescence microscope.[4] Whether the GFP is located inside or outside a structure such as a sarcomere, however, it will fluoresce but not distinguish among structures.

Two-photon excitation fluorescence (TPEF) can be from a specific molecule and thus used for exploring how component molecules are arranged in the myofibrils.[5,6] Second harmonic generation (SHG) is intrinsic to a specific structure and thus enables the study of dynamical assembly of a myofibril without any protein labeling.[7–10] The combination of TPEF and SHG (TPEF-SHG) can provide more structural information in a cardiomyocyte,[11,12] which is ideal for tracking how specific sarcomeric proteins are assembled into the myofibrils during myofibrillgoenesis. Compared with corresponding single-photon excitation microscopy, the double wavelength requirement of TPEF and SHG can achieve a deeper penetration inside biological materials.[13,14] Because both TPEF and SHG require high excitation power, which is distributed only within a very small volume confined at the focal point, TPEF and SHG provide higher three-dimensional (3D) resolution than conventional single-photon excitation microscopy.[15–17]

Dynamic sarcomere contractions in a cardiomyocyte were recorded for up to several minutes using SHG,[8] and the car-diomyocyte remained alive during imaging. However, myofibrillogenesis (e.g., the addition of new sarcomeres) is a process that spans hours to days. Normal physiological conditions (temperature, humidity, pH value, etc.) and appropriate laser power are necessary to maintain normal physiological processes inside the cardiomyocytes while myofibrillogenesis is recorded. Therefore, an incubating system that provides the required physiological conditions is required in the study of myofibrillogenesis.

In this study, we developed a hybrid TPEF-SHG polarization-imaging system with an on-stage incubator that provides normal physiological conditions to isolated cardiomyocytes during imaging of myofibrillogenesis. Here we report the results of using this imaging system to record time-lapse images of single, live cardiomyocytes while they were spreading on the glass substrate.

A schematic of the imaging-system setup is shown in Fig. 1(a). The femtosecond (fs) laser beam from a Ti:Sapphire laser (Tsunami 3960-X1BB pumped by a 10 W Millennia, Spectra-Physics, 100 fs and 80 MHz) was tuned to 810 nm and collimated with optics that also expanded the beam 3 times and enforced the beam's polarization. The expanded beam was directed to the microscope custom-designed and built in our lab using several Olympus microscopic components. The beam was then steered onto the XY scanner (6210H, Cambridge Tech.). After being scanned and passing the scanning lens, the beam was reflected by a dichroic beam splitter and passed the tube lens. The beam then passed the TPEF dichroic beam splitter (FF665, Semrock) and was fed into the back aperture of the objective (W 60X, 1.0NA, Olypus,) to stimulate the sample. The stimulated TPEF signals were delivered through the objective, reflected by the TPEF dichroic beam splitter, and recorded by
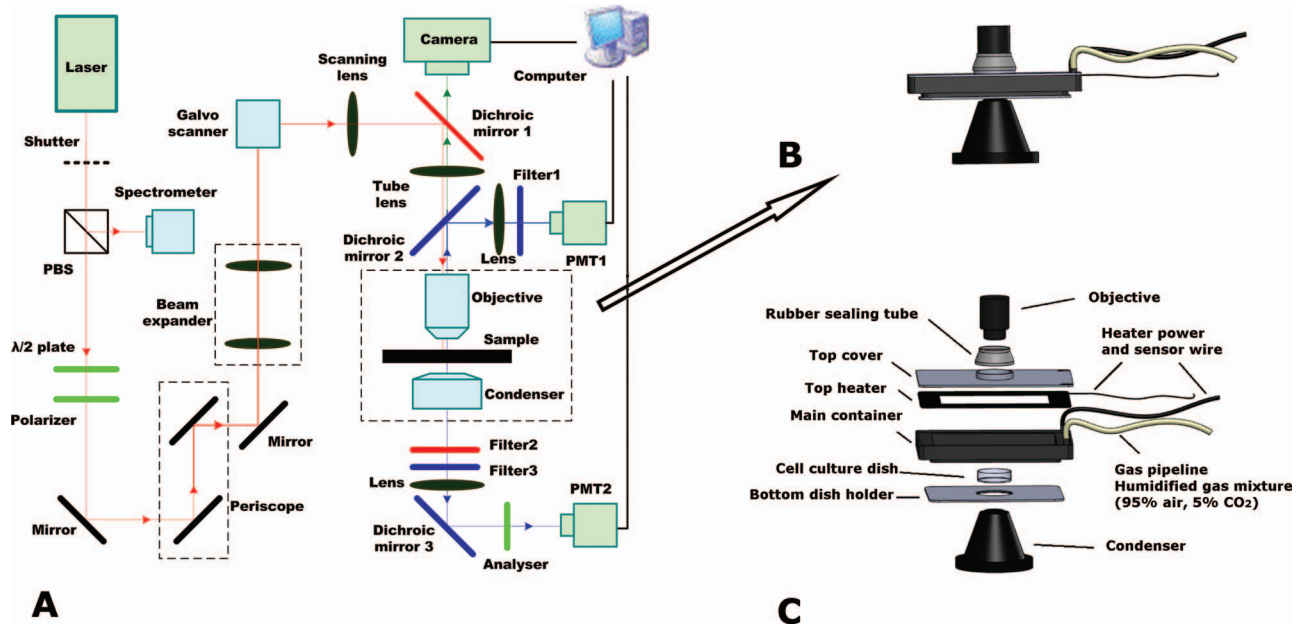
Fig. 1 Schematic of the hybrid TPEF-SHG microscope with an on-stage incubator: (a) Schematic of the imaging system; (b) Location of the on-stage incubator; (c) Components of the on-stage incubator.

a photomultiplier tube (PMT1, H7422p-40, Hamamatsu) with an IR filter (Filter1, FF01–720, Semrock). The SHG signals were collected from the forward direction through an Olympus 1.4 NA oil immersion condenser. The same types of PMT (PMT2) and IR filter (Filter2) were used in addition to a 405 $\pm$ 10 nm bandpass filter (Filter3, FF01-405/10-25, Semrock). The microscope's XY stage and the objective were controlled respectively through three orthogonally mounted motors (MP-285, Sutter). The microscope was equipped with a ThorLab microscope illumination unit (M530L2) with a polarizer, an EXPO fluorescence-illumination system (X-cite 120Q450), and an Andor EM-CCD camera (DU-888E-C00-#DZ) so that it could be used individually as a standard polarized microscope or conjunctionally as a conventional fluorescence microscope.

The lateral scan for 3D imaging was achieved with a pair of orthogonal galvanometers, which bidirectionally raster-scanned the fs laser beam to construct the sectional image. To implement high speed scanning, the turning regions of the triangle waves were smoothed by our custom-designed waveform. The laser scanning, data acquisition (PCI6115, NI), and the optical-shutter switch were controlled using custom-built, LABVIEW-based software developed in our lab. The scanning range was calibrated using a standard-resolution target (Fluorescent USAF 1951, Edmund Optics). The resolution was estimated by scanning a 0.2-μm fluorescent bead, imaging it through the TPEF channel, and fitting the data to the theoretical-point-spread function. The lateral resolution was estimated to be 0.47 μm, and the axial resolution was estimated to be 1.2 μm. The scanning speed was 4 s/frame (spf, 512$\times$512 pixels), and the data were acquired from both channels simultaneously; different frames acquired in one single image-acquisition trial were saved in an image virtual stack. The image virtual stack was then processed by IMAGEJ software (http://rsbweb.nih.gov/ij/).

Although no energy is absorbed during generation of second harmonics,[18] the incident laser may damage cardiomyocytes.[11]

In our experiment, we proved that an incident-laser power of 2.8 mW (adjusted by a pair of polarizers) was not damaging to the cultured neonatal cardiomyocyte at days 1 to 5. The sample was scanned repeatedly, and the images were acquired continuously at the rate of 4 spf in each image-acquisition trial. Approximately 10 to 20 frames were recorded in one virtual stack.

An electric CO_2 microscopic stage incubator (H301-TC1-HMTC, 2GF-MIXER, Okolab) was incorporated into the TPEF-SHG imaging system [Fig. 1(b)]. The glass-bottom culture dish that was used for cardiomyocyte culture was mounted on the on-stage incubator during real-time imaging of live neonatal cardiomyocytes. The temperature inside the incubator was maintained at 37°C, and a mixture of CO_2 (5%) and air (95%) was passed through a humidifying module and pumped into the incubator. The gap between the objective lens and the top cover of the on-stage incubator was sealed with a rubber sealing tube to maintain a stable temperature inside the incubator and to minimize leakage of the gas mixture.

Three-day-old neonatal rats were euthanized by decapitation according to procedures approved by Clemson's IACUC. The heart was isolated and minced into 1 mm^3 pieces with scissors. The tissue pieces were digested with enzyme solution (0.14 mg/ml trypsin-NO EDTA in PBS solution) overnight and then shaken at 75 rpm in the collagenase solution (PBS with 1 mg/ml Collagenase II, GIBCO; 0.24 unit/ml Neutral Protease, Worthington) for 1.5 h. The fibroblasts were removed from the cell solution using an adhesive assay by incubating the cell solution in a 150-cm^2 flask with culture medium (DMEM, HyClone; 20% Fetal bovine serum, HyClone; 1% Penicillin/Streptomycin, Fisher Scientific) for 2 h at 37°C. The cardiomyocytes were diluted to a concentration of 100 k cells/ml. One milliliter of cell solution was seeded into a 35-mm culture dish with a glass bottom coated with laminin (20 μg/ml). The cells were cultured in a conventional incubator (37°C and 5% CO_2). The culture
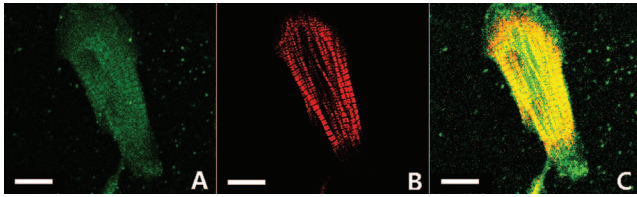
Fig. 2 2D image of *in vitro* cultured neonatal cardiomyocyte (day 3): (a) TPEF image of a live cardiomyocyte with DiO-stained membrane (green); (b) Simultaneous SHG image of the same cell, showing sarcomeric structure (red); (c) Combination of images (a) and (b) (scale bars: 10 μm), showing the relationship between the frontier cell membrane and the myofibril tip.

medium was changed after 24 h to remove dead cells and then was changed every 2 days.

The cell membrane was stained with DiO (Invitrogen) before imaging: The serum-culture medium in the culture dish was removed. The cells were washed with warm PBS solution and then rinsed again with serum-free medium (DMEM, 1% penicillin/streptomycin). Then, 1 ml DiO solution (5 μl DiO per ml serum-free medium) was added to the culture dish, and the cells were incubated in the dark at 37°C for 40 min. The DiO solution was removed from the culture dish, and the cells were washed 3 times with serum-free medium. Then fresh culture medium with serum was added to the culture dish.

After we cultured the cardiomyocytes for 1 to 5 days, the culture dish with DiO-stained cells was moved from the conventional incubator to the on-stage incubator of the TPEF-SHG imaging system. The cell to be imaged was first identified via the fluorescence channel of the imaging system. Then the cells were excited by the 810-nm fs laser beam. The TPEF and SHG signals from the sample were collected simultaneously through two different channels. A two-dimensional (2D) image of the cell was reconstructed from the signals acquired from both channels and displayed on the monitor. The focus was adjusted to find the SHG image of the best quality. Time-lapse images were acquired from both channels and saved at designated time intervals. The 2D images of the selected cells were reconstructed using IMAGEJ software (Fig. 2).

Figure 3 displays TPEF-SHG overlapped, time-lapse images of a myofibril structure from a cardiomyocyte on the second day in culture. These images were acquired when the myofibrils (red) had not extended to the tip of the filopodia (green) while the cell was spreading on the substrate. Each sarcomere is indicated by a white arrow. After 40 min of on-stage incubation, two additional sarcomeres appeared inside the white rectangle. The two series of white dots, which denote the loci of the myofibrils at the cell boundary, form two different inward curves respectively in Figs. 3(a) and 3(b), exhibiting the rotation of the sarcomeres in 40 min. The length of the sarcomeres indicated in Figs. 3(a) and 3(b) by white arrows was estimated using IMAGEJ software and denoted in Figs. 3(c) and 3(d). The displayed loci and indicated sarcomere lengths in Fig. 3 demonstrate that the space required by the newly added sarcomeres was gained from rotation and shrinkage of the existing sarcomeres. The SHG signal has been found to arise from the coiled rod region of myosin thick filaments.[9] Therefore, although there were no striated sarcomeric structures at the tip of the myofibril [the area denoted by the long arrows in Figs. 3(a) and 3(b)], the detection of SHG signals suggested that myosin thick filaments ex-
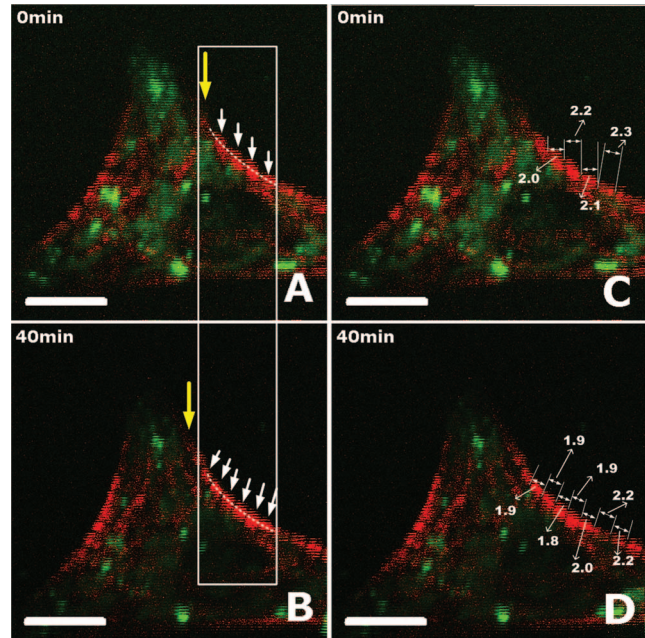

Fig. 3 Addition of new sarcomeres during myofibril growth. DiO-stained cell membranes acquired through the TPEF channel are green; myofibrils detected by SHG are red. (a) and (c) are identical TPEF-SHG overlapped images acquired at 0 min. (b) and (d) are identical TPEF-SHG overlapped images acquired 40 min after the initial image. In (a) and (b), each white arrow denotes one sarcomere; the white box in (a) and (b) shows the addition of two new sarcomeres at 40 min. In (c) and (d), sarcomeric length (μm) is shown. In the area denoted by the long arrows in (a) and (b), the SHG signal shows that striated sarcomeric structure has not yet developed. The scale bars represent 10 μm.

isted in this area before being assembled into myofibrils to form the striated sarcomeric structure. This is consistent with previous research that showed that muscle myosin bundles form separately from the formation of other myofibril components[19, 20] before the assembly of the sarcomeric structure.

In myofibrillogenesis research in which the distribution of alpha-actinin fused with GFP in live embryonic cardiomyocytes was monitored, myofibrils were found to extend more than 10 μm within several hours through the connecting and binding of newly formed myofibrils to existing myofibrils.[4] One sarcomere was found to be added in an hour to effectively compensate for an increase in the resting-sarcomere length while a strain of 10% was applied to neonatal cardiomyocytes.[21] In our experiments, two new sarcomeres were found to appear in the mature myofibril within 40 min while the cardiomyocyte was spreading on the substrate. We noticed that while the cardiomyocytes were cultured *in vitro*, the different microenvironments caused different growth rates of new sarcomeres in each cell in the same culture dish. However, the cells remained physiologically active for at least 10 h (data not shown) in our incubated imaging system, and the SHG imaging rate of our system (4 spf) had provided us sufficient time resolution for the observation of single sarcomere addition.

Our study demonstrates that our TPEF-SHG imaging system including an on-stage incubator is effective in real-time study of long-term structural changes in cardiomyocytes. We observed the addition of new sarcomeres to the mature myofibrils that occurred within a short time. The results demonstrate that our

imaging system could be a useful tool for investigating the prolonged process of myofibrillogenesis of a single cardiomyocyte.

Acknowledgments

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