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Pulse splitter-based nonlinear microscopy for live-cardiomyocyte imaging

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Abstract

Second harmonic generation (SHG) microscopy is a new imaging technique used in sarcomeric-addition studies. However, during the early stage of cell culture in which sarcomeric additions occur, the neonatal cardiomyocytes that we have been working with are very sensitive to photodamage, the resulting high rate of cell death prevents systematic study of sarcomeric addition using a conventional SHG system. To address this challenge, we introduced use of the pulse-splitter system developed by Na Ji et al. in our two photon excitation fluorescence (TPEF) and SHG hybrid microscope. The system dramatically reduced photodamage to neonatal cardiomyocytes in early stages of culture, greatly increasing cell viability. Thus continuous imaging of live cardiomyocytes was achieved with a stronger laser and for a longer period than has been reported in the literature. The pulse splitter-based TPEF-SHG microscope constructed in this study was demonstrated to be an ideal imaging system for sarcomeric addition-related investigations of neonatal cardiomyocytes in early stages of culture.

Keywords

pulse-splitter; SHG; TPEF; live imaging; cardiomyocyte; hypertrophy

1. INTRODUCTION

Pathological cardiac hypertrophy accompanied by sarcomeric addition is an adaptive response to hemodynamic overloads. Understanding the detailed process of sarcomeric addition to existing myofibrils is important to study of the early development of pathological cardiac hypertrophy. Neonatal rat cardiomyocyte culture is a very useful model for the study of sarcomeric addition because new sarcomeric additions can be visualized during cell spread, for example, through second harmonic generation (SHG) microscopy. However, during the early stages of cell culture in which sarcomeric additions occur, neonatal

cardiomyocytes are extremely sensitive to photodamage. The only solution to this problem has been to reduce the power of the incident laser;¹⁻⁵ the tradeoff is greatly compromised image quality. Even with this precaution, internal cellular process within cardiomyocytes may still be affected.³ So far, only a few research groups have reported cardiomyocyte imaging with SHG microscopy.⁶⁻¹⁰

For many optical processes, as excitation intensity decreases, the laser-stimulated signal decreases much more slowly than the photodamage to the sample; this suggests that by reducing the intensity of a single pulse and keeping the overall power of a femtosecond laser, the ratio of signal-to-photodamage can be increased to a level suitable for live-cell imaging.¹¹ This was proved by research by Ji aimed at reducing photobleaching and photodamage in TPEF experiments using passive pulse-splitters.¹² The success of Ji's experiment suggests that the same method can be applied to SHG microscopy, in which the relationship of the signal and photodamage to laser intensity is the same as in TPEF.^{13, 14}

Recently, we have constructed a TPEF-SHG hybrid imaging system and have been exploring its application to dynamic imaging at the subcellular level.^{5, 15-20} SHG is intrinsic to noncentrosymmetric structures, which exist in biological samples such as the coiled-rod myosin filaments within cardiomyocytes. TPEF is fluorescence-based and can be used to reveal additional structural information in live-cell imaging. When a solution to the problem of photodamage exists, the combination of TPEF and SHG microscopy is an ideal imaging tool for sarcomeric-addition studies.^{9, 21} In this study, we added Ji's pulse-splitter unit to our TPEF-SHG hybrid imaging system; data obtained with the modified microscopy showed that the system was suitable for live-neonatal-cardiomyocyte imaging.

2. METHODS

2.1 TPEF-SHG imaging system

Our TPEF-SHG hybrid microscope is described in our previous publications.¹⁻⁴ The only change in the optical setup is the introduction of a passive pulse-splitter upstream of the incident beam. The optical setup of the modified system is schematically shown in Figure 1A. Briefly, a femtosecond (fs) laser beam from a Ti: Sapphire laser (Tsunami 3960-X1BB pumped by a 10W Millennia, Spectra-Physics, 100 fs and 80 MHz) was tuned to 830nm and collimated to a 64X passive pulse-splitter unit. The output beam was then expanded to double the beam diameter and directed at a 2-axis galvo-scanner (6210H, Cambridge Tech). The scanned beam was collected by a scanning lens and expanded again to double the beam diameter with a tube lens so that the diameter was slightly bigger than that of the back aperture of the objective (Olympus 60X 1.0NA water immersion objective). From the tube lens, the beam went through a dichroic mirror (FF665, Semrock) and was focused on the sample by the objective. The excited TPEF signal was collected by the same objective and reflected by the dichroic mirror to a short-pass filter (Filter1, FF01-720/SP-25, Semrock). The filtered light was then recorded by a photomultiplier tube (PMT1, H7422p-40, Hamamatsu). The SHG signal was collected by a forward Olympus 1.4NA oil immersion condenser and filtered by another band-pass filter (Filter2, FF01-414/46-25, Semrock). The filtered light was then recorded by another photomultiplier tube (PMT2) that is the same type as PMT1.

2.2 Passive pulse-splitter design

The passive pulse-splitter design was based on the description in Ji's publication.¹² The optical scheme of the pulse-splitting unit is shown in Figure 1B-C. The fundamental 4X pulse-splitting subunit consisted of two mirrors separated by a low dispersion fused-silica plate and a layer of air. The front surface of the fused-silica plate that touched the layer of air was coated with half-transmission and half-reflection optical coatings to form a beam splitting interface. The back surface of the plate was coated with transmission-enhancing coatings. When one beam entered the 4X pulse-splitting unit from the splitting interface at the designed angle, it was divided into two subbeams, which were then reflected by the two precisely separated mirrors. The thicknesses of the fused-silica plate and the layer of air were designed to assure that the reflected subbeams met at the same point on the interface so that only two subbeams would be formed after the two reflected beams were split by the interface. Thus, the 4X pulse-splitter split one beam with a single pulse train into two beams, each with two temporally separated pulse trains. By incorporating another incident beam on the other side of the 4X pulse-splitter as shown in Figure 1C, it was possible to split two beams, each with one pulse train that was a phase shift from the other, into two beams each with four temporally separated pulse trains. The 64X pulse-splitter unit consisted of two half-transmission, half-reflection beam splitters and two 4X pulse-splitter subunits to achieve a 64X pulse-splitting function. The two 4X pulse-splitter subunits were designed to have different internal pulse delays, and the paths of light were carefully designed to avoid overlay of the subpulses. The two exit beams from the second 4X pulse-splitter were recombined into one beam by a polarized beam splitter. The half-wave plate in the path of the side beam was used to tune the polarization of the side beam to achieve optimal output.

2.3 Primary cell culture

Neonatal rat ventricular cardiomyocytes (NRVMs) were dissociated from ventricles of three-day-old Sprague-Dawley rats using a two-day protocol. Briefly, neonatal rats were euthanized by decapitation following a procedure approved by Clemson University Institutional Animal Care and Use Committee and their hearts were collected. The collected hearts were minced into small pieces in trypsin solution with autoclaved scissors and then stored in 4 °C refrigerator for digestion overnight, which was terminated by the addition of trypsin-inhibitor solution the next day. Subsequently, collagenase type II solution was added for further digestion, and the mixture was shaken at 50 rpm for 1.5 hours in a water bath. After that, the mixture was filtered through a 70µm pore-size film to remove imperfectly-digested heart tissue. The filtered solution was then placed under a biological hood for 40 minutes, after which cells were precipitated in a centrifuge at 800rpm for 3 minutes. The cell pellet was resuspended in 20ml cell-culture media. Fibroblasts adhere to substrate more quickly than cardiomyocytes; the fibroblasts were removed from the mixture by incubation. A 2-hour incubation period was sufficient to remove most fibroblasts and achieve a purity of 90 percent pure cardiomyocytes. A small aliquot of the NRVMs were then separated and stained with DiO for cell membrane. The stained and unstained cardiomyocytes were mixed and seeded onto a glass-bottom petri dish. Culture media was changed daily and before imaging.

2.4 Cell-viability assessment

Cell viability was assessed with a method similar to Liu's, using the sarcomeric structure within a cardiomyocyte as an indicator of potential photodamage caused by laser exposure.³ If the sarcomeric structure was lost after a designated period of laser exposure at a particular (tested) laser intensity, that intensity was considered harmful to cell viability. During the assessment, continuous scanning of a cell for 80 seconds was used as an acquisition trial. A total of 5 acquisition trials were performed during a 1 hour test of laser power, once every 15 minutes. Tested laser power was from 10mW to 50mW, with a 10mW increment. Cardiomyocytes that were cultured for 2 days were used in the assessment because these cardiomyocytes had clearly visible sarcomeric structure and were still sensitive to photodamage. Image of sarcomeric structure taken in the last trial was compared with that taken in the first trial and a 30 percent SHG signal loss was considered as cell damage. An optimum laser power was proposed according to the results of the assessment and was used in the following live-cardiomyocyte imaging.

2.5 Live-cardiomyocyte imaging

Cardiomyocytes were first cultured on glass-bottom petri dishes in regular incubator for 22 hours and were then transferred to on-stage incubator, which was mounted on the TPEF-SHG hybrid microscope. On-stage incubator could provide the same culture environment (5% CO₂, 95% air, RH 95%) as that in regular incubators. A DiO-stained cardiomyocyte surrounded by unstained cardiomyocytes was selected for live-cell imaging so that the shape of the cardiomyocyte of interest could be delineated with fluorescence images reconstructed with signals from the TPEF channel. After 1 hour stabilization, images of the selected cardiomyocyte were taken hourly at the optimum laser intensity determined in the cell viability assessment. During each acquisition, 10 frames of the same layer of the cardiomyocyte were collected and averaged to get a final image. During the experiment, 500μl culture media was added to the petri dish every other hour to compensate for media loss caused by evaporation.

3. RESULTS

Sub beams within the 64X pulse-splitter unit were well aligned when they left each pulse-splitting component and subunit. Each recombined beam was required to have a spot coincidence of less than 1mm after 10 meters' propagation in free space. The lateral resolution of the modified system was measured with 0.2μm fluorescent beads, and it was estimated experimentally to be 0.55μm, sufficient for sarcomeric imaging. Cardiomyocyte viability was assessed as designed for 5 laser powers (10mW, 20mW, 30mW, 40mW, and 50mW). Results showed that there was no noticeable sarcomeric loss during cardiomyocyte examination for laser powers under 50mW. Therefore, all laser powers that were less than 40mW (including 40mW) were considered safe for live-cardiomyocyte imaging. To avoid potential interference with internal cellular processes within cardiomyocytes, 20mW was chosen as the optimum laser power for live-cell imaging, which was used in the subsequent long term live-cardiomyocyte imaging experiment.

A rod-shaped DiO-stained cardiomyocyte was selected for live-cell imaging using the modified TPEF and SHG hybrid imaging system and the imaged cardiomyocyte survived the repeated laser exposure throughout 24 hours. The sarcomere assembly process was captured within this period and the changes of cell shape and sarcomeric structure in the first 7 hours are shown in Figure 2. The shape of the selected cardiomyocyte was clearly visualized in images reconstructed from the TPEF channel and sarcomeric structure within the cardiomyocyte became increasingly clear along with time in images reconstructed from the SHG channel. The normality of the sarcomere assembly process over time demonstrated that internal cellular process was not interfered in live-cell imaging with the pulse splitter-based TPEF-SHG hybrid imaging system.

4. DISCUSSION

The lateral resolution achieved in our current study was $0.55\mu\text{m}$, which was sufficient to observe sarcomeres, the average length of which was approximately $2\mu\text{m}$.^{1, 22} However, the lateral resolution was slightly lower than was reported in our previous publications ($0.47\mu\text{m}$).²³ This possibly resulted from the misalignment of the subbeams during recombination. Even though the subbeams were adjusted to ensure that the recombined beam had a sufficiently small spot coincidence after a sufficiently long propagation path, the parallelism of the recombined beam was not comparable to that of the original beam. The misalignment of the subbeams was then converted by the objective to focus offsets in the focal plan, thus damaging the lateral resolution.

The efficacy of the pulse-splitter strategy for reducing photodamage and keeping signal generation was proved in current study and the results complied with what had been report in Ji's research.¹² Safety laser power for live-cardiomyocyte imaging with TPEF and SHG microscopy was increased to 40mW by introducing a 64X pulse-splitter, which was thirteen times larger than was reported in our previous studies (2.8mW).³ Even at the optimum laser power determined in our previous studies the internal cellular process was likely to be interfered or terminated by the repeated laser exposure. In addition, exposure to laser beams that had a power of 5mW or greater would result in immediate cell death. In current study, the inclusion of a 64X pulse-splitter in the same imaging system enabled the reservation of the sarcomere assembly process within a cardiomyocyte even at a much larger laser power.

The selected cardiomyocyte was live-imaged with the modified microscope for a total of 27 hours. The imaged cardiomyocyte remained active in the first 24 hours; however, from the 24th hour on, it started to degenerate and lose its sarcomeric structure gradually and eventually died at the end of the 27th hour. The death of the cardiomyocyte was believed to be induced by the increasingly tougher exterior environment with increasing osmolality caused by media evaporation and accumulated metabolic waste. A method of changing media during live-cell imaging was supposed to be devised to solve this problem in future experiments. We believed that live-cardiomyocyte imaging with the pulse splitter-based TPEF-SHG microscope could last a longer period once the problem was solved.

5. CONCLUSIONS

In this study, we successfully introduced a pulse-splitter unit into our TPEF-SHG hybrid microscope. Using the pulse-splitting strategy, photodamage to neonatal cardiomyocytes in early stages of culture was dramatically reduced and cell viability was greatly increased. Continuous imaging of live cardiomyocytes was achieved with a stronger laser and for a longer period than has been reported in previous literature. The pulse splitter-based TPEF-SHG microscope constructed in this study was demonstrated to be an ideal imaging system for sarcomeric addition-related investigations of neonatal cardiomyocytes in early stages of culture.

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REFERENCES

1. Liu H, Qin W, Shao Y, Ma Z, Ye T, Borg T, Gao BZ. Myofibrillogenesis in live neonatal cardiomyocytes observed with hybrid two-photon excitation fluorescence-second harmonic generation microscopy. *J Biomed Opt.* 2011; 16(12):126012. [PubMed: 22191929]
2. Liu HH, Qin W, Shao YH, Liu QY, Ma Z, Borg TK, Gao BZ. Live Cardiomyocyte Imaging via Hybrid TPEF-SHG Microscopy. *Imaging, Manipulation, and Analysis of Biomolecules, Cells, and Tissues.* 2012; X:8225.
3. Liu HH, Shao YH, Qin W, Runyan RB, Xu MF, Ma Z, Borg TK, Markwald R, Gao BZ. Myosin filament assembly onto myofibrils in live neonatal cardiomyocytes observed by TPEF-SHG microscopy. *Cardiovasc Res.* 2013; 97(2):262–270. [PubMed: 23118131]
4. Liu HH, Qin W, Shao YH, Wang ZH, Yang HX, Runyan RB, Borg TK, Gao BZ. Disassembly of Myofibrils in Adult Cardiomyocytes during Dedifferentiation. *Imaging, Manipulation, and Analysis of Biomolecules, Cells, and Tissues Xi.* 2013:8587.
5. Qin W, Shao YH, Liu HH, Qu JL, Peng X, Niu HB, Gao BC. Multifocus nonlinear optical microscopy based on SLM. *Multiphoton Microscopy in the Biomedical Sciences Xii.* 2012:8226.
6. Martin TP, Norris G, McConnell G, Currie S. A novel approach for assessing cardiac fibrosis using label-free second harmonic generation. *Int J Cardiovasc Imaging.* 2013; 29(8):1733–40. [PubMed: 23921804]
7. Caorsi V, Toepfer C, Sikkell MB, Lyon AR, MacLeod K, Ferenczi MA. Non-linear optical microscopy sheds light on cardiovascular disease. *PLoS One.* 2013; 8(2):e56136. [PubMed: 23409139]
8. Awasthi S, Matthews DL, Li RA, Chiamvimonvat N, Lieu DK, Chan JW. Label-free identification and characterization of human pluripotent stem cell-derived cardiomyocytes using second harmonic generation (SHG) microscopy. *J Biophotonics.* 2012; 5(1):57–66. [PubMed: 22083829]
9. Wallace SJ, Morrison JL, Botting KJ, Kee TW. Second-harmonic generation and two-photon-excited autofluorescence microscopy of cardiomyocytes: quantification of cell volume and myosin filaments. *J Biomed Opt.* 2008; 13(6):064018. [PubMed: 19123664]
10. Boulesteix T, Beaupaire E, Sauviat MP, Schanne-Klein MC. Second-harmonic microscopy of unstained living cardiac myocytes: measurements of sarcomere length with 20-nm accuracy. *Opt Lett.* 2004; 29(17):2031–3. [PubMed: 15455770]
11. Saytashev I, Arkhipov SN, Winkler N, Zuraski K, Lozovoy VV, Dantus M. Pulse duration and energy dependence of photodamage and lethality induced by femtosecond near infrared laser pulses in *Drosophila melanogaster*. *J Photochem Photobiol B.* 2012; 115:42–50. [PubMed: 22840378]

12. Ji N, Magee JC, Betzig E. High-speed, low-photodamage nonlinear imaging using passive pulse splitters. *Nat Methods*. 2008; 5(2):197–202. [PubMed: 18204458]
13. Campagnola PJ, Clark HA, Mohler WA, Lewis A, Loew LM. Second-harmonic imaging microscopy of living cells. *J Biomed Opt*. 2001; 6(3):277–86. [PubMed: 11516317]
14. Tang S, Krasieva TB, Chen Z, Tempea G, Tromberg BJ. Effect of pulse duration on two-photon excited fluorescence and second harmonic generation in nonlinear optical microscopy. *J Biomed Opt*. 2006; 11(2):020501. [PubMed: 16674172]
15. Qin W, Shao YH, Liu HH, Peng X, Niu HB, Gao B. Addressable discrete-line-scanning multiphoton microscopy based on a spatial light modulator. *Opt Lett*. 2012; 37(5):827–829. [PubMed: 22378407]
16. Shao YH, Liu HH, Qin W, Qu JL, Peng X, Niu HB, Gao BZ. Addressable, large-field second harmonic generation microscopy based on 2D acousto-optical deflector and spatial light modulator. *Applied Physics B-Lasers and Optics*. 2012; 108(4):713–716.
17. Shao Y, Qin W, Liu H, Qu J, Peng X, Niu H, Gao BZ. Multifocal multiphoton microscopy based on a spatial light modulator. *Applied Physics B-Lasers and Optics*. 2012; 107(3):653–657.
18. Shao YH, Qin W, Liu HH, Qu JL, Peng X, Niu HB, Gao BZ. Addressable multiregional and multifocal multiphoton microscopy based on a spatial light modulator. *J Biomed Opt*. 2012; 17(3)
19. Shao YH, Qin W, Liu HH, Qu JL, Peng X, Niu HB, Gao BZ. Ultrafast, large-field multiphoton microscopy based on an acousto-optic deflector and a spatial light modulator. *Opt Lett*. 2012; 37(13):2532–2534. [PubMed: 22743445]
20. Qin W, Shao YH, Liu HH, Peng X, Niu HB, Gao BZ. 1D-Scanning Addressable Multiregional Multifocal Multiphoton Microscopy. *Ultrafast Imaging and Spectroscopy*. 2013:8845.
21. Wallenburg MA, Wu J, Li RK, Vitkin IA. Two-photon microscopy of healthy, infarcted and stem-cell treated regenerating heart. *J Biophotonics*. 2011; 4(5):297–304. [PubMed: 20827682]
22. Yu JG, Russell B. Cardiomyocyte remodeling and sarcomere addition after uniaxial static strain in vitro. *J Histochem Cytochem*. 2005; 53(7):839–44. [PubMed: 15995142]
23. Shao YH, Liu HH, Ye T, Borg T, Qu JL, Peng X, Niu HB, Gao B. 3D Myofibril Imaging in Live Cardiomyocytes via Hybrid SHG-TPEF Microscopy. *Multiphoton Microscopy in the Biomedical Sciences Xi*. 2011:7903.

Figure 1. (A) Optical schematic of modified TPEF-SHG hybrid imaging system. (B) Optical schematic of the 64X passive pulse-splitter. (C) Optical schematic and constitution of a 4X pulse-splitter unit.¹²

Figure 2.

Sarcomere assembly process and cell shape changes in the first 7 hours of live-cardiomyocyte imaging. (A) Changes of cell shape and sarcomeric structure in the first 3 hours. (B) Changes of cell shape and sarcomeric structure from the 4th hour to the 7th hour. DiO-stained cell membrane was recorded in the TPEF channel and sarcomeric structure was recorded in the SHG channel. Cell membrane was manually assigned red and sarcomere was manually assigned green in the merged images. Scale bars=10 μ m.