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Monitoring drug induced changes in cardiomyocyte contractility with second harmonic generation (SHG) microscopy

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ABSTRACT

The cost of taking a drug to market can exceed $2 billion dollars. The escalating cost of drug discovery is a major motivating factor for seeking new methods to predict the safety and efficacy of new compounds as early as possible in the drug development process to avoid drug attrition during late phases of clinical trials or even the withdrawal of approved drugs. Cardiotoxicity accounts for nearly 30% of US post-marketing drug withdrawal and remains a major concern to the point where the US Food and Drug Administration (FDA) is focused on in vitro cardiotoxicity screening to minimize cardiac risks associated with drugs. A technique that can directly quantify interactions between drugs and cardiomyocytes without the interference from exogenous genetic or chemical labels would be highly beneficial for directly screening these new drugs.

Our group has previously shown that second harmonic generation (SHG) signals generated from myosin filaments in cardiomyocytes can be used as a robust label-free optical technique for recording cell shortening dynamics at high spatial and temporal resolution due to the ability of the myosin rod domains in heart muscle cells to emit the frequency-doubled light. The dynamics is recorded without adding any fluorescent labels that may otherwise affect and modify the natural cell contractility of the cell. In this study, we investigated the use of SHG microscopy for measuring drug-induced changes in cardiac cell contractility and discuss its feasibility as a tool for screening drugs and evaluating cardiotoxicity.

Keywords: cardiotoxicity, iPSC, cardiomyocyte, iPSC-CMs, second harmonic generation, SHG, myosin, drug screening assay

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1. INTRODUCTION

Putting a new therapeutic drug on the market is not easy and the discovery process typically costs an enormous monetary figure. On average, even top pharmaceutical firms like Amgen Inc., Novartis, Bristol-Meyers Squibb Co. need to spend more than $ 3 billion dollars per drug in order to successfully launch a prescription medicine. The first of all critical steps during the pre-clinical development is to investigate the effects of these newly synthesized drug candidates at the cellular level in petri dish. This type of in vitro discovery "loop" progresses with identifying and validating targets relevant to the disease, optimizing a hit to lead process to find promising lead compounds, and then profiling the efficacy and safety of the leads by systematically studying their distribution, drug metabolism, and pharmacokinetics (DMPK) repeatedly before animal and human trials. Comprehensive toxicity assessments during this stage of the development include investigating the drug-induced toxicity to brain, liver, kidney, and heart. In fact, not only the adverse drug-induced effect on heart (also called cardiotoxicity) is the main cause for the trial discontinuation at any phase of the discovery process, it accounts for almost one third of the post-market drug withdrawal in the US in the past three decades. It is particularly challenging for some drugs aimed to be chronically administered prescription drugs as they simply do not show any sign of cardiotoxicity until it has had long-term acumination in the heart. Bioanalytical approaches that enables researchers to evaluate toxic responses of cardiac cells are much needed for evaluating both short-term and long-term effects early at the in vitro screening phase. Much more importantly, an ex vivo cellular source that can be manufactured on a large scale and also reflects the physiological features for animals or humans that the drugs are designed to treat is critical for this type of screening process. After all, the goal is to see the effects of drugs at the cellular level that might only show later in the clinical trials in the in vitro testing and hopefully it will reduce the time frame for getting a drug approved, usually a 10-15 year long process.

The year 2020 marks the 14th anniversary of the innovation of induced pluripotent stem cells (also abbreviated as iPSCs), a molecular biology technique capable of converting adult cells from individual patients into pluripotent states and then generating specialized cell type such as cardiomyocytes (iPSC-CMs). For such a short period of time since its invention, it has opened up a whole new window for researchers to explore applications in disease modeling, drug screening, and even cardiac transplantations for repairing damaged hearts [1-4]. Not only the cell lines can be generated from specific patients, it can essentially generate an unlimited amount of cells for laboratory testing. Perhaps the most uplifting discussions in the drug developments have focused on whether or not we can directly test the effects of therapeutic leads directly on these types of cells at the pre-clinical tests to find out any adverse effects that can only be seen during later clinical trials [5]. In addition, cells can be initially taken from both healthy and diseased patients for comparing the differences in drug responses to the same drug for the purpose of the disease modeling. In combination with genetic editing technologies such as clustered regularly interspaced short palindromic repeats (abbreviated as CRISPR), cells can also be programed to show or suppress certain immune activities for the purpose of cardiac tissue engineering and transplantation [6].

The cardiac contraction is the result of a series of cellular physiological events that can be summarized by the excitation-contraction couplings scheme [7,8]. It is a process in which the ion flux of different types of ions such as calcium, potassium, and sodium alters the net membrane voltage and consequently the cell changes the binding site of myosin bundle along the actin filament. The sliding of the binding sides along these two filaments therefore results in a shortening of the cardiac muscle fiber. In vitro cardiotoxicity tests aim to investigate how the drug affects the ionic flux, membrane potential, cardiac rhythms, and contractility. For monitoring ionic flux within cells, many fluorescent indicators have been developed to show fluorescence once these tags bind to target ions. For example, potassium ionic transients can be monitored by Potassium-binding benzofuran isophthalate (PBFI) [9]. Intracellular sodium level can be detected using sodium-binding benzofuran isophthalate (SBFI) [10] by exciting the cell with 340 nm light. If the excitation source if not convenient for the experimental procedure, one can also choose the conventional FITC probe with CoroNa green [11]. There are more fluorescent indicators available for determining calcium concentrations with a variety of selections at different spectral window such as fura-2, fura-4, fura-red, fluo-3, fluo-4, BAPTA, Calcium Green, and X-rhod-1 [12]. Cardiac automaticity and membrane voltage, sometimes called action potential (AP), are important physiological parameters for monitoring net cellular membrane voltage change and the rhythm regulation. Cardiac arrhythmia can be accurately monitored by the change in the fluorescence intensity of voltage sensitive dyes with highly sensitive detectors even when the iPSC-CMs are not contracting at an observable scale. The traditional patch clamp technique can be used to directly record the real time voltage change cell-by-cell with the electrical circuit

through electrodes under microscopes. The modern development of voltage-sensitive fluorescent probes such as FluoVolt and Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol (DiBAC4(3)) also allows researchers to detect the increase and decrease of the membrane voltage by recording the fluorescence intensity from these dyes over time. The response time for FluoVot even reaches a sub-millisecond time range [13,14]. Optical imaging through fluorescent probes also allows researcher to design assays to systematically study these physiological events. With automated microscopes, high content screening platforms can now generate image stacks with a resolution up to ~125 frames per second for monitoring these cellular events and provide the multi-parametric analysis on a large field of view at the single-cell level.

However, assessing the mechanical output with the quantitative cell shortening information for individual cells is a much more challenging task when studying the contracting performance of cardiac cells. Moreover, there is also a significant difference between primary adult and stem cell-derived cardiomyocytes when investigating cardiac contractions due to the drastic difference in myosin filament development. For primary animal cardiomyocytes, a non-invasive imaging techniques is well-established by doing fast Fourier transform analysis on video frames of contracting cells. The sarcomere of health primary cardiomyocytes typically show a striated structure under the bright-field microscope. The transverse pattern of the bright-field image is due to the periodic alternations of isotropic (I band, light patterns) and anisotropic (A band, dark patterns) bands on sarcomeres. The fast Fourier transform algorithm determines the average sarcomere length from these striated patterns and the software plots the sarcomere length over time during the contractions at 10K Hz imaging rates. Several commercial optical systems based on fast Fourier transform technique, such as SarcOptiM, IonOptix, and 1600A permeabilized Myocyte Test System made by Aurora Scientific have been able to generate robust analyses to simultaneously monitor the cellular contractility and physiological events of primary cardiac cells isolated from animals [15]. Unfortunately, the striated patterns are typically nonexistent in the induced pluripotent stem cell derived cardiomyocytes under the bright-field microscope, presumably due to the underdevelopment of the myofilament (**Figure 1**). Several alternative methods for estimating mechanical outputs are recently developed to overcome the lack of sarcomere striations by using indirect measurements. In one design, cells were replated on elastic hydrogel substrates, in which the substrate contains immobilized fluorescent beads [16]. The contractility was then indirectly estimated by analyzing the movements of beads under the fluorescent microscope. A complex computing algorithm is then certainly required to analyze the moments of many beads beneath the cell in order to generate an index for representing the contractility. Another design utilized genomic sequencing techniques to label filamentous actin (F-actin) in the muscle cells with fluorescent markers [17,18]. This new 17-amino-acid peptide was called Lifeact and can be used to generate fusion protiens with fluorescent probes like GFP and mCherry. Although this breakthrough allows a direct visualization of the contraction by simply analyzing the change in gap space between striations, many groups suspected the genetically added fluorophores might have changed the nature of the physiological events in these iPSC-CMs [19]. Not to mention the effects of drugs could be hindered by the potentially intractable interactions between markers, drugs, and cells from different. The ideal tool for studying the cardiac contractility and the associated drug-induced change would be a platform that is capable of directly reflect the change in cell shortening without exogenous fluorescent labels even when the cells are lacking the sarcomere striation.

In this study, we demonstrated a method for using SHG microscopy to directly estimate the drug-induced changes in contractility of iPSC-derived cardiomyocytes. This method not only was able to visualize the sarcomere contraction at the sub-micron resolution with an intuitive observation, potential influences caused by chemical or biological fluorescent probes between the interaction of the drug and cells were totally eliminated.

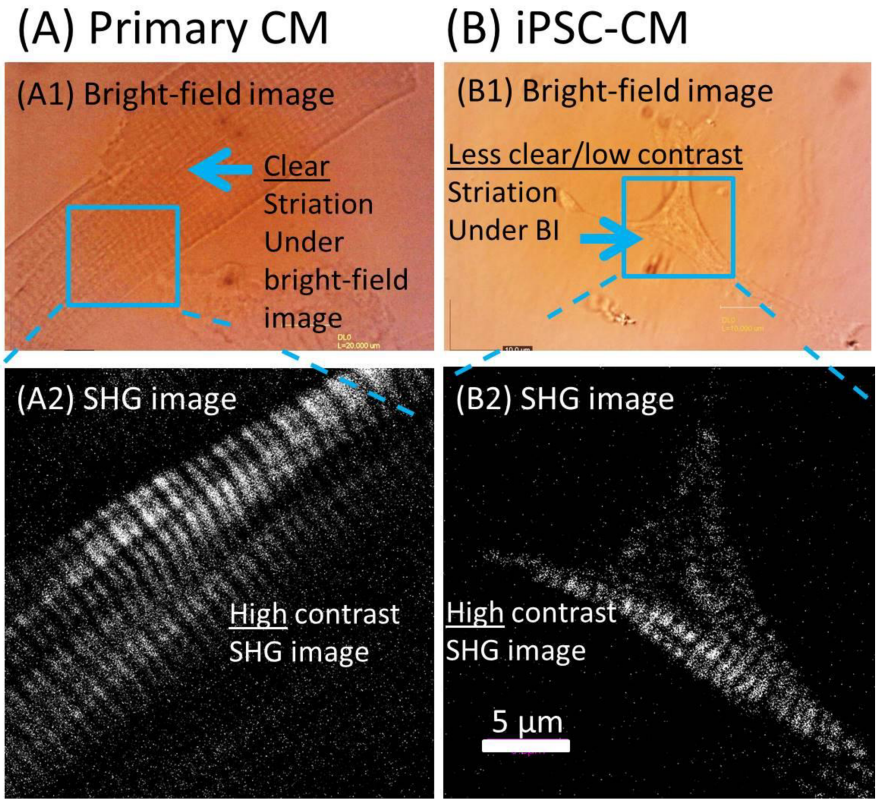


Figure 1. The difference in sarcomere striation under the bright-field microscope (A1, B1) and SHG microscope (A2, B2) between a primary cardiomyocyte and a human induced pluripotent stem cell derived cardiomyocyte. The repeated patterns in SHG images indicate the locations of myosin bundle within the cells.

2. METHODS

2.1 Preparations of induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs)

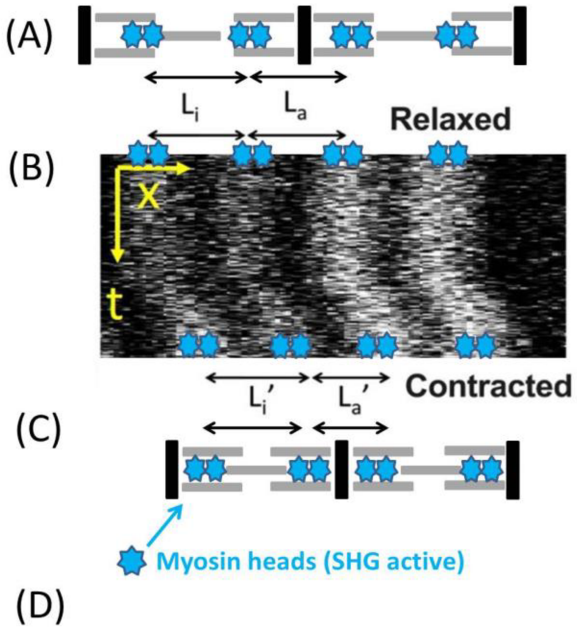
Induced pluripotent stem cell derived cardiomyocytes through directed differentiation with a total maturity of 68 days were used for experiments, in which cells with a maturity of 50 days post differentiation were replated on petri dishes for another 18 days. The details were published following a similar protocol published by co-authors of this article [20]. In short, the 6-9-9T iPSCs from WiCell were culture in a xeno-free cell culture media iPS-Brew XF from Miltenyi Biotech on Engelbreth-Holm-Swarm (EHS) mouse sarcoma based metrigel made by Corning and then cells were passaged using an enzyme-free reagent ReLeSR from Stem Cell Technologies. From day 0-2, the directed differentiation proceeded by treating cells with 6-µM glycogen synthase kinase GSK-3 inhibitor CHIR99021 from Tocris; from day 3-5, cells were treated with 5-µM Wnt pathway inhibitor IWR-1 from Tocris in Roswell Park Memorial Institute (RPMI) 1640 medium with Lglutamine, B-27 supplement without insulin, and dual antibiotics penicillin-streptomycin from. After day 7, iPSC-CMs were maintained in RPMI 1640 with L-glutamine, B-27 supplement, and insulin until day 50 before dissociation with the recombinant cell-dissociation enzymes TrypLE from to Invitrogen to replace porcine trypsin. Cells were further replated as single cells on matrigel-coated glass bottom dishes for 18 days prior to optical measurements. During the second harmonic generation (SHG) recording, the culturing media was replaced with Tyrode's solution and the temperature of media was at 37 degree Celsius.

2.2 Live-cell second harmonic generation (SHG) imaging and shortening analysis

SHG is nonlinear optical phenomena that happens as the intense photons within an ultrashort laser pulse propagating through the non-centrosymmetric materials [21,22]. While the laser passing through the SHG active material, two incident photons within the pulse are combined in the material and then a photon with the energy twice the incident photons is emitted. Ordered non-centrosymmetric cellular structures such as certain types of collagens, myosin bundles in striated muscles, and microtubules are biomolecular structures that are SHG-active [23-25]. Therefore, the SHG signal with ½ λ can be used for the label-free imaging when the excitation laser with a wavelength λ passes through cardiomyocytes. As myosin is capable of emitting SHG signals, fluorescence labels are not required for this type of imaging. It therefore provides a label-free approach to study cardiac physiology that is free of the influence from exogenous fluorescent markers. Moreover, SHG microscopy is intrinsically confocal with a sub-micron lateral resolution as the nonlinear optical process only happens at the focal plane. SHG microscopy also offers three other advantages over conventional fluorescent imaging by eliminating photobleaching, enabling the deep-tissue imaging, and adding the capability for the multicolor imaging [26]. The issue of photobleaching is eliminated because SHG imaging does not require fluorescent probes. Deep-tissue imaging is possible because the wavelength of excitation light is twice longer than the conventional one-photon excitation. It is known that photons with a longer wavelength suffer less in getting scattered by animal tissues, and therefore can penetrate deeper into the tissue to excite the cells. Most importantly, in order to excite an SHG material, the excitation wavelength is not restricted to a specific wavelength, and therefore creates an opportunity for simultaneously collecting SHG signals and two-photon fluorescence by using a same excitation source.

In this study, the optical layout for the instrumentation can be found in our previous publication [27]. In brief, the excitation source is a femtosecond pulsed laser operating at 80 MHz with an ultrashort pulse (~140 fs) from Coherent. The laser was tuned to 940 nm and coupled through free space onto an inverted microscope. The laser was focused onto the sample through a 60X water objective (1.2 NA) and the power at the sample was ~25 mW. To excite the myosin filaments from all directions to increase the SHG photon counts, the excitation laser beam was circularly polarized to ensure the filament was not only specific to one certain polarization. SHG signals were then collected from the forward detecting channel through a condenser and a 470 nm (± 20 nm) filter with a PMT. The line-scan data carpet was collected along the longitudinal axis of the cell at the 3-5 μs/pixel scanning speed for ~30 seconds. Verapamil was first dissolved in the Tyrode's solution and then added to the cells in the recording chamber with a final concentration 50 nM.

Figure 2 shows the process for extracting shortening percentage of the sarcomere during a cardiac contraction. SHG signals emitted by the myosin heads on the thick filaments show the relative locations of each myosin bundle. During the contraction, the distance between myosin bundles within the same sarcomere (ΔL_i = ~0) remains constant, whereas the distance of myosin bundles between two sarcomeres changes ($\Delta L_a = L_a' - L_a$). The maximal shortening percentage is then calculated by computing the ration between ΔL_a relative to the total length ($L_a + L_i$). This process provides a relatively intuitive and label-free method for estimating mechanical outputs of the iPSC-CMs. It is a feasible approach even when myosin filaments are less organized in the immature iPSC-CMs and their striation is not clearly seen under the bright-field microscope.



$$\text{Shortening \%} = (-100\%) \times \left(\frac{\Delta L_i + \Delta L_a}{L_i + L_a}\right)$$

Figure 2. A schematic description for extracting the shortening percentage from line-scan SHG data carpet. The blue dots represent the myosin heads in the thick filaments and they form cross linkages with actin filaments. During a contraction, the myosin heads slide along the actin filaments and it results in the shortening between adjacent sarcomeres.

3. RESULTS AND DISCUSSION

Adult cardiomyocytes have a relatively well-developed sarcomere structure and typically show repeated patterns of transverse dark and light bands, resulting a clear contrast under the bright-field microscope (Figure 1, A1). This type of striated pattern can be converted to the average length of the sarcomere by open-source codes or commercial imaging software capable of doing the fast Fourier transform algorithm [28]. This repeating pattern, however, is usually not seen in iPSC-CMs (Figure 1, B1), presumably due to the less organized filaments or the under-development of sarcomere structure. After all, the ultrastructure of iPSC-CMs may not be exactly resembling that of adult cardiomyocytes. It has been shown that iPSC-CMs are relatively smaller in size and have structures similar to that of underdeveloped primary cardiomyocytes [29]. Our previous work has also shown that the SHG intensity will increase once iPSC-CMs become mature, suggesting a positive correlation between SHG intensity and the development of myofilaments [1,30]. Fortunately, the SHG images of both adult cardiomyocytes and iPSC-CMs exhibit a high contrast to indicate the locations of myosin heads. Most important of all, the myosin bundles in iPSC-CMs are sensitive enough to emit bright SHG signals despite its poor development in overall tubular myofibrils. The relative locations of myosin heads changes during the contraction, and the shortening percentage can be extracted directly from the line-scan data carpet. The result showed that the iPSC-CMs with a total maturity of 68 days post differentiation had a shortening percentage ~ 11% (**Figure 3**). Verapamil, a negative inotrope, is a calcium channel blocker that has been used to treat high blood pressure by reducing the mechanical output of cardiomyocytes [31-33]. The corresponding decrease in the mechanical performance induced by verapamil is reflected on the SHG line-scan data when cells were put in the 50-nM, showing a smaller movement and a shortening percentage ~7%.

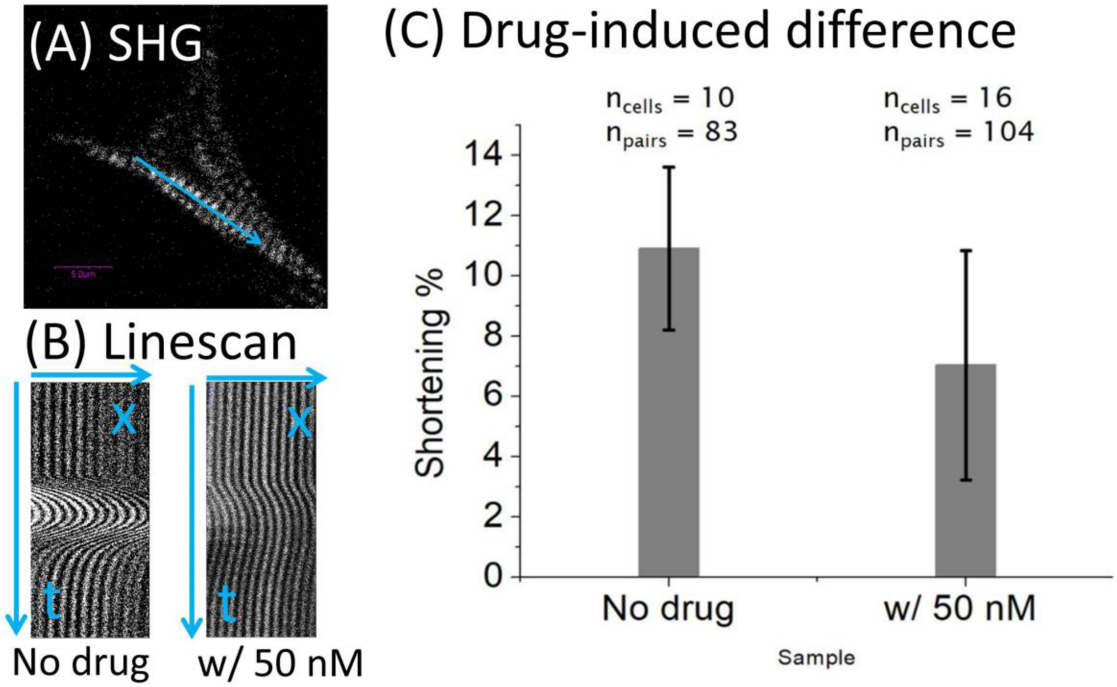


Figure 3. Verapamil-induced changes in cardiac contractility. (A) SHG image of an iPSC-CM. (B) The contrast in contractility after Verapamil was introduced to the system. (C) The average shortening percentages with standard deviations in each case.

4. SUMMARY

Specialized cells derived from stem cells such as iPSC-CMs can become an unlimited ex vivo source of cells for testing the effects of both cardiac and non-cardiac drugs early in the pre-clinical trials. SHG microscopy can be used as a reliable label-free tool for quantifying mechanical outputs of iPSC-CMs without introducing any fluorescent markers to the system. Therefore, the shortening data estimated from SHG signals truly reflects the drug-induced effects on the contractility of cells. Approved inotropes such as verapamil showed an expected decrease in the mechanical performance in this study. Before iPSC-CMs becomes a reliable platform for drug testing, more careful planning and experiments are still required to address concerns such as whether or not primary cardiomyocyte and iPSC-CMs have the same response to the same drug [34].

5. ACKNOWLEDGMENTS

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