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Cardiac applications of second harmonic generation (SHG) microscopy

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ABSTRACT

Human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) are an unlimited *ex vivo* supply of heart cells for cardiac applications. The establishment of pure iPSC-CMs populations is crucial for downstream medical applications such as human disease modeling, patient-specific stem cell therapy, human transplantation, and drug development. However, a significant challenge is the lack of an established purification method to isolate populations of iPSC-CMs by their phenotype, maturity, and subtype due to the lack of specific iPSC-CM markers. The ability to remove potentially teratoma forming pluripotent stem cells, arrhythmia inducing immature and pacemaking cells, and other non-CMs is extremely important for engineering tissues with desired cell compositions that are both safe for human transplantation and that can accurately replicate cardiac functions. Contemporary purification techniques have either low specificity or require genetic modification. We have proposed that second harmonic generation (SHG) signals, which are known to originate from the sarcomeric myosin filaments in cardiomyocytes, can be a highly specific, label-free marker for identifying iPSC-CMs. Here, we demonstrate the use of SHG microscopy for characterizing iPSC-CMs and their subtypes.

Keywords: induced pluripotent stem cells, cardiomyocytes, action potential, second harmonic generation, myosin, heart muscle

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1. INTRODUCTION

The idea of using pluripotent stem cells for cardiac applications has attracted great attention over the past decade due to increasingly refined advances in regenerative and molecular medicine[1]. Treating cardiovascular diseases by replacing dysfunctional cells with specialized stem cells or using these versatile cells for disease modeling and drug discovery not only extend our understanding of the cardiovascular physiology from the bottom up, but also directly provide feasible solutions to tackle cardiovascular diseases[2, 3]. As an alternative strategy to utilizing embryonic stem cells for such applications, induced pluripotent stem cells (iPSCs) that are genetically converted from human skin or blood cells offers a non-controversial opportunity to potentially be used to create a wide range of cells or tissues of the human body. The capacity to generate a pure population of specialized induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) with highly controlled maturity, phenotype, and cardiac subtype is a crucial step for cardiovascular applications. For example, the outcome of any current differentiation method for generating iPSC-CMs yields a heterogeneous population including unwanted cells such as undifferentiated PSCs and non-CMs. To date, typical protocols for generating iPSC-CMs are estimated to yield < 70% of pure cardiomyocytes. The double-edged sword of utilizing these unpurified iPSC-CMs mixed with undesired stem cells is that iPSC-CMs have versatile functions in regenerative medicine on the upside, while some undesired populations could become problematic. In some cases, these undesired stem cells may become teratomas to form tumors as the result of their intrinsic pluripotency[4-6]. In addition, native iPSC-CM populations are a mixture of cardiac subtypes including ventricular-like, atrial-like, and pacemaking-like cells. In the case of repairing damaged left ventricular dysfunction with ventricular-like iPSC-CMs, it has been known that immature pacemaking-like populations could introduce arrhythmia after the transplantation[7-9]. A highly purified population of iPSC-CMs not only is necessary for clinical research in tissue transplantation but also critical for research in molecular medicine such as drug discovery or cardiac toxicity tests to examine the impact of a chemical toward specific types of cardiomyocytes.

A critical bottleneck for acquiring highly defined iPSC-CM populations is the lack of a reliable and established purification technology. The urgency and importance of developing new iPSC-CM purification methods has been highlighted by leading researchers in regenerative medicine [10, 11]. Broadly speaking, the difficulty with iPSC-CM purification is because cardiomyocytes have no highly specific chemical or biological markers to identify them. In addition, the identification and discrimination of contractile and pacemaking cells is extremely challenging. Efforts from two different angles have been explored to either modulate culturing environments or signaling pathways to direct CMs toward a specific subtype, or to develop chemical or biological fluorescence markers to identify CMs. The former requires a clear elucidation of underlying molecular mechanisms, although generating a 100% subtype population is still very difficult. The latter requires fluorescent markers with high specificities for labeling. The inconsistency in screening results by using these developed markers have been reported recently to discuss the complexity involved among different cell lines and cell types. A non-genetic purification technique that can also be potentially combined with high-throughput cell sorting techniques is yet available [10, 12-18].

We previously demonstrated that second harmonic generation (SHG) signals can be generated from immature iPSC-CMs and have the sensitivity and specificity to discriminate iPSC-CMs from iPSCs and other non-CM cell types. Our work showed the potential of SHG signals as a label-free, non-genetic, and non-invasive optical marker for iPSC-CMs. Second harmonic generation is a nonlinear optical process in which the SHG-active material such as collagen, microtubules, and myosin filaments, combine two incident photons and emit a photon at twice the energy of the one incident photon. Since myosin is specific to cardiomyocytes, the SHG intensity not only can be used to identify cardiomyocytes but also intrinsically reflects the development of myosin filaments in the cell. We have previously reported that iPSC-CMs as immature as 7 days post-differentiation could generated SHG signals and showed that the SHG signal intensity can be used to discriminate iPSC-CMs of different maturity [19]. In this work, we expand upon our previous study to test the hypothesis that SHG signals can be used to identify cardiac subtypes.

2. METHODS

2.1 Culture and differentiation of cardiomyocytes

The hiPSC-derived cardiomyocytes (19-9-7T, WiCell, Madison, USA) were cultured in mTeER1 and passaged using ReLeSR (Stem Cell Technologies, Vancouver, Canada). The cell differentiation process followed a previously published protocol by treating cells with glycogen synthase kinase inhibitor (GlaxoSmithKline plc), Wnt inhibitor, PRMI 1640 media with L-glutamine, B-27 supplement without insulin, and penicillin-streptomycin (Thermo Fisher Scientific). The cells were then incubated in RPMI 1640 media mixed with L-glutamine, B-27 supplements, and insulin. Cells were cultured to 80 days and then plated on matrigel-coated (BD Bioscience) gridded glass bottom dishes (ibidi) for 10 days prior to optical measurements[19, 20].

2.2 Optical recording of action potential

The iPSC-derived cardiomyocytes seeded on gridded dishes with a total maturity of 90 days were used for the optical recording of the spontaneous action potential[20]. Before the optical recording, the cells were incubated with a voltage sensitive fluorescence probe FluoVolt (5 uM, Thermo Fisher Scientific, F10488), Pluronic F127 (0.04%, Sigma-Aldrich, CAS 9003-11-6), probenecid (2 mM, Sigma-Aldrich, CAS 57-66-9) in media for 20 minutes. After being washed thoroughly with the media, the cell were immersed in Tyrode's solution with an excitation-contraction inhibitor blebbistatin (Sigma-Aldrich, CAS 857925-71-8) to stop cell contraction during the recordings. A high-speed spinning disk confocal microscope (SDCM, Olympus IX71 equipped with the Yokogawa CSU10 unit and an Andor iXon 987 EMCCD) was used to record the time-lapsed membrane action potential images at ~100 fps for 6000 frames with a FITC filter set (Semrock). The excitation laser (Coherent Sapphire SF single-frequency continuous 488-nm laser, linewidth < 1.5 MHZ) source was focused on the sample through an Olympus 10X objective (Plan N, 0.25 NA) with a post-objective power ~ 1 mW with a power fluctuation < 2%. The time-lapsed fluorescence data for each cell was logged into a database for analyses.

2.3 Second-harmonic generation intensity recording

The same cells that were used for optical action potential recordings were also used for SHG measurements. Since the location of each cell has been recorded on the gridded dish, the correlation between SHG intensity and the subtype can be directly established after the subtype classification. After the optical recordings of the spontaneous action potentials were completed, the Day-90 iPSC-derived cardiomyocytes were immediately fixed with 4% paraformaldehyde (Sigma-Aldrich, CAS 30525-89-4) for 15 minutes at room temperature and then maintained with phosphate buffered saline (Thermo Fisher Scientific) for the SHG imaging[19]. For the SHG imaging, a femtosecond tunable Coherent Chameleon laser was tuned to 940 nm with ~140 fs pulses at the repetition rate at 80 MHz. The laser beam was free-space coupled to an Olympus FluoView 300 scanning unit. The laser beam was focused on the sample through an Olympus Super Achromat UPLSAPO 60X objective (water immersion, 1.2 NA). SHG images was acquired by a photomultiplier tube through an optical condenser with a 0.55 NA and a 470 ± 20 nm filter at the scanning speed of ~10 μs/pixel. The whole-cell SHG intensity was collected through Z-stacking over ranges of 10-20 μm with a 500-nm step size. The zoom was set to a constant throughout all SHG images analyzed in this project. To obtain the whole cell SHG intensities, the boundary of each cell was manually circled. The net signal intensities were summed across all Z-stacks after subtracting the background noise.

2.4 Classification of iPSC-CM subtypes

The time-lapsed fluorescence data was used for the classification of cardiomyocyte subtypes including pacemaking-like, atrial-like, and ventricular-like cells following a published protocol. The protocol compared the electrophysiology and action potential kinetics from the optical recordings and the traditional patch-clamp recordings and found the

clustering result to be consistent between two methods[20]. Briefly, the background subtracted time-lapsed fluorescence data sets are analyzed through the a home-made script utilizing the Mclust package of R computing software, a programming language developed by R Foundation for Statistical Computing. The averaged action potential profile for each cell within 6000 frames is used to generate the duration-slope-curvature (DSC) parameter for further clustering analyses. The DSC parameter collectively describes the action potential duration at 50% of repolarization (APD50), the slope between APD50 and APD90, and the curvature of action potential profile along APD10 and APD30. The outcome of the DSC clustering generates the ventricular-like and non-ventricular-like groups. The non-ventricular-like group is further clustered with the frequency of cardiac automaticity into high- and low- frequency groups. Pacemaking-like cells are cells with the action potential amplitude < 75 mV from the high-frequency group. The rest of the cells in the high-frequency group and all cells from the low-frequency groups are classified as atrial-like cardiomyocytes.

3. RESULTS AND DISCUSSION

Adult ventricular cardiomyocytes typically have strong second harmonic generation signal due to the amount of developed myosin networks within the cell (Figure 1A). The myosin heads (A bands) being the source of the SHG signals are on average ~ 0.8-1.2 μm apart from each other. Although the human induced pluripotent stem cell-derived cardiomyocytes (Figure 1B) may not have fully developed sarcomeric myosin filaments across the whole cell compared to adult cells, the pattern-like SHG feature from the myosin bundle can already be used to examine the development of sarcomeric structures or be used to purge non-cardiomyocytes from the mixture of all types of cells. Moreover, our group has previously established the method for using SHG signals to discriminate hiPSC-CMs with different degrees of maturity showing a strong dependence between the SHG intensity and the cell maturity[19].

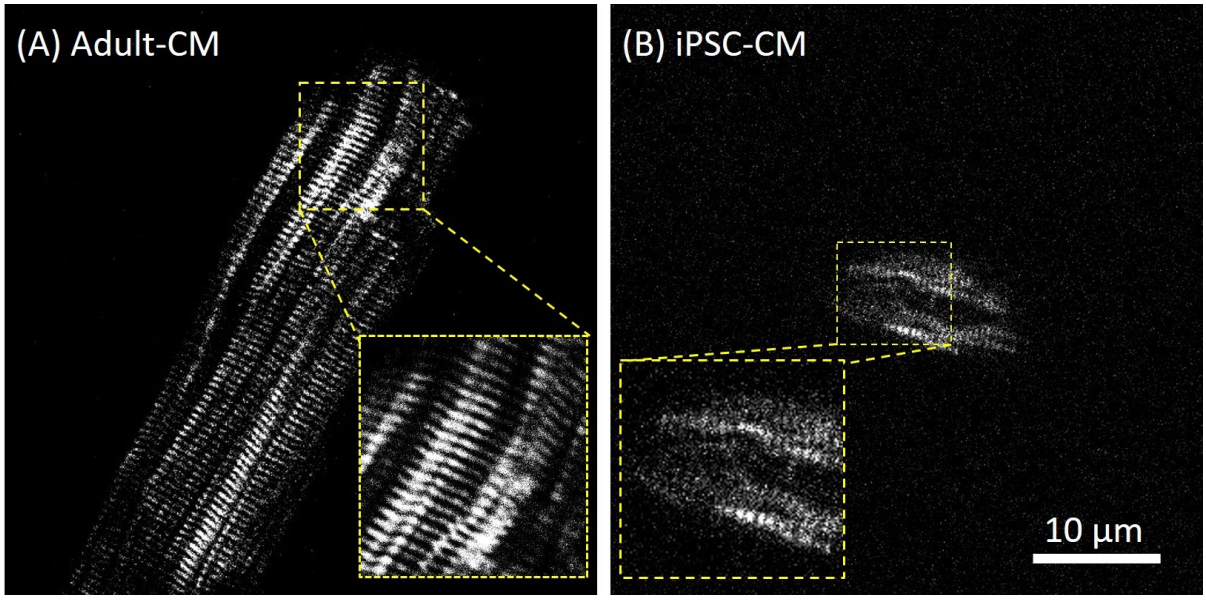

Figure 1. Representative second-harmonic generation images of an individual (A) adult mouse ventricular cardiomyocyte and (B) a ventricular-like induce pluripotent stem cell cardiomyocyte. The adult ventricular cardiomyocyte was isolated from the ventricle of a mouse heart; the ventricular-like hiPSC-CM was identified by its action potential profile. Sarcomeric patterns from the thick myosin filaments can be seen at the submicron resolution without applying any fluorescence molecular labels. The myosin filaments in the adult cardiomyocytes typically are fully developed and therefore have a stronger SHG intensity, whereas iPSC-CMs may not have fully developed thick filaments across the whole cell. The excitation laser was 940 nm with a post-objective laser power ~30 mW. SHG images were collected through a 470 ± 10 nm filter at the scanning speed 10 μs/pixel.

In order to correlate the SHG intensity among different cardiac subtypes (Figure 2) for cells at the degree of maturity, we first used the voltage-sensitive fluorescence probe to acquire the cardiac action potential profiles for ~200 cells. Fast-response potentiometric fluorescent probes such as FluoVolt or ANEP (AminoNaphthylEthenylPyridinium) enable researchers to optically record the changes in membrane potential voltage through optical imaging. The changes in membrane potential plays a crucial role in many biological cellular processes such as neuron impulse propagation and muscle contraction and the profile of the membrane voltage over time can be reflected by using these fluorescent probes. When the fluorescent probes in cells experience a change in membrane potential, it generates corresponding changes in either spectral profile or the intensity of their fluorescence. In conjunction with the spinning disk confocal imaging technique, the cardiac action potential profiles of iPSC-CMs reflected by the time-lapsed fluorescence intensity, were recorded at the imaging speed of ~ 100 fps (frames per second). The background-subtracted, averaged action potential profile of each cell was further used for the subtype classification following an established protocol as summarized in the Methods section. Among all 220 cells, the pacemaking-like hiPSC-CM represents the smallest population ~ 10%, whereas ventricular-like and atrial-like cells account for 44% and 46%, respectively. Ventricular-like cells show a more prominent plateau phase (Figure 2) than the atrial-like cells, which is reflected by the shape of the action potential. Moreover, ventricular-like cells have an average duration (APD90) near 520 ms, which is considerably longer than that of atrial-like cells (~380 ms). In contrast, pacemaking-like cells have a ~ 300 ms APD90 and an average membrane voltage change ~ 55 mV[20].

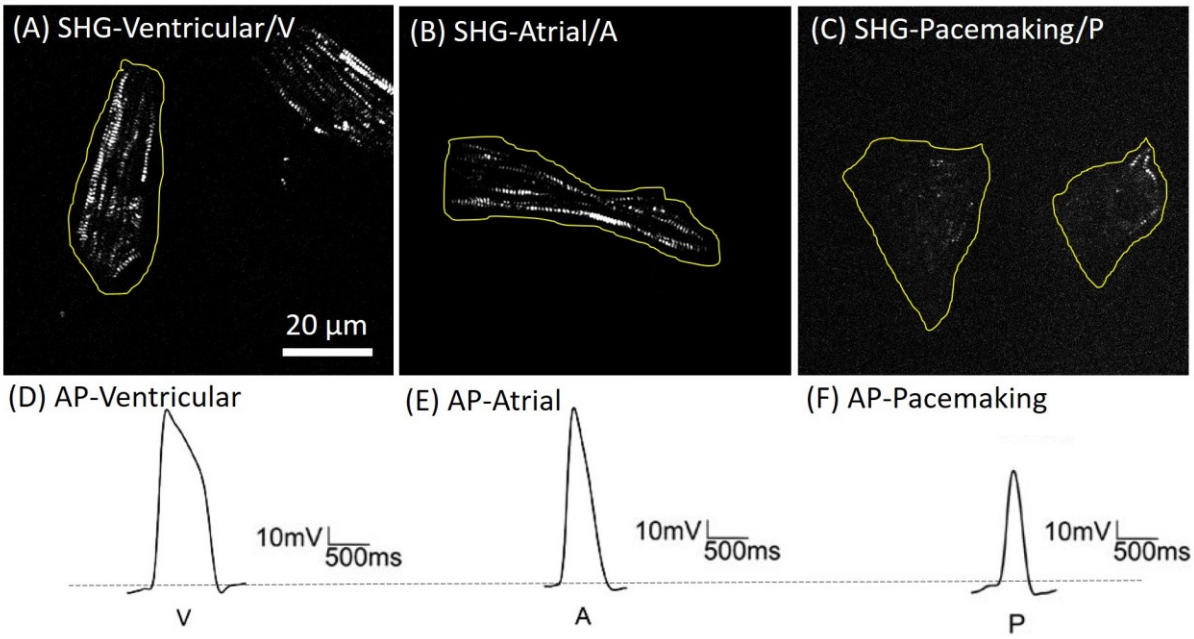

Figure 2. Representative (A-C**)** second-harmonic generation images and (D-F) optical action potential recording of Day-90 human induce pluripotent stem cell cardiomyocytes (hiPSCs-CMs) for all three subtypes (Ventricular-like, Atrial-like, and pacemaking-like cells). The sarcomeric patterns originated from myosin filament can be seen in all three subtypes, in which pacemaking cells typically have weakest SHG intensities. Ventricular-like cells typically have more well-developed myosin filaments than the other two subtypes across a whole cell.

The whole-cell SHG intensity was acquired through Z-sectioning with a 1-µm step. The beginning and stopping Z locations were defined based on the two-photon fluorescence (2PF) images that were simultaneously recorded with SHG modality. On average, contracting cells such as ventricular-like and atrial-like cells exhibit a higher SHG photon counts than pacemaking-like cells (Figure 2-3). The dependence of the whole-cell SHG intensity on the filament development in different subtype was sensitive enough to allow the visualization for this difference. Ventricular-like and atrial-like cells typically form a pattern-like feature originated from the myosin filament network. In contrast, SHG

signals of pacemaking-like cells have a much lower SHG counts. Moreover, the distribution of SHG photons usually originates from a partially developed filament (Figure 2C, right) or from less-organized filament network across the whole cell (Figure 2C, left). On average, contracting cells such as ventricular-like and atrial-like cells generate > 3.5X more SHG signal than pacemaking-like cells (Figure 3).

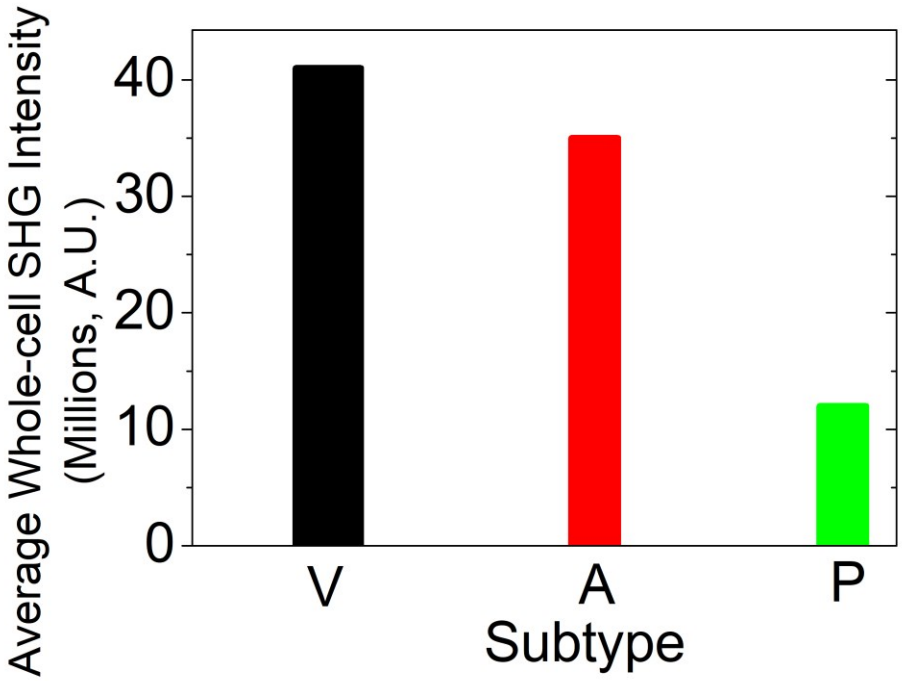

Figure 3. The average whole-cell second harmonic generation (SHG) intensity for each subtype (Ventricular-like, Atrial-like, and pacemaking-like cells) of human induced pluripotent stem cell cardiomyocytes (hiPSCs-CMs) among 220 cells. The percent population characterized by using optical action potential recordings is 44% (V), 46% (A), and 10% (P).

4. SUMMARY

We have shown that second harmonic generation microscopy can be a potential label-free and non-genetic technique for charactering cardiomyocytes. Because the SHG signal is specific to sarcomeric myosin in CMs, we found that the signal intensity can be used to purge undifferentiated stem cells, and moreover, is a reliable tool for identifying and characterizing human induced pluripotent stem cell-derived cardiomyocytes based on their maturity level and cardiac subtypes (ventricular-like, atrial-like, pacemaking-like). The experiments outlined in this report were conducted on the microscope to discriminate cell types. The result lays the foundation for purifying and sorting iPSC-CMs based on the factors mentioned above with the SHG-based high-throughput flow cytometer without adding any fluorescent probes[21].

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