

Label-free imaging for cardiomyocyte differentiation from human pluripotent stem cells

Tongcheng Qian (✉ tqian5@wisc.edu)

Morgridge Institute for Research <https://orcid.org/0000-0002-9071-3543>

Amani Gillette

Morgridge Institute for Research

Melissa Skala (✉ mcskala@wisc.edu)

Morgridge Institute for Research <https://orcid.org/0000-0002-6320-7637>

Method Article

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Abstract

Human pluripotent stem cell (hPSC)-derived cardiomyocytes provide a promising regenerative cell therapy for cardiovascular patients and an important model system to accelerate drug discovery. However, cost-effective and time-efficient platforms must be developed to evaluate the quality of hPSC-derived cardiomyocytes during biomanufacturing. Here, we develop a non-invasive label-free live cell imaging platform to monitor hPSC differentiation into cardiomyocytes. Autofluorescence imaging of metabolic co-enzymes is performed under varying differentiation conditions (cell density, concentration of Wnt signaling activator) across five hPSC lines.

Introduction

Despite advances in treatment, cardiovascular disease is the leading cause of death worldwide¹. Globally, about 12% of adults are diagnosed with cardiovascular disease and over 30% of all deaths are caused by cardiovascular disease¹. The excessive demand of heart transplantation has outpaced the limited number of healthy and functional heart donors². Cell-based regenerative therapy provides a promising treatment for patients suffering from cardiac tissue injury^{3,4}. However, cardiomyocytes (CMs) are terminally differentiated cells with no regenerative capacity⁵. Hence, cost-effective and time-efficient platforms to generate functional CMs with high quality has emerged as an urgent need for cardiac medicine in drug screening, toxicity testing, disease modeling, and regenerative cell therapy.

Human pluripotent stem cells (hPSCs) can differentiate into cells from all three germ layers⁶⁻⁸. A variety of methods have been established to generate CMs from hPSCs⁹⁻¹¹. These hPSC-derived CMs exhibit similar functional phenotypes to their *in vivo* counterparts¹¹, including self-contraction and action potentials. hPSC-derived CMs have been used in disease modeling^{12,13} and drug screening¹⁴, and hold great potential for regenerative medicine^{15,16}. However, batch-to-batch and line-to-line variability in the differentiation process of hPSCs into CMs has impeded the scale-up of CM manufacturing¹⁷. For safety, the quality of clinical-graded hPSC-derived CMs must be rigorously evaluated before they can be used for regenerative cell therapy in patients¹⁸. Current approaches to quantify CM differentiation rely on low-throughput, labor-intensive, and destructive immunofluorescence labelling and electrophysiological measurements¹¹. New technologies that can non-invasively monitor CM differentiation in real time and evaluate the differentiation outcome at early stages are needed to effectively optimize biomanufacturing of CMs from stem cells.

Previous studies indicate that hPSC-derived CMs undergo dramatic metabolic changes throughout differentiation¹⁹. Reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and oxidized flavin adenine dinucleotide (FAD) are autofluorescent cellular metabolic co-enzymes that can be imaged to

collect metabolic information at a single-cell level²⁰. The ratio of NAD(P)H to FAD intensity is the “optical redox ratio”, which reflects the relative oxidation-reduction state of the cell. The fluorescence lifetimes of NAD(P)H and FAD are distinct in the free and protein-bound conformations, so changes in these fluorescence lifetimes reflect changes in protein-binding activity^{21, 22}. Optical metabolic imaging (OMI) quantifies both NAD(P)H and FAD intensity and lifetime variables. Several groups have demonstrated that autofluorescence imaging can non-invasively track stem cell metabolic activities in real time, including monitoring mesenchymal stem cell differentiation into adipocytes^{23, 24}, osteocytes^{24, 25}, and chondrocytes²⁵, distinguishing differentiation of hPSCs into dermal and epidermal lineages²⁶, metabolic difference between hPSCs and feeder cells²⁷, and hematopoietic stem cells at different stages²⁸. These prior studies indicate that OMI is suitable to detect the metabolic changes that occur during CM differentiation.

The goal of this study is to build a predictive model based on OMI to determine whether OMI can monitor CM differentiation process. We demonstrate a facile method to non-invasively monitor metabolic changes during hPSC differentiation into CMs by combining OMI with quantitative image analysis. OMI is performed at multiple time points during a 12-day differentiation process under varying conditions (cell density, concentration of Wnt signaling activator) and different hPSC lines (human embryonic pluripotent stem cells and human induced pluripotent stem cells). Differentiation efficiency is quantified by flow cytometry with cTnT labelling on day 12. During the differentiation process all 13 OMI variables, including both NAD(P)H and FAD intensity and lifetime variables are collected. This label-free and non-destructive method could be used for quality control for CM manufacturing from hPSCs.

Reagents

B-27 supplement (Life Technologies, cat. no. 17504-044)

B-27 supplement without insulin (Life Technologies, cat. no. 0050129SA)

CHIR99021 (Selleckchem, cat. no. S1263-25 mg) (Steps 12A and 12B only)

DMEM (Life Technologies, cat. no. 11965-092)

DMEM/F12 (Life Technologies, cat. no. 11330-057)

DMSO (Sigma, cat. no. D8418)

Human pluripotent stem cell lines, e.g., 19-9-11, IMR90C4, H9, H13

IWP2 (Tocris, cat. no. 3533-10 mg)

RPMI (Life Technologies, cat. no. 11875-119)

mTeSR1 (STEMCELL Technologies, cat. no. 05857)

Matrigel (BD Biosciences, cat. no. 354277)

Bovine serum albumin (Fisherscientific, cat. no. BP1600-100)

PBS (Sigma, cat. no. D8537)

Triton X-100 (Sigma, cat. no. T8532)

StemPro Accutase (ThermoFisher, cat. no. A1110501)

Versene (Life Technologies, cat. no. 15040-066)

Y27632 (Tocris, cat. no. 1254)

Formaldehyde (16%, vol/vol; Polysciences, cat. no. 18814)

Liquid nitrogen

Ethanol, 70% (vol/vol)

Milli-Q water

Equipment

Conical tubes (15 and 50 ml; BD Biosciences, cat. nos. 352095 and 352073)

6-well Plates (Nunc, cat. no. 140675)

24-well plates (ibidi, 82406)

Sorvall ST8 centrifuge (Thermo Scientific, cat. no. 75007200)

Sterile biosafety cabinets

Liquid waste disposal system

Flow cytometry FACSCalibur (Becton Dickinson)

Humidified tissue culture incubator (37 °C, 5% CO₂)

Sterilized Pasteur pipettes (Fisher, cat. no. 13-678-20D)

Hemocytometer (Hausser Scientific, cat. no. 3200)

Flow round-bottom tube (5 ml; BD Biosciences, cat. no. 352052)

EVOS XL Core Imaging System (ThermoFisher)

Ultima two-photon imaging system (Bruker)

Procedure

1. Take hPSCs cultured on 6-well plates in mTeSR1 medium at 80–90% confluence.
2. Aspirate the medium and add 1 mL of room-temperature Accutase to each well. Put the plate in a 37 °C, 5% CO₂ incubator and wait for 8 min.
3. Add 1 ml of mTeSR1 into each well and pool all of the cells in a 15-ml conical tube. Count the total cell number with a hemocytometer. Centrifuge the cells at 200*g* for 5 min at room temperature. Aspirate the supernatant, resuspend the cells in mTeSR1 + 10 µM Y27632, and replate at a cell density ranging from 1.3×10^5 cells/cm² to 2.5×10^6 cells/cm² onto Matrigel-coated 24-well ibidi u-plate. This time point corresponds to day -2 of differentiation.
4. On the next day, aspirate the medium in each well and replace it with 2 mL of fresh room temperature mTeSR1 medium. Repeat this medium replacement until day 0 before initiating differentiation.
5. On differentiation day 0, initiate cardiomyocyte differentiation by change medium to RPMI + B27 minus insulin supplemented with CHIR99021 (ranging 6 µM to 12 µM).
6. 24 hours post treatment of CHIR99021, aspirate the medium and replace it with fresh room temperature RPMI + B27 minus insulin.
7. On differentiation day 3, aspirate the medium and replace it with RPMI + B27 minus insulin supplemented with 5 µM IWP2.
8. On differentiation day 5, aspirate the medium and replace it with RPMI + B27 minus insulin.
9. On differentiation day 7, aspirate the medium and replace it with RPMI + B27. Repeat this process every other day until differentiation day 12.
10. Autofluorescence dynamics of NAD(P)H and FAD by OMI during the process of hPSC differentiation into cardiomyocytes are recorded by an Ultima two-photon imaging system (Bruker). Cells are imaged on differentiation day 0 (immediately pre-treatment with CHIR99021, a Wnt signaling activator), day 1 (24 hours post-treatment with CHIR99021), day 3 (immediately pre-treatment with IWP2, a Wnt signaling inhibitor), and day 5 (48 hours post-treatment with IWP2). OMI is performed at these time points based on the biphasic role of Wnt signaling activation and inhibition in the cardiomyocyte differentiation protocol. The ultrafast tunable laser source enables sequential excitation of NAD(P)H at 750 nm and FAD at 890 nm. NAD(P)H and FAD emission is isolated using 440/80 nm and 550/100 nm bandpass filters

(Chroma), respectively. The laser power at the sample for NAD(P)H and FAD excitation was approximately 2.3 mW and 7.9 mW, respectively. Fluorescence lifetime decays with 256 time bins were acquired across 256×256 pixel images with a pixel dwell time of 4.8 μ s and an integration period of 60 seconds. All samples are illuminated through a 40 \times /1.15 NA objective (Nikon). The instrument response function was acquired from the second harmonic generated signal of urea crystals at 890 nm and was measured for each imaging session. (Figure 1). Imaging steps are illustrated below

Turning on the system:

1. Turn on the master switch
2. Turn off room lights
3. Turn on the power for the PMTs 1 and 2
4. Turn on the computer
5. Open Prairie View Software
6. Turn on the laser
7. Open the shutter
8. Turn on power meter
9. Set the Nikon TI-E microscope to E100 and BF
10. Turn on the bright field light
11. Place a drop of water on the objective
12. Place your sample in the holder
13. Bring the objective up to your sample and focus onto cells
14. Turn off bright field light
15. Switch microscope to 720LP and R100
16. Turn off the display light on the microscope
17. Close the light box
18. On the computer:

- a. Change the save path so the images go to the right folder
- b. Check that acquisition mode is set to FLIM
- c. Check that averaging is set to 60s
- d. Check resolution (usually 256×256)
- e. Check pixel dwell time (usually 4.8 μ s)
- f. Check optical zoom (usually 1)

For Imaging NADH:

1. On Imaging window select Ch 2
2. Under Laser set wavelength at 750 nm
3. Under Power/Gain Set PMT 2 at 800
 - a. Set Pockels to 80
4. Check that power meter is set to 750
5. Click Live Scan
6. Adjust Pockels and field of view
7. Click Single Scan and wait for it to finish

For Imaging FAD:

1. On Imaging window select Ch 1
2. Under Laser set wavelength at 890 nm
3. Under Power/Gain Set PMT 1 at 800
 - a. Set Pockels to 100
4. Check that power meter is set to 890
5. Click Live Scan

6. Adjust pockels to brighten image
 - a. DO NOT ADJUST FIELD OF VIEW
7. Click Single Scan and wait for it to finish

Imaging Notes to record:

- Objective
- Resolution
- Pixel Dwell time
- Optical zoom
- PMT Setting
- NADH
- o Starting Pockels
- o Power
- FAD
- o Starting Pockels
- o Power

Other things to remember:

- Try to keep the power the same, not necessarily the pockels
- Check that the CFD is between $1e5$ and $1e6$ while imaging
- Acquire an IRF with urea crystals
- o FAD wavelength (890)
- o NADH PMT 2 set to 800

§ Pockels set low (15 max)

Turning off the system:

1. Make sure microscope is set to E100 and BF
2. Take off the sample
3. Clean the objective
4. Turn off the light
5. Turn off the PMTs
6. Close the laser shutter
7. Turn off the laser
8. Close out of Prairie View Software
9. Turn off the computer
10. Flip the big switch on the back of the computer stand

Troubleshooting

Time Taken

14 days to complete the whole differentiation and imaging sessions.

Anticipated Results

Non-invasively monitoring metabolic changes during the whole process of stem cell differentiation into cardiomyocytes from the same location.

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Figures

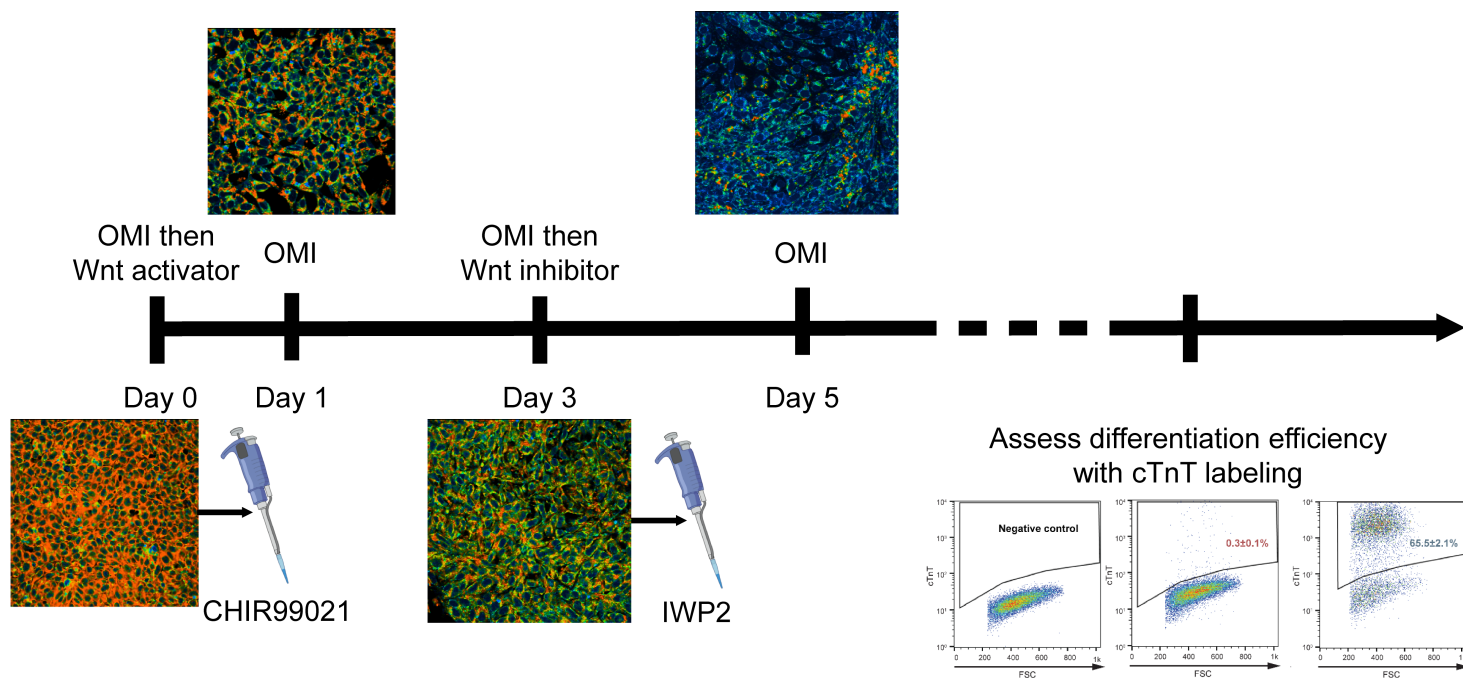


Figure 1

Diagram of autofluorescence imaging of cardiomyocyte differentiation from human pluripotent stem cells.