

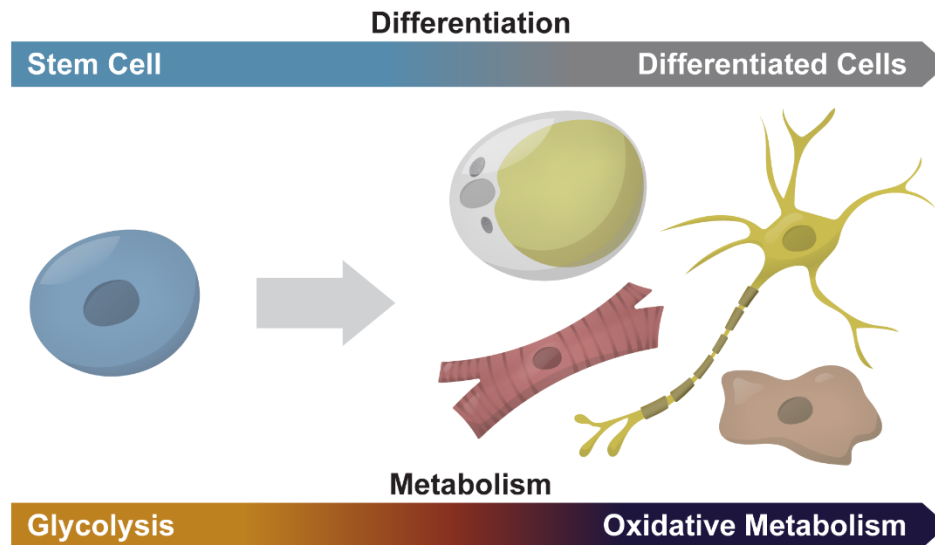
## **Predicting cardiomyocyte differentiation with label-free imaging**

Tongcheng Qian, Matthew Stefely, Melissa Skala

In Melissa Skala's laboratory, we develop non-invasive optical imaging technologies for applications in biology and medicine. Reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and oxidized flavin adenine dinucleotide (FAD) are autofluorescent metabolic co-enzymes that provide sources of contrast for metabolic imaging at a single-cell level<sup>1</sup>. Previously, our group has shown that autofluorescence imaging of the intensities and lifetimes of NAD(P)H and FAD (optical metabolic imaging, or OMI) can detect drug response in patient-derived cancer organoids<sup>2</sup> and metabolic changes with T cell activation<sup>3</sup>. When Tongcheng Qian joined the laboratory, he brought expertise in stem cell technology and cardiovascular engineering. He thought these optical imaging technologies could impact optimization efforts in cardiomyocyte biomanufacturing from human pluripotent stem cells (hPSC).

Cardiovascular disease is the leading cause of death worldwide<sup>4</sup>. hPSC-derived cardiomyocytes hold promise for cardiac disease modeling, drug screening, and cell therapy in patients. However, generating cardiomyocytes from hPSCs suffers from batch-to-batch and line-to-line variability, and this limitation hinders the development of potent and reproducible hPSC-derived cardiomyocytes<sup>5</sup>. Previously, flow cytometry has been used to quantify stem cell differentiation outcome. However, flow cytometry is destructive, labor intensive, and therefore poorly suited for quality control of cell biomanufacturing. Other laboratories have used autofluorescence imaging to monitor stem cell differentiation<sup>6</sup>, but no method has been developed to predict stem cell differentiation outcome at an early stage. Compared to terminally differentiated cells, hPSCs heavily rely on glycolytic metabolism<sup>7</sup> (Figure 1). Therefore, we tested whether OMI was suitable for real-time, non-invasive monitoring of metabolic changes during stem cell differentiation into cardiomyocytes. Our goal was to develop a single-cell computational model to predict cardiomyocyte differentiation efficiency at an early time point based on NAD(P)H and FAD fluorescence

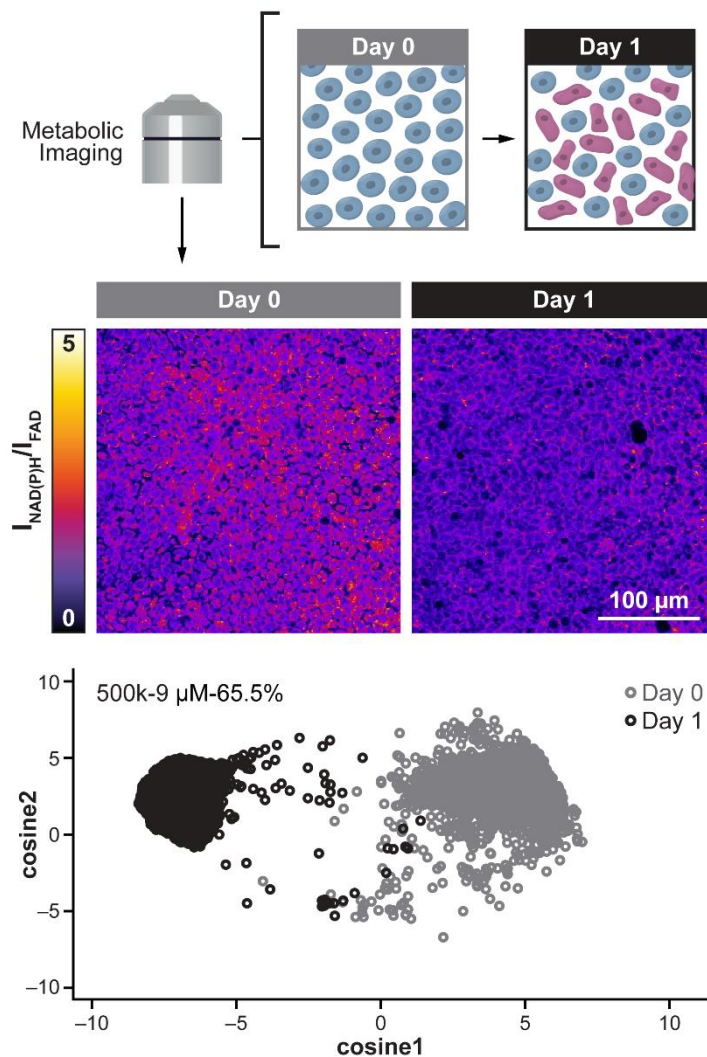
changes during the differentiation process. Eventually this method could lower manufacturing costs and personnel time for biomanufacturing by flagging samples for timely interventions.



**Figure 1. Illustration of metabolic switch from glycolysis to oxidative metabolism during stem cell differentiation.** Adapted from reference 7.

In this project, we differentiated cardiomyocytes from hPSCs by following a well-established cardiomyocyte differentiation protocol with sequential Wnt signaling manipulation<sup>8</sup>. A variety of conditions were investigated to achieve a full range of differentiation efficiencies. Single-cell OMI data were collected from over 100 thousand cells at multiple key time points during the differentiation process to train our prediction model. Prediction models included logistic regression and other classifiers, and uniform manifold approximation and projection (UMAP) clustering based on 13 OMI variables. We first evaluated our prediction model in two different hPSC lines and found that day 1 population exhibited distinct separation from other time points for high differentiation efficiency (> 50%) while all populations for low differentiation efficiency (< 50%) clustered together (Figure 2)<sup>9</sup>. We then verified that day 1 OMI is sufficient for predict cardiomyocyte differentiation outcome. After we received the reviewers'

comments, we further added two more hPSC lines to our prediction model. The additional data were acquired two years apart from the original dataset. Surprisingly, even with drastic changes of the microscope settings over two years, our method still provided reproducibly high prediction accuracy. We are happy and confident that we established a robust predictive model for cardiomyocyte differentiation efficiency with non-invasive imaging.



**Figure 2. Multivariate analysis of NAD(P)H and FAD autofluorescence reveals day 1 population forms distinct cluster from day 0 stem cell population.** Results from reference 9 figure 1 and figure 2.

The optical redox ratio is defined as the fluorescence intensity of NAD(P)H divided by that of FAD

( $I_{\text{NAD(P)H}} / I_{\text{FAD}}$ ). Representative UMAP cluster from the differentiation condition 500k-9  $\mu\text{M}$ -65.5% (500k

cells seeding density in each well of a 12-well plate with 9  $\mu$ M CHIR99021 treatment, and final differentiation efficiency is 65.5%). The UMAP cluster is based on 13 autofluorescence intensity and lifetime variables from NAD(P)H and FAD.

In summary, we developed a proof-of-concept method, which can potentially streamline cell manufacturing from stem cells. Currently, our method provides a binary prediction (high vs low differentiation efficiency). We plan to introduce more variables, such as cell morphology, into the prediction model in future to attain finer prediction metrics. Eventually, we want to extend these methods to a universal platform that benefits stem cell biomanufacturing across different types of cells. We are excited to share our research with scientists in regenerative medicine, engineers in biomedical optics, and experts in the biomanufacturing industry with the hope that these methods can improve the reproducibility and potency of cell therapies for regenerative medicine and disease modelling.

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