Mending the Myocardium: Tracking Cardiomyocyte Differentiation with Milo

INTRODUCTION

Once damaged, the heart can do very little to replace lost cells, meaning that a decline in heart function and cardiovascular disease (CVD) progression are inevitable. To address this problem, various cell replacement approaches are being developed as potential therapeutic solutions. Cardiomyocytes (CMs), derived from human induced pluripotent stem cells (hiPSCs), enable a novel and promising regenerative strategy for both treating and modeling CVD. Based on the pioneering work of Yamanaka *et al.*, researchers can now generate hiPSCs from various sources and then differentiate the hiPSCs into cardiomyocytes.[1,2] Compared with native (*in vivo*) human adult CMs, however, lab-grown human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) may vary in their state of maturity, molecular expression profile, and function, thereby limiting the consistency of their usefulness. To reach their full potential for regenerative treatments, hiPSC-CM cultures must be rigorously characterized to demonstrate their uniformity. Systematic, single-cell transcriptional analyses can provide these important characterizations.[3]

Analysis of hiPSC-CM heterogeneity and advancement in the regenerative medicine field depends on accurate single-cell profiling techniques. Single-Cell Westerns on Milo™ have the resolving power to measure cell-to-cell differences in protein expression and cellular identity, and they can reliably monitor cellular differentiation into distinct cell subpopulations. Milo also has great utility in the manufacturing of cardiomyocytes differentiated from mixed populations of stem cell-derived tissue. In this application note, we validate Milo for the analysis of hiPSC-CM cultures. Further, we demonstrate how Single-Cell Western analysis can track phenotypic marker heterogeneity over time and monitor the relative proportion of cell subsets during culture differentiation

HOW MILO MOVES TO THE BEAT

The heart of the Single-Cell Western system is the scWest chip, a pre-cast polyacrylamide gel patterned with 6,400 microwells (**Figure 1**). The scWest chip captures ~1,000 single cells from a cell suspension with one cell per microwell based on Poisson statistics. Milo then lyses the captured cells and performs simultaneous 1-minute SDS-PAGE separations on each single cell lysate. After separation, proteins are immobilized by ultraviolet (UV) light and UV-sensitive chemistry. You can probe the scWest chip with off-the-shelf Western blot-qualified antibodies to measure protein expression for up to 12 targets per cell and use any open format microarray scanner to obtain chip fluorescence images. You'll finish with automated data analysis using Scout™ software which detects proteins in each single-cell separation by generating fluorescence intensity plots along the separation lane. Scout then quantitates peak area for each protein target of interest in each single cell.

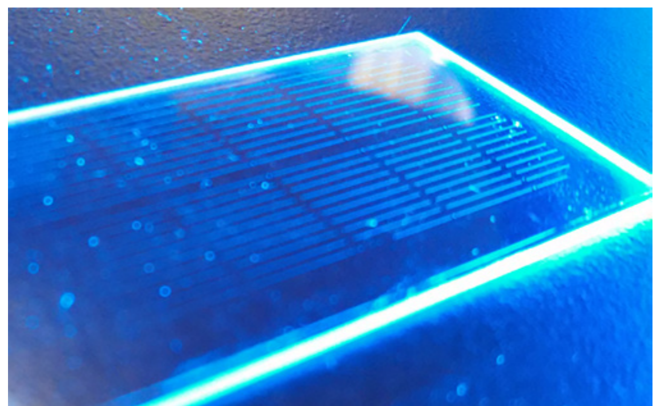


FIGURE 1. Each scWest chip contains thousands of microwells that are approximately the size of a single cell, patterned into a pre-cast polyacrylamide gel.

MATERIALS AND METHODS

hiPSC-Cardiomyocyte Differentiation with GMP-grade Reagents

iBJ6 human induced pluripotent stem cells (iPSC) were differentiated into cardiomyocytes using GMP-grade reagents from Bio-Techne following previously published methods (**Table 1**).[4-6] iBJ6 cells were harvested on Day 0, suspended in serum-free stem cell expansion media containing GMP Y-27632, and plated at 4 x 10^4 cells/cm^2 in 24-well plates coated with Cultrex® Stem Cell Qualified RGF Basement Membrane Extract. Cultures were grown to 80% confluence without Y-27632. On Day 1 of differentiation, iBJ6 cells were overlaid with Cultrex Stem Cell Qualified RGF BME (1:60 dilution) and cultured overnight. On Day 2, definitive endoderm differentiation was initiated by the addition of GMP Recombinant Human Activin A and N21-MAX Insulin Free Media Supplement. From Day 3 – 7, cells were cultured in media containing GMP Recombinant Human BMP-4, GMP CHIR 99021, and GMP Recombinant Human FGF-2. For the final stage of differentiation (Day 8 – 9), cells were cultured in media containing GMP Recombinant Human Dkk-1 and N21-MAX Insulin Free Media Supplement. Dkk-1 was removed on Day 10, and cells were cultured in media containing N21-MAX Insulin Free Media Supplement. iPSC-derived cardiomyocytes were matured and maintained (Day 15 – 21) in media containing N21-MAX Media Supplement.

Single-Cell Western Analysis

Single-Cell Western assays were run with the Large scWest Kit from ProteinSimple (PN K700) which includes eight scWest chips (large well size) and sufficient wash, lysis/run, suspension, and blocking buffers to run eight chips. scWest chips were run with either hiPSC samples or differentiated CM samples. Approximately 100,000 cells in 1 mL of 1X Suspension Buffer were loaded per scWest chip, for both samples. Cells were settled for 4 minutes. After washing off uncaptured cells, settled cells were lysed for 10 seconds, protein electrophoresis was initiated for 70 seconds @ 240 V, and proteins were immobilized by UV exposure for 4 minutes. After washing, scWest chips were probed for 2 hours at room temperature with one of two primary antibody cocktails ("hiPSC assay" or "hiPSC-derived CM assay") diluted in SDS Milk-Free Antibody Diluent (ProteinSimple 043-542) at the following concentrations: rabbit Oct4 (Cell Signaling 2750) at 1:10, goat Sox2 (R&D Systems, AF2018) at 100 µg/mL, mouse Nanog (Novus Biologicals NBP2-22632) at 1:250, sheep Histone H3 (Novus Biologicals NB100-747) at 100 µg/mL ("hiPSC assay"); or mouse MLC2V (Novus Biologicals NBP1-28871) at 1:10, rabbit MLC2A (Pierce PA530789) at 1:10, rabbit Cardiac Troponin T (Abcam ab45932) at 100 µg/mL, and sheep Histone H3 (Novus Biologicals NB100-747) at 100 µg/mL ("hiPSC-derived CM assay"). Following 3 x 15 minute washes in 1X Wash Buffer, the chips were probed in the dark for 1 hour at room temperature with fluorescent-labeled secondary antibodies donkey anti-Rabbit IgG Alexa Fluor® 488 (Thermo Fisher A-21206), donkey anti-Goat IgG Alexa Fluor 555 (Thermo Fisher A-21432), donkey

REAGENT	CATALOG #	BIO-TECHNE BRAND
GMP Recombinant Human Activin A	338-GMP	R&D Systems
GMP Recombinant Human BMP-4	314-GMP	R&D Systems
GMP Recombinant Human FGF-2	233-GMP	R&D Systems
GMP Recombinant Human Dkk-1	5439-GMP	R&D Systems
GMP CHIR 99021	TB4423-GMP	Tocris
GMP Y-27632 dihydrochloride	TB1254-GMP	Tocris
Cultrex® Stem Cell Qualified Reduced Growth Factor (RGF) Basement Membrane Extract (BME)	3434-005-02	R&D Systems
N21-MAX Insulin Free Media Supplement (50X)	AR010	R&D Systems
N21-MAX Media Supplement (50X)	AR008	R&D Systems

Table 1. Summary of reagents used to generate hiPSC-derived cardiomyocytes.

anti-Mouse IgG Alexa Fluor 594 (Thermo Fisher A-21203), and donkey anti-Sheep IgG Alexa Fluor 647 (Thermo Fisher A-21448). Secondary antibodies were diluted at 1:20 in SDS Milk-free Antibody Diluent (ProteinSimple, 043-524). Troponin T was probed separately following stripping of the MLC2A and MLC2V antibodies (see our Technical Note on Stripping and Reprobing Your Single-Cell Westerns). Sheep Histone H3 and the secondary anti-sheep antibodies were probed separately following stripping of the other antibodies. Chips were imaged using a Genepix® 4400a four-color microarray scanner (Molecular Devices) and images were analyzed using Scout software (ProteinSimple).

RESULTS AND DISCUSSION

Using Milo To Identify & Study Pluripotent Cell Phenotypes

You can use Milo's multiplexing ability and single-cell resolution to identify and quantify specific cell phenotypes in your sample. The multiplexed Single-Cell Western data shown in **Figure 2** demonstrate the simultaneous detection of the transcription factors Oct4 (39 kDa), Sox2 (34 kDa), and Nanog (40 kDa) plus Histone H3 (15 kDa) in a single-hiPSC. Co-expression of these transcription factors in a single cell confirms that this specific cell exhibits a hiPSC phenotype. By tracking co-expression patterns across all the cells analyzed in a sample, Milo enables the identification and quantification of distinct cellular phenotypes within a heterogeneous sample.

Assay optimization for the hiPSC and differentiated CM samples was accomplished by selecting an electrophoresis run time of 70 seconds, which ensured that the larger proteins fully entered the gel and that H3 did not migrate beyond the end of the lane and into the next lane. For a detailed description of selecting appropriate electrophoresis times for multiplexed Single-Cell Western experiments, check out our Technical Note, Selecting an Electrophoresis Time for Multiplexed Single-Cell Western Assays.

The gel images shown in **Figure 2A** were derived from the same lane of a single cell separation and imaged in distinct spectral channels using a four-color microarray scanner. Scout software performs the data analysis and creates fluorescence intensity plots from these lane views for each spectral channel by plotting fluorescence intensity along the separation axis (**Figure 2B**). Protein peaks are then identified by Scout software by correlating a canonical peak shape with the one-dimensional fluorescence intensity plots for each cell. The histograms in **Figure 2C** indicate the cellular heterogeneity of protein expression within the population analyzed. Multiplexing with Milo gives you expression data for multiple proteins in one experiment, enabling you to track multiple phenotypic markers in individual cells to stratify cells based on subtle differences in marker co-expression!

Using Milo To Monitor Cardiomyocyte Differentiation

You can use Milo's multiplexing capability to confirm that your protocol is effective for driving undifferentiated cells into differentiated CMs. **Figure 3** shows Milo's analysis of a cell culture before and after 21 days of cardiomyocyte differentiation. Data analysis is done with Scout software and data are presented as scatterplots with individual dots corresponding to single cells. As hiPSCs differentiate into cardiomyocytes, the pluripotency markers Sox2 and Oct4, which are abundant in a large percentage of hiPSCs, become undetectable in cardiomyocytes, as expected. Furthermore, analysis of the cardiomyocyte marker Troponin T shows low level expression in a small number of cells in the hiPSC sample contrasted with a large increase in the number and per-cell Troponin T expression in the CM sample. **Figure 4** further quantifies this difference in Troponin T expression in the two samples by measuring a dramatic 24-fold increase in mean per cell Troponin T expression (**Figure 4A**) as well as a marked increase in the measured percentage of Troponin T positive cells from 10.5% of cells in the undifferentiated iPSC sample to 65% in the differentiated cardiomyocyte sample (**Figure 4B**). The basal levels of Troponin T in the hiPSC sample could be indicative of spontaneous differentiation.

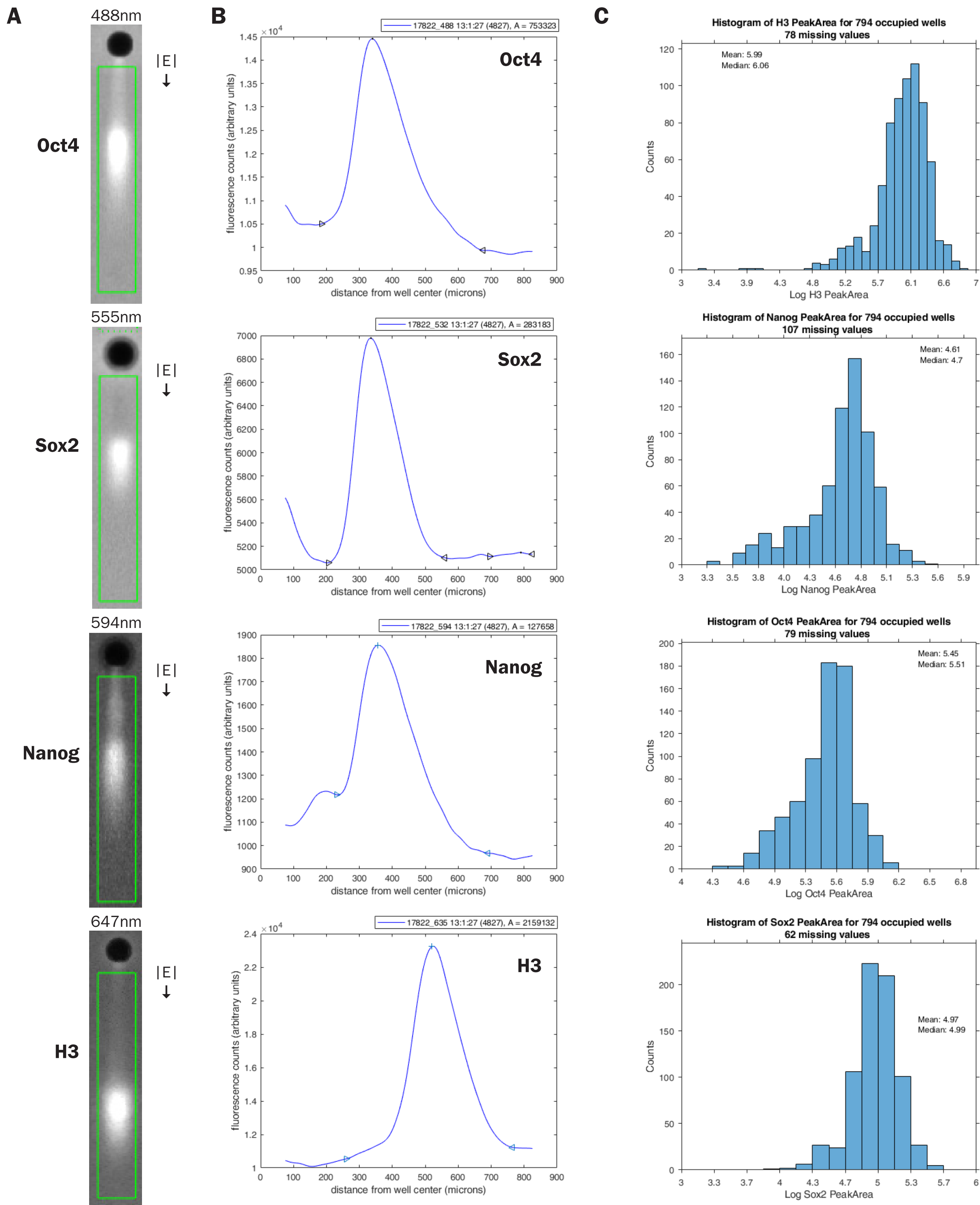


FIGURE 2. Multiplexed induced pluripotent stem cell assay enables identification of cells that co-express pluripotency markers in a hiPSC sample. (A) Lane views of a single-cell separation imaged in four different spectral channels, (B) fluorescence intensity plots of the separation lane imaged in each spectral channel, and (C) histograms of peak area for pluripotency markers Oct4, Sox2, Nanog, and H3 across all cells analyzed on the scWest chip. Histone H3 is included as a loading control. The lane views and fluorescence intensity plots of all four targets were multiplexed in the same hiPSC, while the expression histograms represent the entire population of 794 occupied wells. The high abundance of these pluripotency markers is typical for undifferentiated hiPSCs.

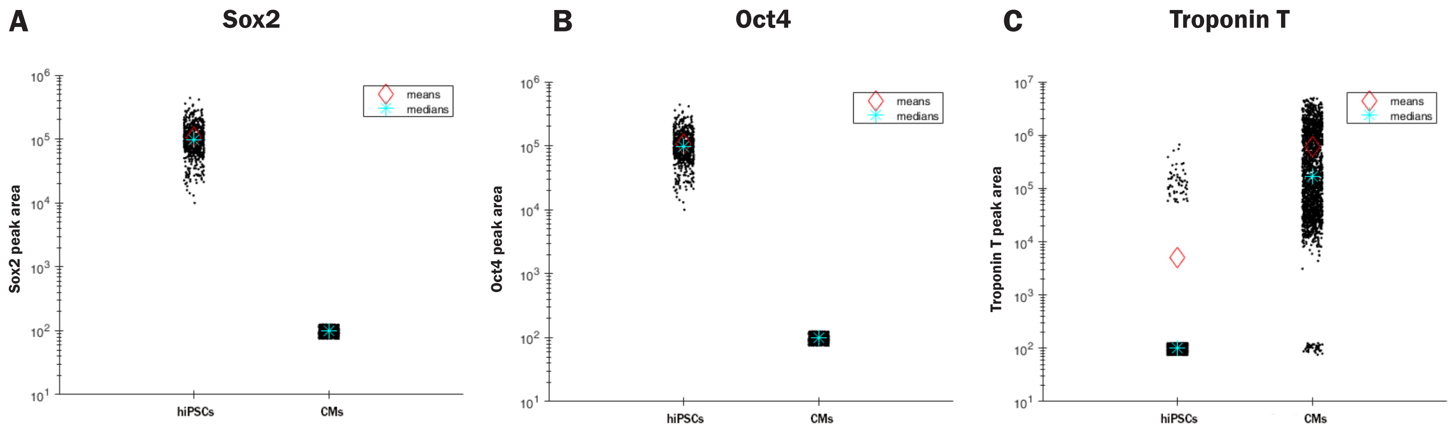


FIGURE 3. Milo tracks the change in phenotypic marker expression as differentiation progresses. One-dimensional peak area scatterplots of (A) pluripotency marker Sox2, (B) pluripotency marker Oct4, and (C) cardiomyocyte marker Troponin T at Day 0 (hiPSCs) and Day 21 (CMs) of the differentiation protocol. Sox2 = 732 occupied wells, Oct4 = 1139 occupied wells, Troponin T = 1805 occupied wells.

A **Day 0**

Histogram of Troponin T PeakArea for the subset of 505 wells
(53 Troponin T+ / 452 Troponin T-)

Mean: 6.04E+04
Median: 6.02E+04

Counts
Log Troponin T PeakArea

Day 21

Histogram of Troponin T PeakArea for the subset of 754 wells
(488 Troponin T+ / 266 Troponin T-)

Mean: 1.45E+06
Median: 1.27E+06

Counts
Log Troponin T PeakArea

B **Day 0**

	Troponin T+	Troponin T-	Total
Histone H3+	53	452	505
Histone H3-	0	0	0
Total	53	452	505

	Troponin T+	Troponin T-	Total
Histone H3+	10.5%	89.5%	100.0%
Histone H3-	0.0%	0.0%	0.0%
Total	10.5%	89.5%	100.0%

Day 21

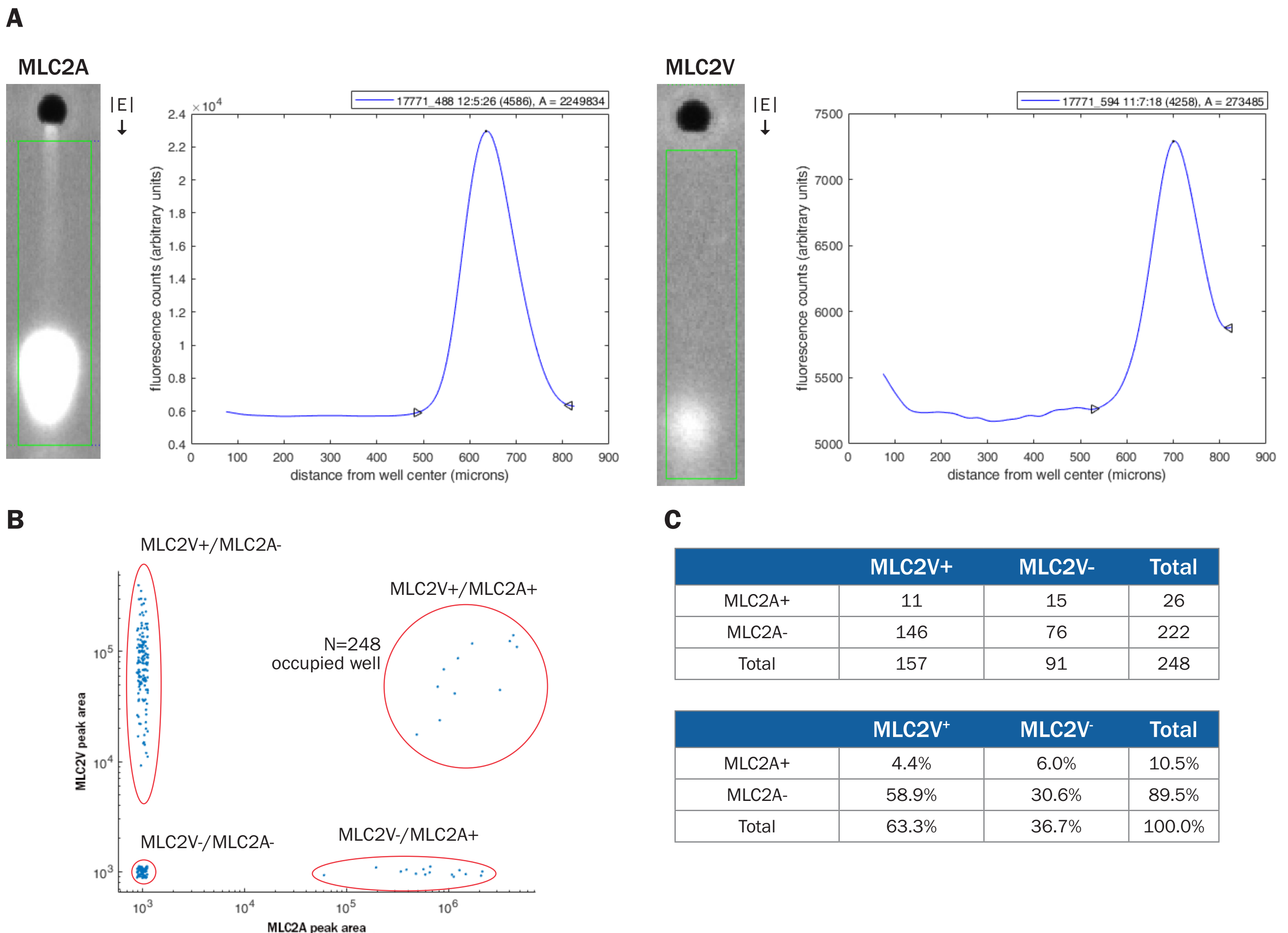
	Troponin T+	Troponin T-	Total
Histone H3+	488	266	754
Histone H3-	0	0	0
Total	488	266	754

	Troponin T+	Troponin T-	Total
Histone H3+	64.7%	35.3%	100.0%
Histone H3-	0.0%	0.0%	0.0%
Total	64.7%	35.3%	100.0%

FIGURE 4. Mean per-cell expression of Troponin T and the percentage of cells expressing Troponin T increase dramatically as cardiomyocyte differentiation progresses. (A) Histograms of Troponin T expression as a measure of peak area in a hiPSC population at Day 0 (left) and a hiPSC-CM population at Day 21 (right). (B) Enumeration tables assessing the number (top) and percentage (bottom) of cells expressing each marker measure Troponin T positive hiPSCs at Day 0 (left) and hiPSC-CMs at Day 21 (right).

Using Milo To Assess Atrial And Ventricular Phenotypes

The atrial marker MLC2A and the ventricular marker MLC2V are also important proteins for hiPSC-CM characterization, as they can illustrate the relative proportion of atrial and ventricular cell phenotypes in the differentiated hiPSC-CM sample. As such, the expression of these markers was evaluated over a 21 day period of cardiomyocyte culture. Figure 5 shows Single-Cell Western detection of cells co-expressing MLC2V and MLC2A as well as cells enriched for one marker relative to the other. Historically, the discrimination between cell subpopulations based on single or co-expression of two different proteins has not been possible with conventional Western blots. Milo can detect discrete cell populations by measuring multiple proteins within individual cardiomyocytes, allowing you to track cellular differentiation and maturity over time in culture. The three cell phenotypes identified in these cultures, namely MLC2A+, MLC2V+ and MLC2A+/MLC2V+, were further defined according to the expression level of each marker (**Figure 5B, C**). The largest population of cells (59%) was MLC2V+/MLC2A- indicating a ventricular phenotype, while only 6% of cells were of an atrial phenotype (MLC2V-/MLC2A+).



	MLC2V+	MLC2V-	Total
MLC2A+	11	15	26
MLC2A-	146	76	222
Total	157	91	248

	MLC2V+	MLC2V-	Total
MLC2A+	4.4%	6.0%	10.5%
MLC2A-	58.9%	30.6%	89.5%
Total	63.3%	36.7%	100.0%

FIGURE 5. Single-Cell Westerns demonstrate the relative proportion of atrial and ventricular phenotypes within a differentiated hiPSC-derived cardiomyocyte sample. (A) Representative MLC2A (atrial marker) and MLC2V (ventricular marker) lane view images and their accompanying fluorescence intensity plots. (B) Two-dimensional scatterplot of MLC2A peak area versus MLC2V peak area in a hiPSC-CM sample with 248 occupied microwells. (C) Enumeration tables quantifying the number (top) and percentage (bottom) of cells in the hiPSC-CM sample that exhibit atrial and ventricular phenotypes.

CONCLUSION

With applications in both basic and translational cardiac research, hiPSC-CMs are poised to pave the way to a better understanding and treatment of CVD, enhanced drug discovery and development, and improved insight into normal heart development. However, bulk sample analysis does not provide adequate resolution to characterize the molecular heterogeneity of induced pluripotent stem cells or hiPSC-CM cell culture subpopulations and the fluctuation of protein expression during hiPSC-CM maturation. Single-Cell Westerns on Milo enable single-cell resolution profiling of protein expression and can help you determine subpopulation heterogeneity. In this study, we've shown Milo's power for the analysis of hiPSC and hiPSC-CM samples and demonstrated his capacity to track phenotypic changes over time. This exciting capability will help you improve the consistency of your cardiomyocyte production and accelerate the development of robust tissue regeneration treatments.

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