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APPLICATION NOTE

Attune NxT Flow Cytometer

Flow cytometry analysis of transcription factor expression during differentiation of hPSC-derived cardiomyocytes

Introduction

The ability to direct human pluripotent stem cells (hPSCs) towards differentiated cell phenotypes offers tremendous potential for personalized and regenerative medicine [1,2]. The identification of key transcriptional regulators of pluripotency, as well as chemically defined media and cell culture conditions that drive PSCs towards distinct cell fates, have enabled researchers to derive a multitude of differentiated cell types with a high degree of control and precision [3]. One of the hallmarks of the transition from pluripotency towards terminal differentiation is the orchestrated nuclear expression of various transcription factors that act as regulators of cell fate determination. In the case of hPSC-derived cardiomyocytes, the down-regulation and eventual loss of pluripotency markers is followed by the sequential expression of other factors that act to restrict cell fate potential [4]. The cells initially become committed to a general mesodermal lineage, followed by specification to cardiovascular progenitors, and finally to differentiated cardiomyocytes that exhibit many of the physiological properties of primary cardiac cells, including spontaneous electrical activity and contractility [5]. For researchers studying cardiovascular biology or cardiotoxicity of potential drug candidates, the ability to reliably generate cell cultures with these properties can greatly accelerate discovery and screening.

Quantification of the dynamic expression patterns of transcription factors that underlie cardiomyocyte differentiation often relies on detection of mRNA transcripts via quantitative reverse transcription PCR (RT-qPCR) in cell and tissue lysates made from heterogeneous populations of cells. While this approach is highly sensitive and can be performed using small amounts of input material, it does not provide resolution at the level of the individual cells. An alternate approach is to use specific antibodies for detection and quantification of transcription factor expression at the single-cell level using either high-content imaging and analysis, or multiparameter flow cytometry. A major challenge for image-based analysis of differentiating PSCs is the complex, three-dimensional nature of the cell colonies formed in culture that confounds automated focus and acquisition (Figure 1).

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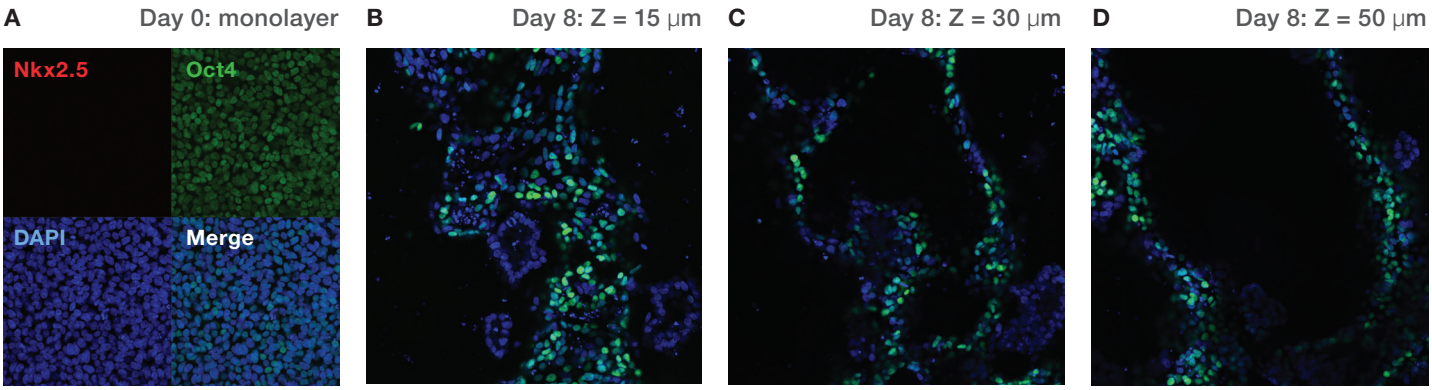

Figure 1. Confocal images of hPSCs prior to differentiation (A) and 8 days after induction (B–D) of cardiomyocyte differentiation. (A) Cells are labeled with antibodies against Oct4 (Invitrogen™ Alexa Fluor™ 488 dye, green) and Nkx2.5 (Alexa Fluor™ 568 dye, red), and with DAPI (blue) to label nuclei. Nearly all cells show positive green nuclear staining for Oct4, consistent with maintenance of a pluripotent state. No red labeling was detected for Nkx2.5, indicating that cells have not begun to differentiate along a cardiac mesodermal fate. Cells form a confluent monolayer at this stage and can be easily imaged using a single plane. **(B–D)** Cells were labeled with anti-Nkx2.5 (Alexa Fluor 488 dye, green) and DAPI (blue). These represent individual optical slices taken from 15, 30, and 50 µm depths within a 105 µm Z-stack confocal image (B, C, and D, respectively). This demonstrates that the differentiated hPSCs, many of which are Nkx2.5$^+$, are distributed throughout the 105 µm thickness. Because of the three-dimensional nature of these cultures, accurate analysis of Nkx2.5$^+$ cells cannot easily be achieved using wide-field, high-content analysis imaging systems.

Here, we describe a flow cytometric method for the simultaneous quantification of Oct4, a canonical marker of pluripotency, and Nkx2.5, a marker of cardiac fate, in hPSCs induced to differentiate towards cardiomyocytes. Analysis was performed on an Invitrogen™ Attune™ NxT Flow Cytometer, which is ideally suited for use with fragile and large cell types like stem cells and cardiomyocytes, as it allows for gentle and safe analysis without clogging the instrument or wasting cells. This is partly due to the fact that the Attune NxT Flow Cytometer was developed with acoustic-assisted hydrodynamic focusing technology, and advanced fluidics was designed to minimize clogging and effectively handle a broad range of cell types. This allows for a higher degree of data, detail, and throughput that enables processing of a large range of sample types, including large clumpy cells, samples with a low concentration of cells, and precious samples, more quickly and accurately than ever before with no loss in data quality.

Methods

Materials

- WiCell™ human iPSCs (Cat. No. WA09)
- Gibco™ Vitronectin protein (Cat. No. A14700)
- Gibco™ Essential 8™ Medium (Cat. No. A15170-01)
- Gibco™ PSC Cardiomyocyte Differentiation Kit (Cat. No. A29212-01)
- Gibco™ TrypLE™ Express enzyme (Cat. No. 12605-010)
- Polysciences Formaldehyde, 16%, methanol-free (Cat. No. 18814)
- Cell Signaling Technology Rabbit Anti-Oct4, clone C30A3, Alexa Fluor™ 488 conjugate (Cat. No. 5177)
- Cell Signaling Technology Rabbit Anti-Nkx2.5, clone E1Y8H (Cat. No. 8792)
- Invitrogen™ Donkey Anti–Rabbit IgG, Alexa Fluor™ 647 conjugate (Cat. No. A-31573)
- Jackson ImmunoResearch Normal donkey serum (Cat. No. 017-000-121)
- Jackson ImmunoResearch Rabbit IgG (Cat. No. 011-000-002)
- BD Pharmingen™ Stain Buffer (Cat. No. 554657)
- Sigma Aldrich Triton™ X-100 (Cat. No. X100)
- Gibco™ Phosphate-Buffered Saline (PBS) (Cat. No. 10010)
- Invitrogen™ Countess™ II Automated Cell Counter (Cat. No. AMQAX1000)
- Invitrogen™ EVOS™ XL Core Imaging System (Cat. No. AMEX1000)
- Attune NxT Flow Cytometer, 4-laser configuration (Cat. No. A24858)
- Thermo Scientific™ Nunc™ 6-well tissue culture plates (Cat. No. 140675, or equivalent)
- Thermo Scientific™ 12 x 75 mm round bottom tubes (Cat. No. S40122, or equivalent)

Cell culture and antibody labeling

The PSC Cardiomyocyte Differentiation Kit is a complete, ready-to-use, xeno-free system for the efficient differentiation of hPSCs into contracting cardiomyocytes within 10 days of initiating differentiation. Differentiated cardiomyocytes can be maintained in Gibco™ Cardiomyocyte Maintenance Medium for >30 days.

Undifferentiated H9 hPSCs were cultured and expanded in Essential 8 Medium on vitronectin-coated 6-well plates, according to the product manuals [6,7]. On day 0, the medium was exchanged with Cardiomyocyte Differentiation Medium A. On day 3, the medium was exchanged with Cardiomyocyte Differentiation Medium B. On day 5, the medium was exchanged with Cardiomyocyte Maintenance Medium, and cells were maintained for an additional 5–7 days (Figure 2). By day 12, contractility could be observed using the EVOS XL Core Imaging System with phase-contrast optics, consistent with cardiac cell differentiation.

On each day during the differentiation process, cells were detached from plates using TrypLE Express enzymatic dissociation solution and triturated to a single-cell suspension. Cell counts and viability measurements were taken using a Countess II Automated Cell Counter, and the typical cell diameter was determined to be 13 µm. A total of 1 x 10^6 cells from each time point were transferred to centrifuge tubes, and cells were fixed in freshly prepared 4% formaldehyde in PBS for 15 min. Cells were then centrifuged at 400 x *g* for 5 min and washed in PBS. Fixed cells were stored in PBS at 4°C until all time points were collected. Cells were permeabilized and blocked in BD Pharmingen Stain Buffer containing 5% normal donkey serum and 0.1% Triton X-100 surfactant for 20 min at room temperature. Rabbit anti-Nkx2.5 antibody was added at 1:200 in blocking buffer, and cells were incubated overnight at 4°C. Following washes in stain buffer, cells were incubated with donkey anti–rabbit IgG with Alexa Fluor 647 conjugate at 4 µg/mL in blocking buffer for 2 hr at room temperature in the dark. Following a wash step in stain buffer, 10 µg/mL of rabbit IgG in blocking buffer was added for 20 min to saturate rabbit IgG–binding sites. Rabbit anti-Oct4 antibody with Alexa Fluor 488 conjugate was added at 1:50 in a solution containing blocking buffer and rabbit IgG for 2 hr at room temperature. Cells were washed in stain buffer and acquired on the Attune NxT Flow Cytometer.

Flow cytometry acquisition

Data were acquired using the BL1 and RL1 detectors for the anti-Oct4 antibody (Alexa Fluor 488 conjugate) and anti-Nkx2.5 antibody (Alexa Fluor 647 conjugate), respectively (Table 1), and forward scatter (FSC) and side scatter (SSC). Data were collected at a flow rate of 200 µL/min with stop criteria set on 10,000 total events, using a FSC threshold.

Table 1. Optical configuration.

Antibody	Fluorophore	Excitation laser (nm)	Emission filter (nm)	Attune NxT detector
Oct4	Alexa Fluor 488	488	530/30	BL1
Nkx2.5	Alexa Fluor 647	638	670/14	RL1

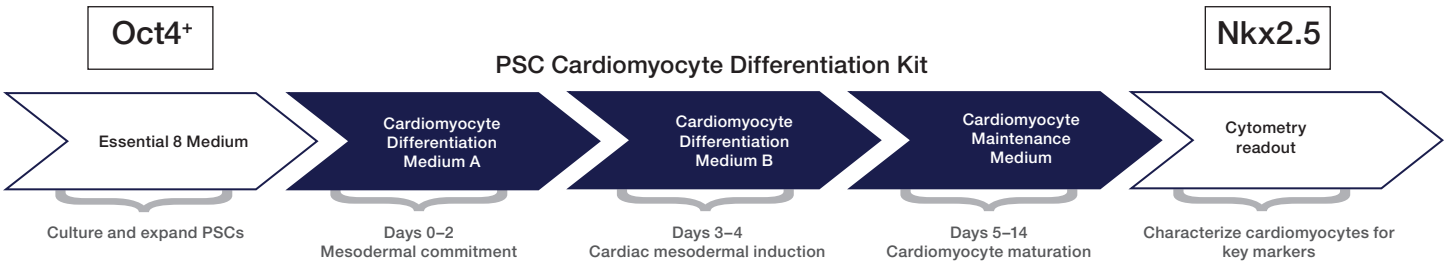

Figure 2. Workflow of cardiomyocyte differentiation.

Results

A dual-parameter plot of FSC-width vs. FSC-height was used to identify singlet cells, and a gate was drawn around the single-cell population (Figure 3). Using the singlet gate, a dual-parameter plot was created for Oct4 fluorescence vs. Nkx2.5 fluorescence. A quadrant gate was used to identify the cell population as it differentiates the Oct4$^+$ events (green), the Nkx2.5$^+$ events (red), and dual negative events (blue) (Figure 4). The plots were displayed in the precedence-density format, and percent positive for each target was recorded (Figure 5). Prior to differentiation, nearly 100% of all cells were Oct4$^+$ and Nkx2.5$^-$, consistent with a pluripotent state. After day 3, the frequency of Oct4$^+$ cells began to decline, consistent with a loss of pluripotency and a transition to a terminally differentiated cardiomyocyte phenotype, with robust expression of Nkx2.5 detected by day 8.

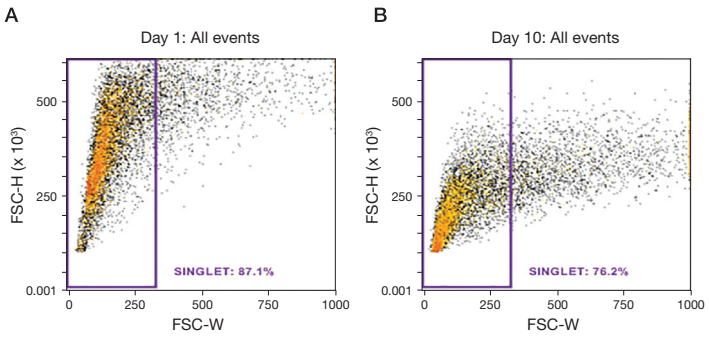

Figure 3. A dual-parameter plot of FSC-width vs. FSC-height was used to identify single cells. A gate labeled "singlet" was drawn around the single-cell population. Data from **(A)** day 1 and **(B)** day 10 show changes in forward scatter profiles with cell differentiation.

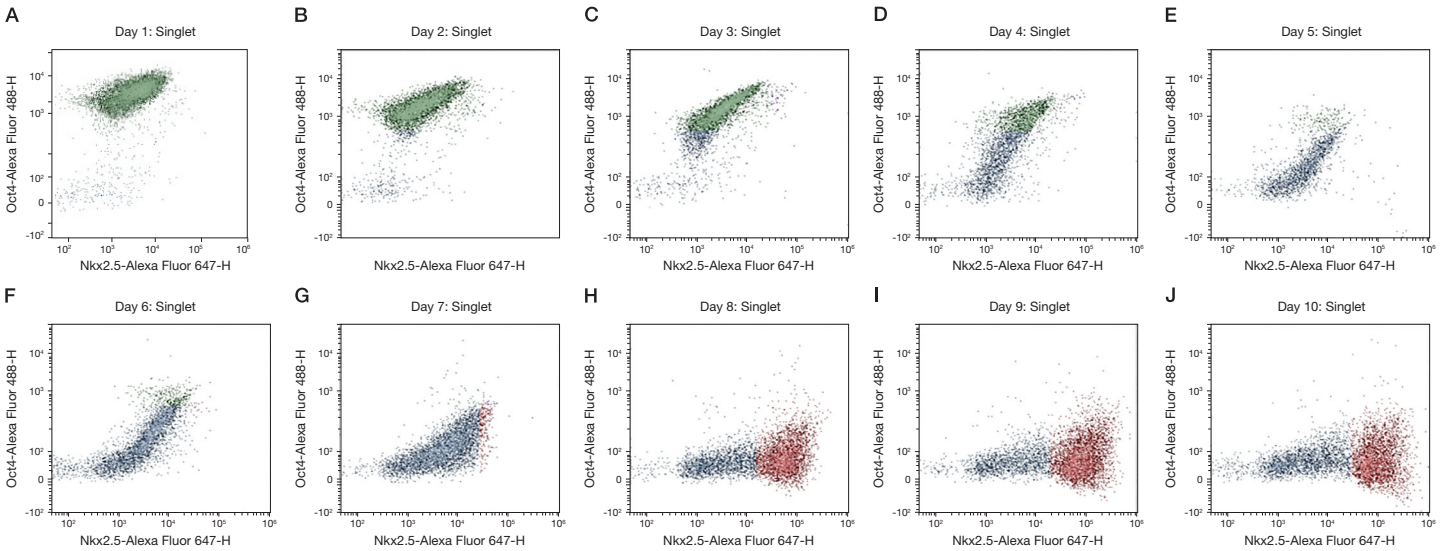

Figure 4. Two-parameter plots representing staining profiles for Oct4 and Nkx2.5 in H9 hPSC cells during cardiomyocyte differentiation. All plots were gated on singlet cells. At day 1 **(A)**, nearly all cells are Oct4$^+$ and Nkx2.5$^-$, consistent with a pluripotent state. During the time course of differentiation, with data shown for each day of differentiation **(B–J)**, cells lose Oct4 expression and begin to express the cardiac marker Nkx2.5. The precedence-density plot display is used, with the red-colored population representing Nkx2.5$^+$ cells, and the green-colored population representing Oct4$^+$ cells.

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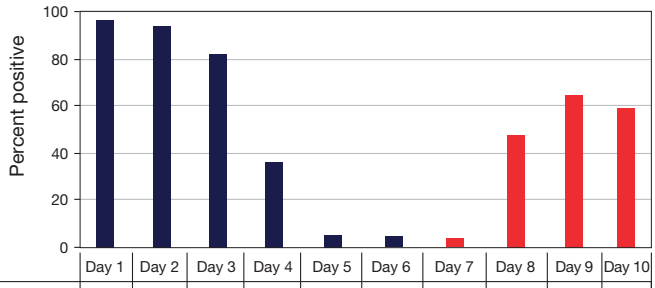
Conclusion

The Attune NxT Flow Cytometer enables single-cell quantification of cells expressing markers of both pluripotency (Oct4) and cardiomyocyte specification (Nkx2.5) in H9 hPSCs as they are induced to differentiate using defined media that are included in the Cardiomyocyte Differentiation Kit. Results obtained using immunolabeling with specific antibodies against these transcription factors are consistent with published data using RT-qPCR quantification of Oct4 and Nkx2.5 mRNA transcripts [4,8] with the added advantage of analysis at the single-cell level. Because hPSCs form dense three-dimensional clusters as they differentiate, flow cytometric quantification has an advantage over image-based, high-content analysis of these cultures.

Stem cells and cardiomyocytes represent traditionally challenging samples for flow cytometry testing due to their size, fragility, and scarcity. Equipped with acoustic-assisted hydrodynamic focusing, the Attune NxT Flow Cytometer is able to achieve sample throughput rates up to 10 times faster than those of traditional cytometers. This means that you can process samples more quickly and acquire sufficient events even from very dilute samples. Researchers using challenging samples such as tumor and stem cells are particularly interested in this feature of the Attune NxT Flow Cytometer because the quality of the data is not compromised by this very rapid sampling rate, unlike with traditional flow cytometers, where the data quality degrades as sample rate is increased. Many cell types and samples are now within your reach with the Attune NxT Flow Cytometer.



	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Oct4 % pos	97	95	83	37	5	5	0	0	0	0
Nkx2.5 % pos	0	0	0	0	1	1	4	48	65	60

Figure 5. Oct4 and Nkx2.5 expression over 10 days in culture. Prior to differentiation, 97% of all cells expressed the Oct4^{+}/Nkx2.5^{-} phenotype, consistent with a pluripotent state. With induction, expression of Oct4 declines, consistent with a loss of pluripotency, and a transition to a terminally differentiated cardiomyocyte phenotype is seen as expression of Nkx2.5 increases. These results were obtained by placing quad gates on an Oct4 vs. Nkx2.5 dual-parameter plot and looking at percent positive (gated on singlet cells).

References

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