Single Cell Analysis of Transcription Factor Expression during Differentiation of hPSC to Cardiomyocytes using Flow Cytometry

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Abstract

The ability to direct human Pluripotent Stem Cells (hPSCs) towards differentiated cell phenotypes offers great potential for personalized and regenerative medicine. The identification of key transcriptional regulators of pluripotency enables researchers to derive a variety of differentiated cell types with a high degree of control and precision. One of the hallmarks of the transition from pluripotency towards terminal differentiation is the orchestrated expression of various nuclear transcription factors, which 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. Quantification of the dynamic expression patterns of transcription factors that underlie cardiomyocyte differentiation often relies on the detection of mRNA transcripts via qRT-PCR 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 material, it does not provide resolution at the level of individual cells. Because hPSCs form dense three-dimensional clusters as they differentiate, flow cytometric quantification is advantageous to image-based high content analysis of these cultures. An alternate approach is to use specific antibodies for detection and quantification of transcription factor expression at the single cell level, flow cytometry is ideally suited for this type of analysis. Stem cells and cardiomyocytes represent traditionally challenging samples for flow cytometric testing due to their size and fragility. Here, we describe a flow cytometric method for quantification of Oct4, a recognized marker of pluripotency, and Nkx2.5, a marker of cardiac fate, in hPSCs induced to differentiate towards cardiomyocytes. Undifferentiated hPSCs were cultured and expanded, and induced to differentiate in a cardiomyocyte fate. Cells were tracked over a 10 day culture after differentiation was initiated. Antibodies to OCT4 and Nkx2.5 with a fluorescent tag were used to label the cells simultaneously, followed by detection using flow cytometry. Prior to differentiation nearly all cells expressed the $Oct4^{pos}/Nkx2.5^{neg}$ 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.

For research use only. Not for use in diagnostic procedures.

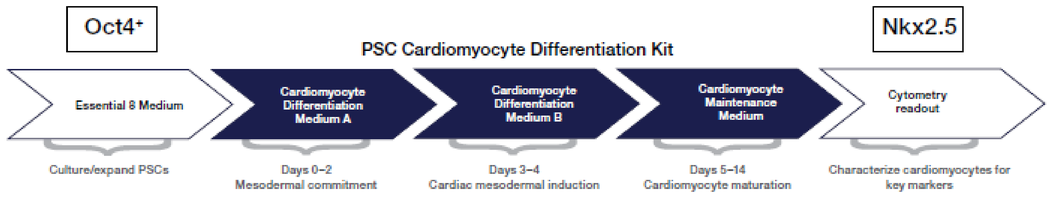

Figure 1. Workflow of cardiomyocyte differentiation from hPSC

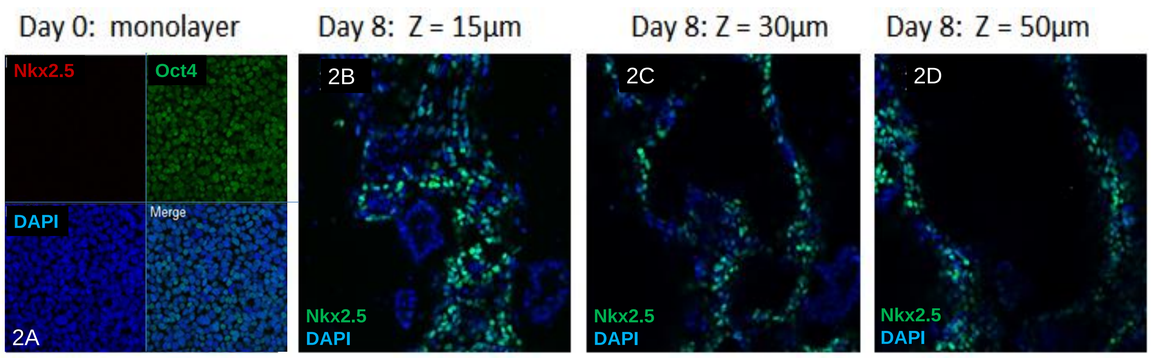

Figure 2. Confocal images of hPSC on day 0, and 8 days after induction of cardiomyocyte differentiation

Figure 2A: image of hPSCs at Day 0, prior to the onset of differentiation.
Cells are labeled with antibodies against Oct4 (Alexa Fluor™ 488, green) and Nkx2.5 (Alexa Fluor™ 568, 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.

Figure 2B - 2D: Confocal image of hPSCs 8 days after induction of cardiomyocyte differentiation.
Cells were labeled with anti-Nkx2.5 (Alexa Fluor 488, green) and DAPI (blue). These represent individual optical slices taken from 15, 30 and 50 um depths within a 105 um Z-stack confocal image (1B, 1C, and 1D, respectively). This demonstrates the differentiated hPSCs, many of which are Nkx2.5+, are distributed throughout the 105 um 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.

Antibody	Clone	Fluorophore	Excitation Laser	Emission Filter	Attune NxT Detector
4-Oct	C30A3	Alexa Fluor 488	488 nm (50 mW)	530/30	BL1
Nkx2.5	E1Y8H	Alexa Fluor 647	638 nm (100 mW)	670/14	RL1

Figure 3. Target, clone and fluorophore used with Attune NxT excitation laser and emission band pass.

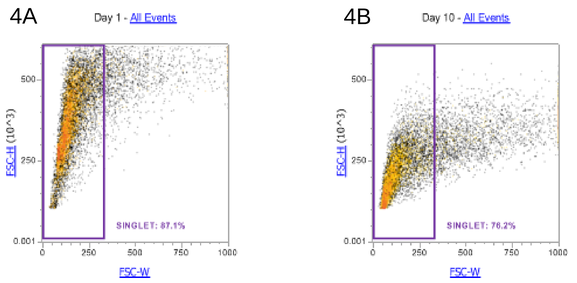

Figure 4. A dual parameter plot of FSC-Width vs. FSC-Height was used to identify single cells.
A gate labeled SINGLET was drawn around the single cell population. Data from day 1 (A) and from day 10 (B) show changes in forward scatter profiles with cell differentiation.

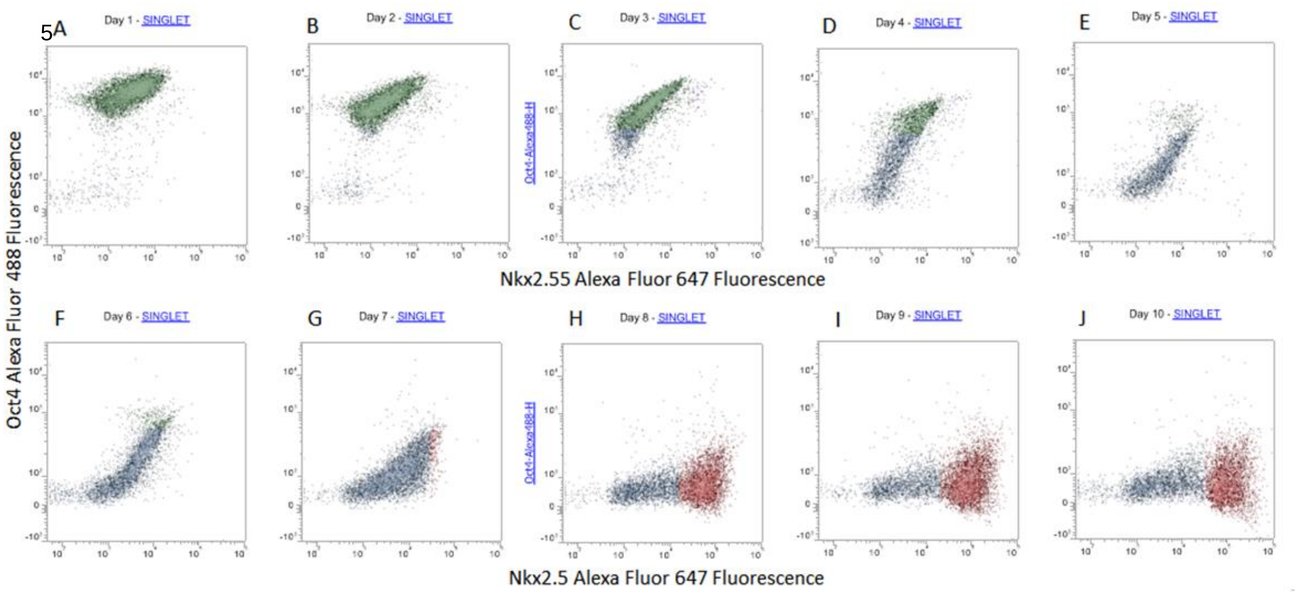

Figure 5. Two parameter plots representing staining profiles for Oct4-Alexa Fluor 488 and Nkx2.5-Alexa Fluor 647 in hPSC cells during cardiomyocyte differentiation. All plots were gated on singlet cells. At Day 1 (A), nearly all cells are Oct4 positive and Nkx2.5 negative, consistent with a pluripotent 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 plots display is used, with red colored dots representing Nkx2.5 positive cells, green colored dots representing Oct4 positive cells.

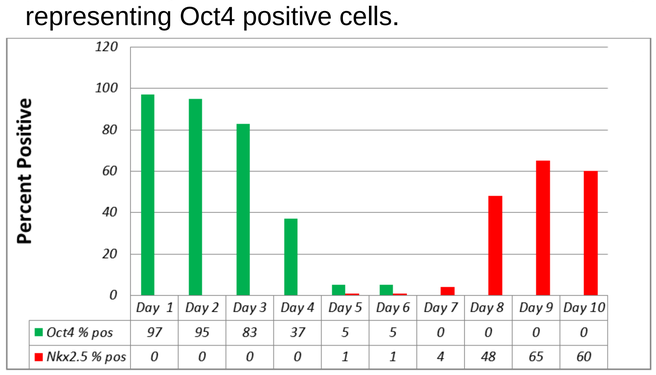

Figure 6. Graph showing the percentages of Oct4+ (green) and Nkx2.5+ (red) expression over 10 days in culture. Prior to differentiation nearly all cells expressed the $Oct4^{+}/Nkx2.5^{-}$ phenotype, consistent with a pluripotent state. With induction, expression of Oct4 declines, consistent with a loss 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 the Oct4 vs Nkx2.5 dual parameter plot and looking at percent positive.

Conclusions

- The use of flow cytometry enables the quantification of cells expressing markers of both pluripotency (Oct4) and cardiomyocyte specification (Nkx2.5) in hPSCs as they are induced to differentiate using defined media included in the Invitrogen Cardiomyocyte Differentiation kit. Results obtained using specific antibodies against these transcription factors are consistent with published data using qRT-PCR quantification of Oct4 and Nkx2.5 mRNA transcripts, with the added advantage of analysis at the cellular level.
- Because hPSCs form dense three-dimensional clusters as they differentiate, flow cytometric quantification is advantageous to image-based high content analysis of these cultures.
- Stem cells and cardiomyocytes represent traditionally challenging samples for flow cytometric testing due to their size, fragility, and scarcity. Equipped with acoustic-assisted hydrodynamic focusing, the Invitrogen Attune NxT Flow Cytometer is able to achieve sample-throughput rates up to 10 times faster than traditional cytometers, which allows the acquisition of very dilute samples in reasonable times. Researchers using challenging samples such as tumor and stem cells can benefit by using the Attune NxT Cytometer as the quality of the data is not compromised by this very rapid sampling rate.