Monitoring Differentiation of Embryonic Stem Cells by Automated Flow Cytometry Sample Preparation on the Biomek® NX^P Laboratory Automation Workstation

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Abstract

The well-documented ability of stem cells to differentiate into various cell lineages generates tremendous potential for cell-based treatments. For example, differentiated cardiomyocytes from embryonic stem cells can be used in drug discovery processes and therapeutic cardiac treatments. Experimentation to optimize differentiation to enhance the yield of cardiomyocytes is enabled by efficient analysis of differentiation. Downstream detection technologies for such experiments vary in time, complexity, and the ability to quantitatively determine the efficiency of differentiation. Flow cytometry is a common method for detection of various cell types, but requires automated sample preparation for use in higher throughput situations. We have employed the Biomek NX^P Span-8 Automation Workstation for high throughput flow cytometry sample preparation to determine the efficiency of cardiomyocyte differentiation from murine embryonic stem cells. The workflow includes fixation, permeabilization, blocking, and antibody staining in a 96-well plate format. The Agilent Microplate Centrifuge has been integrated to the system to reduce user interaction for executing the multiple washes required. The automated workflow and results from the analysis are described.

Introduction

To understand the changes that occur during differentiation of murine embryonic stem cells (mESCs), a selection of markers was monitored throughout the differentiation process. This work is intended to gain a better understanding of how the cells progress through differentiation by monitoring the presence of markers for stem cells, endoderm, ectoderm, mesoderm, and cardiomyocytes (Figure 1). Gaining an understanding of how and when the cells differentiate, may lead to a better understanding on how to control and direct this differentiation process. This work focuses on directed differentiation of cardiomyocytes using various cardiac inducing treatments.

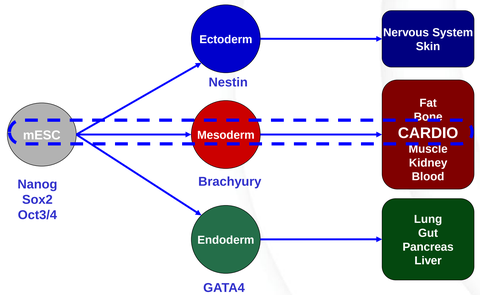


Figure 1: Differentiation pathways stemming from mESC, including markers for each of the lineages. The focus will be on cardiomyocyte differentiation.

Antibodies were combined into four cocktails for multicolor flow cytometry. This includes two panels for isotype controls, a panel for Cardiomyocyte/Stem Cell Markers, and a panel for early differentiation markers Figure 2.

Cardiomyocyte/ESC Panel :

- **Nanog-PE** (BD Biosciences)
- **Sox2-Alexa 647** (BD Biosciences)
- **Oct-3/4-PerCP-Cy5.5** (BD Biosciences)
- **Myosin Heavy Chain(MF20)-Alexa488** (ebiosciences)

Differentiation Panel:

- **GATA4-Alexa 488** (BD Biosciences)
- **Nestin-Alexa 647** (BD Biosciences)
- **Brachyury-PE** (R&D Systems)

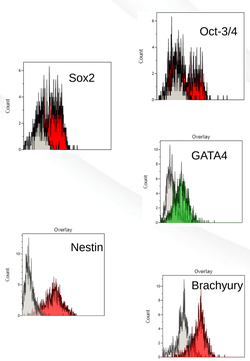
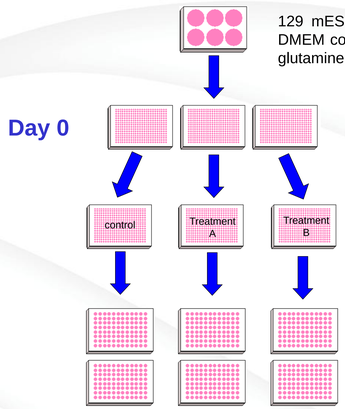


Figure 2: (top to bottom) Isotype Controls (gray) and pure mESC populations stained with Sox2 and Oct3/4 antibodies. Day 6 differentiated cells stained with isotype controls (gray) and GATA4, Nestin, and Brachyury antibodies.

Materials and Methods



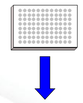
129 mESC (Invitrogen) were maintained in gelatin coated 6 well plates in knockout DMEM containing 15% knockout serum replacement (KSR), non essential amino acids, glutamine, LIF, BME and pen/strep .

500 cells/well (in 40 µL) were plated in differentiation media (same as ES cell media minus LIF, and FBS in place of KSR), in a 384-well polypropylene plates (Nunc). The cells, unable to adhere, coalesce to form Embryoid Bodies (EBs).

Cells were treated with 80 µL of additional media, and cardiac inducing treatments were also added at this time.

To ensure sufficient cell numbers for flow cytometry, 2 EBs were plated in each well of 96-well gelatin coated plates. The EBs adhered to this surface overnight. Additional differentiation media was also added to the EBs at this time.

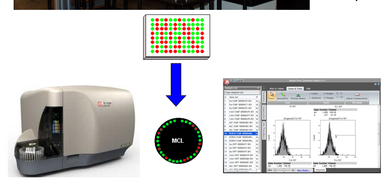
Days 6-9



A sampling of the cells were taken on subsequent days to analyze for differentiation progress. Cells that were not used were fed as needed. Cells for analysis were detached using Accumax (Millipore) until a single cell suspension was achieved. 3 wells of adherent cells were combined into one well of a Costar 96-well round bottom plate for flow processing.

Fix
Wash
Perm
Wash
Block
Wash
Stain
Wash
Resuspend

Cell suspensions in Accumax were placed on the Biomek NX^P for flow cytometry preparation. An initial spin was conducted in the on deck Microplate Centrifuge (Agilent), and the supernatant was removed. Cells were fixed (Invitrogen) for 5 min then washed. Wash steps include adding 200 µL PBS or PBS+0.1%Tween, followed by a spin and supernatant removal. Cells were permeabilized (Invitrogen) for 20 min, then washed. The cells were blocked with PBS + 2% Goat Serum for 30 min, then washed. Antibody cocktails were added, and incubated at RT for 1 hr. A final wash was completed and cells were resuspended in 200 µL of PBS. All conditions were run in triplicate



Samples were transferred to a multi-carousel loader (MCL) and analyzed using the Gallios™ Flow Cytometer**. Data was analyzed using Kaluza™ Software**.

Gating Strategy: Isotype controls for each of the antibodies were used as a gating tool. Using Fluorescent intensity vs. side scatter, a gate was drawn around the negative population (Figure 3A). These gates were then used for all positively stained samples (Figure 3B). This methodology was found to be more consistent than gating on histograms due to the relatively small change in fluorescent intensity for positively stained cells (Figure 3C).

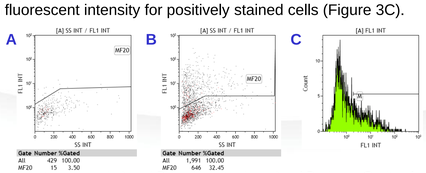
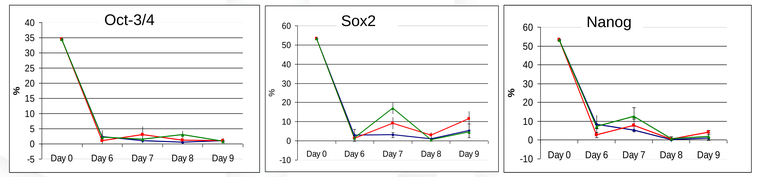


Figure 3: A) Isotype control for MF20 antibodies used to for gating. B) Day 8 differentiated cells. C) Histogram of the same data.

Monitoring Differentiation

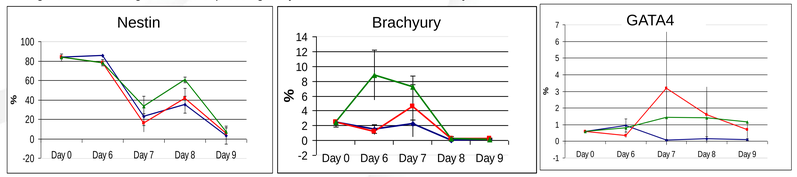
Stem Cell Markers: A dramatic drop in the three stem cell markers can be seen, with the biggest drop occurring in the first 6 days of differentiation. Beyond 6 days, Oct-3/4 remains less than 5% for all conditions. Sox2 shows a small spike on Day 7, as does Nanog. By Day 9, Nanog is less than 5% for all conditions, and Sox2 varies between approximately 5-12% (Figure 4).

Figure 4: Percentages of cells expressing Stem Cell markers on Day 0, 6, 7, 8 and 9.



Early Differentiation Markers: Interestingly, the majority of ES cells express Nestin (~80%). This percentage drops dramatically around day 6 and increases on day 8; this could be indicative of neuronal cell differentiation. Brachyury shows a significant increase in Treatment B, peaking around day 6 before dropping to basal levels (Figure 5); MF20-positive cells peak when brachyury reaches basal levels (Figure 7). GATA4 expression remains low in all experimental conditions; earlier time points may be more informative for GATA4 expression.

Figure 5: Percentages of cells expressing early differentiation markers on Day 0, 6, 7, 8 and 9.



Cardiomyocytes: MF20 expression stays low for the first 6 days of differentiation, and then starts to climb in all three conditions (Figure 6). Treatment B shows the most dramatic cardiomyocyte formation, and peaks on Day 8, at around 35%.

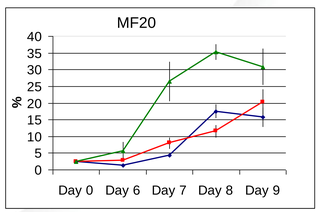
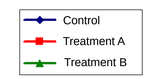


Figure 6: Percentages of cells expressing early differentiation markers on Day 0, 6, 7, 8 and 9.



Conclusions

- The integration of the Biomek NX^P Span-8 automation workstation with a Microplate Centrifuge is a fully automated system capable of multicolor flow cytometry preparation.
- A minimal number of cells express Nanog, Sox2 and Oct-3/4 by day 6 of differentiation indicating differentiation to cell lineages is well underway by this time point.
- Treatment B results in ~35% of cells forming into cardiomyocytes. This condition shows significant increase in cardiomyocyte formation over the control. Treatment A does not appear to enhance cardiomyocyte differentiation.