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

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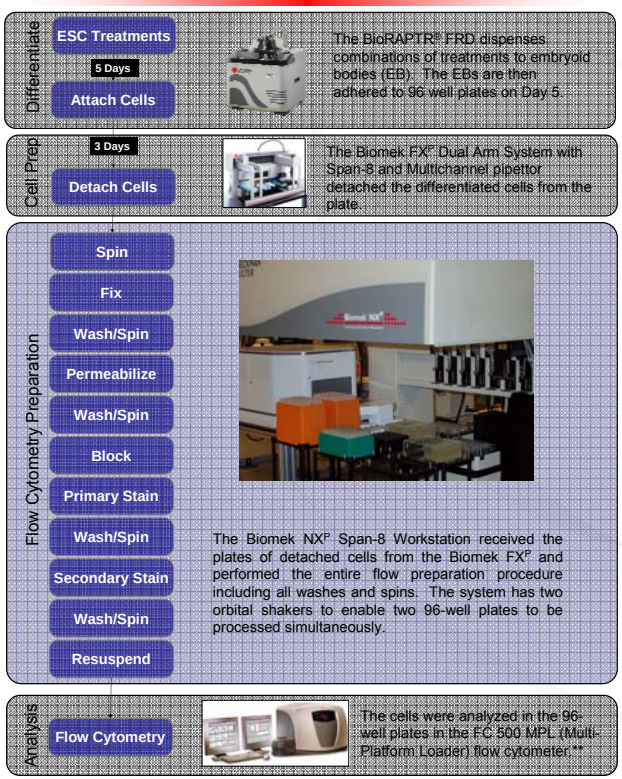
Abstract

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.

Workflow

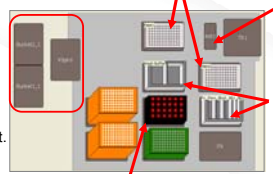


Walk Away Configuration

Process up to 192 samples simultaneously in approximately 5.5 hours or a single 96-well plate in approximately 4 hours.

Wash Station for automatic removal of liquid waste from the deck.

Microplate Centrifuge is integrated to accommodate the multiple wash steps which require centrifugation before removing the supernatant.



All buffers and reagents on deck.

Up to 24 positions for Primary and Secondary antibodies which can be added to samples in any permutation using a worklist.

Cardiomyocyte Staining

Cells were fixed and permeabilized using IntraPrep Permeabilization Kit. PBS + 0.2% Tween was used for all washes with the exception of the wash after fixation which used PBS. The samples were blocked in PBS + 2% Goat Serum. The Primary Antibody was Goat anti- α -Actinin (Sigma) and was diluted 1:500. A The secondary antibody was Mouse anti Goat-Alexa 488 (Invitrogen) and diluted at 1:500. The same procedure was used on slides of mouse cardiomyocytes to determine that the staining showed sarcomeres, indicating that staining is specific (Figure 1).

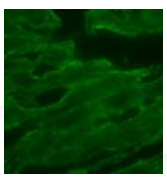
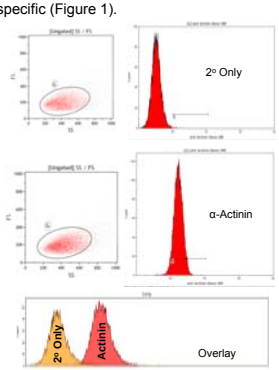


Figure 1: Actinin stained Mouse Cardiomyocytes show clear striations of the sarcomeres



Pure cardiomyocyte populations were tested (Mouse Cardiomyocyte line from Celprogen), and analyzed on flow cytometry to demonstrate that a significant shift in fluorescent intensity is evident (Figure 2). Peaks were analyzed for x-mean, and % CVs were calculated between replicates. Low CVs for the secondary stain indicate a high degree of reproducibility between replicates. Higher CVs for actinin stained cells are accounted for in variability due to cell size. (Table 1).

Figure 2: Pure mouse cardiomyocyte population with only secondary antibody (Top). Same Cells with Actinin staining (Middle) and the overlay of the histograms indicates a significant shift in fluorescent intensity (Bottom).

Table 1: Data summary for replicates of mouse cardiomyocytes.

	X-mean average	Variation between replicates (%CV)
Secondary only (n=16)	0.400 ± 0.036	9.1 %
Actinin Stained (n=16)	1.60 ± 0.29	18.2 %

Differentiation Screen

Mouse Embryonic Stems cells were treated with varying combinations of compounds using the BioRAPTR FRD to induce cardiomyocyte directed differentiation. Cells were plated in suspension to induce differentiation and embryoid body formation; compounds were added on day 0 and day 2, and cells adhered to a 96-well plates on day 5. Contracting cells were observed shortly thereafter and cells detached on day 8 (Michael Kowalski, et al. MP75). Cells were stained with α -actinin antibody using the Biomek NX^P system and detected using the FC 500 flow cytometer. Results indicate that actinin staining on the Biomek NX^P laboratory automation workstation can differentiate between cardiomyocyte and non-cardiomyocyte populations. As a screening tool, this methodology proves to be effective at showing differences in cardiomyocyte counts well to well (Figure 3).

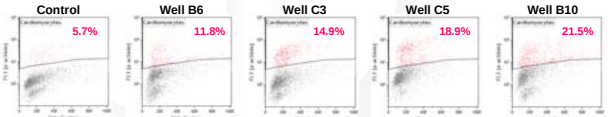


Figure 3: Control wells shows 5.7% differentiation to cardiomyocytes. Four additional wells from the screen were chosen to demonstrate a gradient of cardiomyocyte differentiation can be detected using this staining protocol.

Other Cardiac Markers

Other markers can also be used for this process. Connexin-43 is in the gap junction and is crucial for synchronizing contractions in cardiac tissue. The high turnover of this molecule often leads to reserves stored in the endoplasmic reticulum or golgi. Specific staining of this antibody (BD Biosciences) was confirmed through immunohistochemistry. Connexin-43 was diluted 1:200, and the same secondary antibody was used as previously (Figure 4). Though separation of positive and negative samples is not as great as actinin stains, a significant shift in peaks can still be observed reproducibly (Table 2).

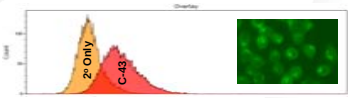


Figure 4: Pure mouse cardiomyocyte population stained with Connexin-43

Alpha Sarcomeric Actin is specific to skeletal and cardiac muscle actins which are part of the contractile function. This antibody (abcam) was diluted 1:200, and the same secondary antibody was used (Figure 5).

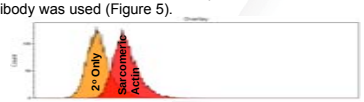


Figure 5: Pure mouse cardiomyocyte population stained with α-Sarcomeric Actin

Table 2: Replicates of Connexin-43 α -Sarcomeric Actin produce acceptable CVs.

	X-mean average	Variation between replicates (%CV)
Secondary only (n=8)	0.657 ± 0.053	8.1%
Connexin-43 (n=8)	1.21 ± 0.164	13.5 %
Sarcomeric Actin (n=8)	1.60 ± 0.13	8.2 %

Conclusions

- The Biomek NX^P laboratory automation workstation with integrated Microplate Centrifuge creates a fully walk away solution for automation of flow preparation.
- The system produces reproducible results on known populations with multiple antibodies.
- This system in combination with the Biomek FX^P laboratory automation workstation, and BioRAPTR FRD provides a fully automated solution to differentiate embryonic stem cells, manipulate the populations, and prepare them for analysis by flow cytometry.