Abstract

Automation with the Biomek 4000 platform can improve the throughput and reproducibility of conventional staining of adherent cells. Applicability is demonstrated using mouse embryonic stem cells (mESCs) to detect the expression of developmental antigens in fixed cells following differentiation to cardiomyocytes which is relevant to stem cell biology research. The cardiomyocyte model resembles approaches used for the differentiation of other cell types. Automation protocols using the Biomek 4000 Laboratory Automation Workstation were developed using PerFix-nc fixation and permeabilization reagents with a no-wash protocol that provide a well resolved and reproducible detection of intracellular markers in murine embryonic stem cells and differentiated cardiomyocytes.

Materials and Methods

mESC Culture and Differentiation

Mouse embryonic stem (mES) cells (Invitrogen, Carlsbad, CA) were maintained in growth media containing leukemia inhibitory factor (LIF) and 15% knockout serum replacement (KSR). For differentiation, cells were cultured in 15% FBS without LIF in a 384-well round bottom polypropylene plate (Nunc, Roskilde, Denmark) in 40 µL differentiation medium (various treatments for 0 to 5 days). Embryoid bodies formed by Day 5 were transferred to gelatin-coated 96-well plates in 100 µL fresh media. After Day 6, a portion of the adherent cells showed visible contraction. Control mES cells were treated in parallel without differentiation factors. Cells were harvested by Trypsin and dispensed into 96-well plates for processing.

Biomek 4000 Laboratory Automation Workstation Configuration

Figure 1 shows images of the instruments used in this information bulletin. Figure 2 shows the configuration of the Biomek 4000 Laboratory Automation Workstation used for cell staining experiments.

Figure 1. Workflow of automated microtiter plate-based cell staining process.
Figure 2. Automated cell staining method on the Biomek 4000 Laboratory Automation Workstation. A) Screen shot of the Define Pattern step in the automation method. Users can freely choose the sample wells on a 96-well plate format. B) Screen shot showing the Deck Layout. Tools, labware and tips are located as indicated. C) Screen shot of User Interface, where users can customize all the parameters, including reagent volumes, washes, incubation times and antibody information, etc. D) Screen shot showing the reagent volumes based on the parameters from the User Interface. E) Screen shot showing the custom defined antibody transfer step, where users can define the antibody locations manually.
**mES Cells and Cardiomyocytes Characterization**

Differentiated cells were harvested on Day 8 using Accumax (Millipore, Billerica, MA). Then mES cells were mixed with either pooled differentiated cells or feeder cells (Invitrogen), and seeded into 96-well imaging plates and allowed 24 hours to attach. Using a Biomek 4000 Laboratory Automation Workstation with multi-probe tool, the mixed cells were fixed and permeabilized with PerFix-nC reagents (B10825, Beckman Coulter, Inc., Brea, CA). The mixture of differentiated cells and mES cells were stained with myosin heavy chain-Alexa Fluor 488 (clone MF20, eBioScience, San Diego, CA) and anti-Sox2-Alexa Fluor 647 (clone O30-678, BD Biosciences, San Jose, CA) (**Figure 3**). The mixture of mES cells and feeder cells were stained with either anti-Nanog-Alexa Fluor 488 (clone eBioMLC-51, eBioScience, San Diego, CA) or anti-Sox2-Alexa Fluor 647 (clone O30-678, BD Biosciences, San Jose, CA). All conjugates were titered for optimal performance, and relevant isotype controls were used to control for non-specific staining. Cells were fixed by adding 5 μL of PerFix-nC reagent 1 for 15 minutes, followed by permeabilization and staining with 50 μL of PerFix-nC reagent 2 containing the antibody conjugates for 30 minutes. After the removal of the supernatant and replacement with 50 μL reagent 3 for 5 minutes, cells were identified by nuclear staining with mounting medium which contains DAPI (Invitrogen).

**Imaging**

All samples were analyzed on an ImagXpress system with MetaXpress software (Molecular Devices, Sunnyvale, CA).

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**Figure 3.** Intracellular staining of stem cell marker Sox2 and myosin heavy chain using PerFix-nC reagents. PerFix-nC fixation and permeabilization reagents are optimized for no-wash detection of intracellular markers for flow cytometry, but also provide excellent sample preparation for imaging applications. The PerFix-nC kit was tested on undifferentiated and Day 8 differentiated mES cells for staining. Mixtures of mES cells and Day 8 differentiated mES cells (ratio 1:2) were detached and re-seeded into a 96-well imaging plate, then allowed 24 hours to plate out. PerFix-nC reagents and antibodies were added into the sample wells. All liquid handling steps were performed on a Biomek 4000 platform. Sixteen wells required less than an hour to prepare for imaging. Images were taken using the 10X objective on an ImageXpress high content imager and processed with MetaXpress software (Molecular Devices). Cells were stained with either isotype controls (**B.a.**) or with myosin heavy chain (**A.b.**), Sox2 (**A.c.**), DAPI (**A.d.**) was used to mark the locations of nuclei. The group treated with cardiomyocyte-inducing reagents (**B.c.**) has more MF20 positive staining than the non-treated group (**B.b.**).
Results

Expression of myosin heavy chain in differentiated cardiomyocytes (Figure 3) and stem cell markers (Figure 4) were clearly demonstrated in the cell preparation processed with the Biomek 4000 Laboratory Automation Workstation. Automated cell staining for 58 wells on the liquid handler required about one hour without user intervention. The PerFix-nc sample preparation system provided good intracellular staining results with good reproducibility and accuracy without washing, greatly facilitating assay automation.

![Figure 4](image)

**Figure 4.** Mixtures of mES cells and feeder cells were seeded into a 96-well imaging plate, then allowed 24 hours to plate out before being treated with PerFix-nc reagents and stained with either anti-Nanog-Alexa Fluor 488 (A.b, green) or with anti-Sox2-Alexa Fluor 647 (A.c, blue). DAPI (A.d, red) was used to mark the locations of nuclei. The percentages of positive staining (B) were quantified using the MetaXpress software. The standard deviation coefficient of variation are the average values of 58 samples.

<table>
<thead>
<tr>
<th>Antibody Staining</th>
<th>Positive Yield</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation</th>
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<tbody>
<tr>
<td>Anti-Nanog (58)</td>
<td>57.1%</td>
<td>3.8%</td>
<td>6.6%</td>
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<tr>
<td>Anti-Sox2 (58)</td>
<td>63.2%</td>
<td>4.8%</td>
<td>7.6%</td>
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Summary

This work demonstrates that cell staining sample preparation workflows can be automated using standard components on our new Biomek 4000 Laboratory Automation Workstation. Automation can achieve preparation timesavings with large numbers of samples while maintaining equivalent results and precision compared with manual processing. As a result, it is possible to perform large cell staining studies in an expandable manner with walk-away capability.