

Abstract

The automated Cell Staining Application on the Biomek 4000 Workstation can improve throughput and reproducibility of conventional staining of live and fixed adherent cells. This application provides a walk-away solution for staining cells in a variety of workflows, including antibody staining, nuclear dyes, and viability assays in a 96-well plate. Flexibility is central to this application, allowing users to choose and customize any combination of steps (fixation, permeabilization, washing, blocking, and staining) by configuring options in a simple User Interface. Manual staining of cells, either to characterize antigen expression or to assess cell health, is labor-intensive and time-consuming. Here we demonstrate several automation strategies using the Cell Staining Application on the Biomek 4000 Workstation.

Cell staining applicability is demonstrated with three models using mouse embryonic stem (mES) cells (Life Technologies, Carlsbad, CA) in monolayers: the detection of apoptosis following induction with staurosporine, the expression of stem cell markers in an undifferentiated population, and the expression of developmental antigens following differentiation of mES cells into cardiomyocytes. These models are relevant to stem cell biology research. The cardiomyocyte model resembles approaches used for the differentiation of other cell types. Apoptosis markers are widely used to assess cell quality in culture systems. Results were analyzed on an Imaxpress system with MetaXpress software.

Imaging shows well-resolved and specific staining of nuclear and sarcomeric structures in differentiated cardiomyocytes at 40X magnification. Across 58 replicate wells containing a mixture of mESCs and Feeder cells, dual staining with embryonic stem cell markers anti-Nanog-Alexa Fluor 488 (eBioscience, San Diego, CA) and anti-Sox2-Alexa Fluor 647 (BD Bioscience, San Jose, CA) yielded CV's of 6.6% and 7.6%, respectively. This staining was conducted in approximately one hour for all 58 samples using the Cell Staining Application on the Biomek 4000 Workstation in combination with PerFix-nc reagent (Beckman Coulter, Miami, FL), a no-wash fixation and permeabilization reagent system. Staining of mESCs treated with apoptosis-inducing staurosporine were stained with Hoechst 33258 and Annexin V-FITC. The results clearly demonstrate the expected increase in apoptotic cells as staurosporine concentration increases, as well as consistency between manual and automated preparations.

The Biomek 4000 Workstation and the Cell Staining Application are not intended or validated for use in the diagnosis of disease or other conditions.

Biomek 4000 Workstation

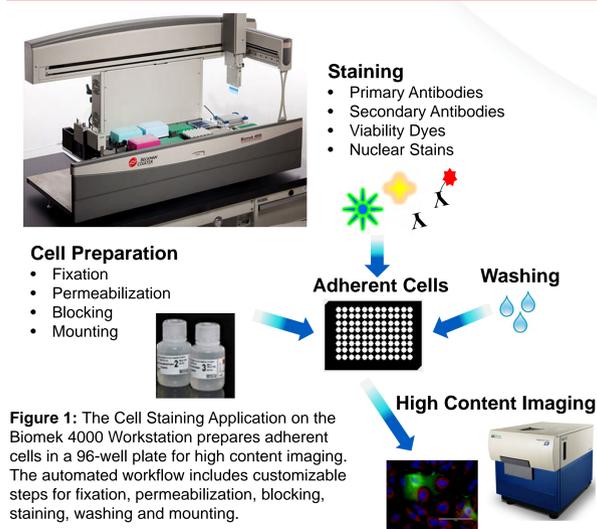


Figure 1: The Cell Staining Application on the Biomek 4000 Workstation prepares adherent cells in a 96-well plate for high content imaging. The automated workflow includes customizable steps for fixation, permeabilization, blocking, staining, washing and mounting.

Cell Staining Application

User Interface

Sample Processing

- All steps are optional according to your workflow.
- Volumes and incubation times customizable for each step (Figure 2).

Staining

- Volumes and incubation times customizable for each step
- Staining Reagents
 - Antibodies (primary, secondary or cocktails)
 - Dyes (nuclear, apoptosis, cell cycle, viability)
- Screening
 - Up to 8 stain cycles
 - All wells treated with same staining reagents
 - Multichannel tool utilized for speed
- Custom Antibody Selection
 - Up to 24 staining reagents held in a tube rack
 - User chosen stain/well combinations
 - Single Channel tool for single well selection (Figure 2).

Figure 2: User Interface for the Cell Staining Application enables customization of sample processing and staining parameters.

Additional Features

Guided Reagent Setup

- Indicates volumes and locations of all reagents required for the staining process (Figure 3)

Process 1-96 Samples

- User defined sample number and location (Figure 4)
- No user intervention required once method begins.
 - All Samples, Tools, Reagents, and Tips fit on the deck simultaneously (Figure 5)

Template Method

- Unlocked for user modification
 - Further customize steps
 - Change Plate types
 - Change Workflow Order
 - Optimize pipetting

8-Column Reagent Reservoir:	
R1:	5320 µL Fix
R2:	2920 µL Perm
R3:	5800 µL Block
R4:	5800 µL Final Wash
R5:	0 µL Mounting
R6:	Empty Empty
R7:	Empty Empty
R8:	Empty Empty

8-Column Antibody Reservoir:	
A1:	1480 µL Ab1
A2:	1960 µL Ab2
A3:	2920 µL Ab3
A4:	3880 µL Ab4
A5:	4840 µL Ab5
A6:	5800 µL Ab6
A7:	6760 µL Ab7
A8:	8680 µL Ab8

Full Modular Reservoir:	
F1:	129800 µL PBS

Figure 3: Volumes for each reagent are calculated based on user inputs in the User Interface.

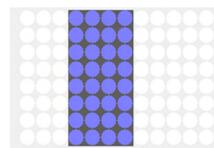


Figure 4: Sample number and location defined at method run time.

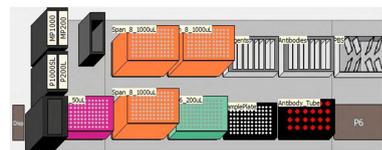


Figure 5: (Right) Biomek 4000 Deck Layout houses all samples, tools, tips, and reagents simultaneously.

Materials and Methods

Murine Embryonic Stem Cell (mESC) Growth and Differentiation

Mouse embryonic stem (mES) cells (Life Technologies) 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 fresh 96-well plates, allowed to adhere for 24 hours prior to staining.

Cellular Staining

Cells were fixed by adding 5 µL of PerFix-nc reagent 1 (B10825, Beckman Coulter, Brea, CA) 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 (Life Technologies).

Apoptosis Characterization

Stem cells were seeded into 96-well imaging plates as above, and then treated with doses of staurosporine (Life Technologies) for 7 hours. The treated cell cultures were stained with the annexin V-FITC / 7-AAD (IM3614, Beckman Coulter). Cells were identified by nuclear staining with Hoechst 33342 (Life Technologies).

Imaging

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

Results

mES Cell Staining

The mixture of mES cells and feeder cells were stained with either anti-Nanog-Alexa Fluor 488 (clone eBioMLC-51, eBioScience) or anti-Sox2-Alexa Fluor 647 (clone O30-678, BD Biosciences) (Figure 6). All conjugates were titrated for optimal performance, and relevant isotype controls were used to control for non-specific staining. Staining was done manually and on the Biomek 4000 Workstation using the Cell Staining Application. The percentages of positively stained cells (Table 1) were quantified using the MetaXpress software. The standard deviation and coefficient of variation are based on 24 replicate samples.

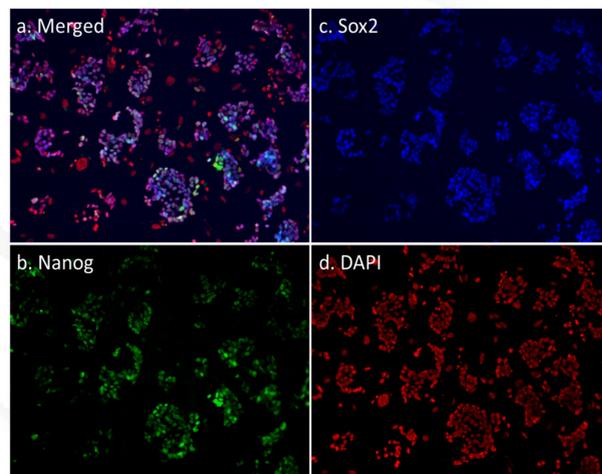


Figure 6: Mixtures of mES cells and feeder cells were seeded into a 96-well imaging plate, then allowed 24 hours to plate out before being treated with PerFix-nc reagents and stained with either anti-Nanog-Alexa Fluor 488 (b., green) or anti-Sox2-Alexa Fluor 647 (c., blue). DAPI (d., red) was used to mark the locations of nuclei.

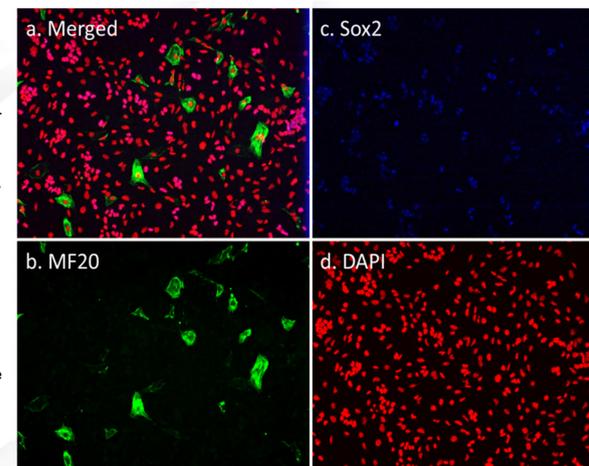
	Antibody	Positive Yield	Standard Deviation	Coefficient of Variation
Cell Staining Application	Anti-Nanog	50.1%	2.6%	5.3%
	Anti-Sox2	55.5%	3.4%	6.2%
Manually Stained Cells	Anti-Nanog	57.0%	4.2%	7.3%
	Anti-Sox2	49.3%	4.9%	9.9%

Table 1: Comparison of Manually stained cells and Cells stained using the Biomek 4000 Cell Staining Application. Percentage positively stained cells for stem cell markers Nanog and Sox2. The standard deviation and coefficient of variation are based on 24 replicate samples.

Cardiomyocyte Staining

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 (Life Technologies), and seeded into 96-well imaging plates and allowed 24 hours to attach. The mixed cells were fixed and permeabilized with PerFix-nc reagents. The mixture of differentiated cells and mES cells were stained with myosin heavy chain-Alexa Fluor 488 (clone MF20, eBioScience) and anti-Sox2-Alexa Fluor 647 (Figure 7). All liquid handling steps were performed on a Biomek 4000 Workstation. Sixteen wells required less than an hour to prepare for imaging. Images were taken using the 10X objective on an Imaxpress high content imager.

Figure 7: Cells were stained with either isotype controls (a.) or with myosin heavy chain (green, b.) and Sox2 (blue, c.), DAPI (red, d.) was used to mark the locations of nuclei.



Annexin V / 7-AAD Staining

The staurosporine treated cells were stained with Annexin V-FITC/7-AAD on the Biomek 4000 Workstation using the Cell Staining Application (Figure 8). Ten replicates of each staurosporine concentration (0 µM, 0.5 µM, 1 µM and 2 µM) were analyzed for a total of 40 samples (Figure 9). The automated staining process for these 40 samples required approximately 1.5 hours with no user intervention.

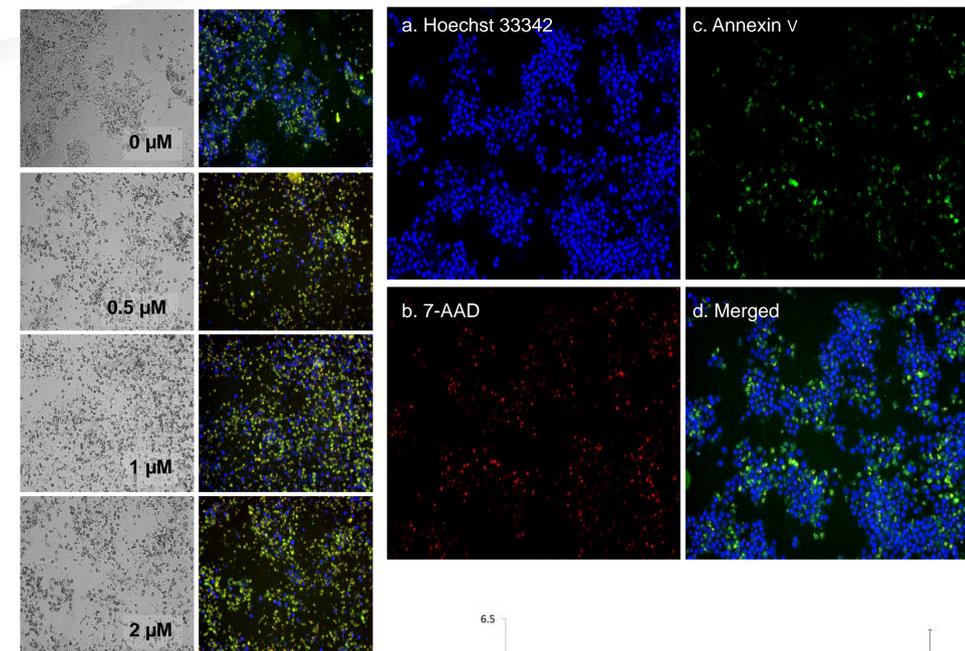


Figure 8: (right) Example Images of Annexin V-FITC/7-AAD staining on staurosporine treated mES cells. (left) Staurosporine (0, 0.5, 1 & 2 µM) treated mES cells images: transmitted light images; and composite images with Annexin V-FITC (green), 7-AAD (red) and Hoechst 33342 (blue)

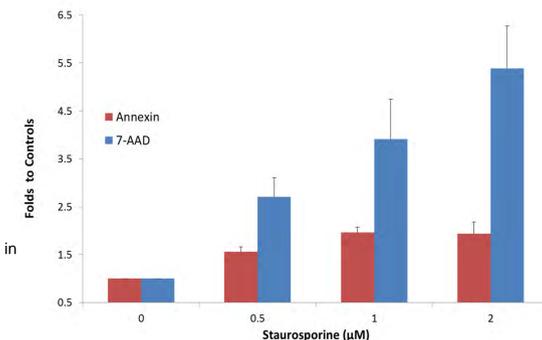


Figure 9: Fold increase in apoptosis with increase in staurosporine concentration. n=10 for each concentration.

Conclusion

- Cell staining workflows can be automated using standard components on the Biomek 4000 Workstation.
- The flexible Application can be customized for a variety of staining workflows.
- Automation can achieve preparation time savings with large numbers of samples while maintaining equivalent results and precision compared with manual processing.
- It is possible to perform large cell staining studies in an expandable manner with walk-away capability.