Automated Solutions for Cellular Analysis Through Flow Cytometry

Abstract

Flow cytometry is a powerful tool to analyze cell populations at the single-cell level. This ability is crucial for researchers that focus on heterogeneous cellular populations, as in the case of oncology research. Unfortunately, to assay cells in this fashion frequently involves a complex array of manipulations. Here we demonstrate how automation can assist with all steps of flow cytometry sample preparation—from cell culture and stimulation to generation and staining of cell suspensions.

Introduction

The more we learn about human physiology and disease states, the more we appreciate the complexity that is present in all conditions. The revelation of tumor heterogeneity is just one example where biomedical inquiry is moving toward a requirement for single-cell analysis and how achieving "well-level" phenotypes of cell populations is no longer sufficient for cellular assays. Flow cytometry has long been used as a means of investigating cell populations at the single-cell level. However, the typical fluorescent labeling of cellular subpopulations frequently involves numerous liquid handling and centrifugation steps for reagent exchange.

Flow cytometry-based assays typically involve a host of steps and challenges (Figure 1). These steps can include any or all of the following: 1) cell culture and/or stimulation; 2) preparing a single-cell suspension; 3) creating cocktails of antibodies; and, 4) staining cells for analysis. Each of these steps can be challenging and time-consuming, particularly as sample throughput increases. Cell staining in particular can be laborious if fixation and permeabilization steps are required or if a variety of antibodies are used. Automation can dramatically reduce the time at the bench that is required for flow-based assays while also improving reproducibility by reducing user-to-user variability and human errors that are common in complex workflows.

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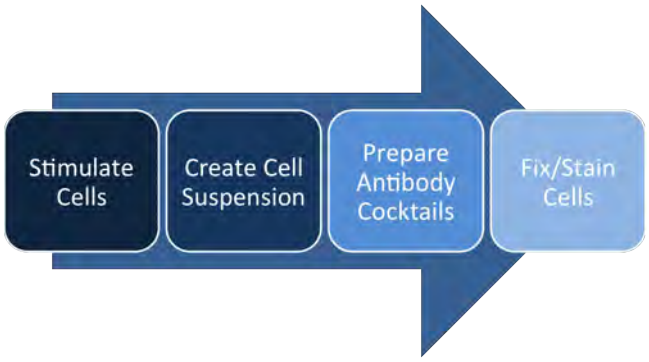

Fig. 1. Typical workflow for flow cytometry sample preparation.

Automation Solutions

The family of Biomek Workstations (Figure 2a) provides the basis for flexible automated solutions for flow cytometry. As the majority of flow cytometers process samples in tubes, the Span-8 pipettors of the Biomek NX^{P} and FX^{P} Workstations can accelerate processing as multiple tubes can be accessed at once, while the single pipetting tool of the Biomek 4000 Workstation is appropriate for lower-throughput applications. In addition, the multichannel head (96 or 384) of the Biomek FX^{P} Workstation can add flexibility and increase throughput for plate-based sample prep. If maintaining sterility of the cells is essential (i.e., cell sorting), then the Workstations can use sterile pipette tips and be contained in HEPA-filtered enclosures or—in the case of the Biomek 4000 Workstation—be placed in a standard laminar flow hood.

As most flow-based workflows require steps beyond liquid transfers, the ability of Biomek Workstations to integrate with numerous devices is essential for complete workflow automation. Since flow cytometry assays cells in suspension, many applications require centrifugation steps for reagent exchange. Figure 2b shows a rendering of a Biomek NX^{P} Workstation with a commonly integrated centrifuge that can be used for tube- or plate-based applications. Additional workflow steps, such as incubations during cell culture or stimulation, can be automated using integrated incubators, and higher sample throughput can be facilitated with integrated plate and tip storage hotels. We next describe a variety of ways that Biomek Workstations have been used in sample preparation steps for flow cytometry assays.

a.

Biomek 4000 Workstation

Biomek NX^{P} Workstation

Biomek FX^{P} Workstation

b.

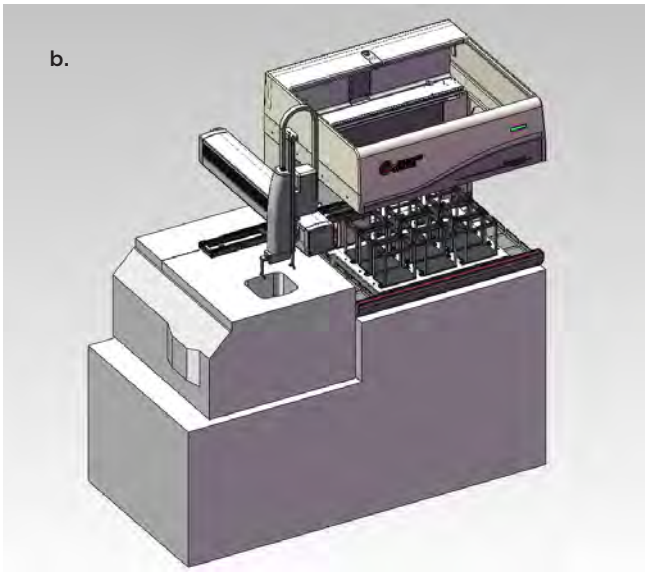

Fig. 2 a. The family of Biomek Workstations. **b.** Rendering of a Biomek NX^{P} Workstation with an integrated centrifuge that can be utilized to pellet cells in plates or tubes in an automated fashion.

Demonstrations

Cell Stimulation

While long-term cell culture and treatments can be automated by integrating an incubator to a Biomek Workstation, short-term cell stimulation can be accomplished on deck of the liquid handler. To demonstrate this, lipopolysaccharide (LPS) or phorbol-12-myristate-13 acetate (PMA) was added to whole blood samples using a Biomek NX^{P} Workstation with Span-8 pipettors. These samples were then incubated at 37°C using a Peltier device on the Biomek Workstation. Following stimulation, samples were fixed and stained to detect phosphorylation of p38 MAP kinase and ERK in CD14^{+} monocytes. The flow cytometry results (Figure 3) show that LPS induced detectable phosphorylation of both p38 MAPK and ERK, while only phosphorylated ERK was detected in PMA-stimulated cells. Automating both the cell stimulation and fixation on the deck of the Biomek Workstation enables the investigation of rapid cellular responses by flow cytometry.

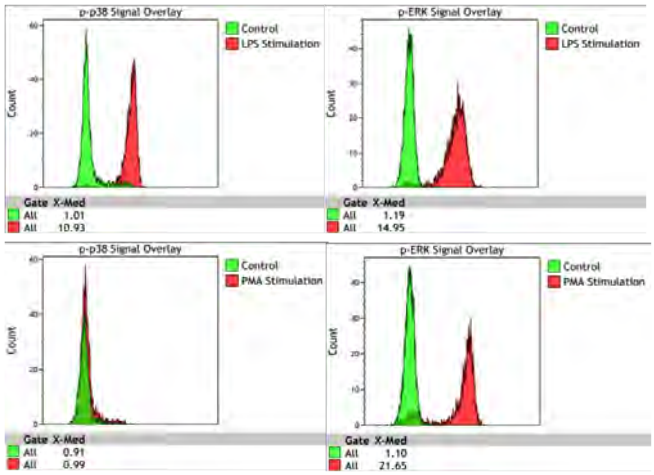

Fig. 3. Automated cell stimulation. Whole blood was treated with lipopolysaccharide (LPS) and phorbol-12-myristate-13 acetate (PMA) and fixed and stained on a Biomek Workstation. Flow cytometry data comparing the phospohorylation state of unstimulated CD14^{+} T cells (green peaks) and those stimulated (red peaks) with LPS (upper histograms) and PMA (lower histograms). LPS induced phosphorylation of p38 MAPK and ERK while PMA only induced phosphorylation of ERK.

Creating Cell Suspensions

When cells are cultured under adherent conditions, they must be released from the tissue culture plate—and from one another—to form a single-cell suspension for flow cytometry analysis. This is typically accomplished via enzymatic digestion of extracellular matrices such as through the addition of trypsin. To illustrate the value of automating this process, we automated the dissociation of embryoid bodies (EBs) formed during the differentiation of murine embryonic stem cells. The spherical nature of EBs, such as those grown in hanging drops, makes them more challenging to dissociate than typical two-dimensional monolayer cultures. After growing murine embryonic stem cells in suspension culture for 5 days to form EBs, we adhered the EBs to 96-well tissue-culture plates for an additional 3 days for further cellular differentiation (Figure 4a, left). On day 8, we automated the media removal and reagent (Accumax) addition, room temperature shaking, and repeated pipetting using the Biomek FX^{P} multichannel head to form a single-cell suspension (Figure 4a, right). This method utilized the ability of the Biomek software to finely control all aspects of liquid handling, such as aspirating the media around the circumference of a flat-bottomed well without disturbing the adherent cells. The cells were then stained for a cardiomyocyte marker (myosin heavy chain) and analyzed by flow cytometry. Figure 4b shows the increase in cardiomyocytes from the differentiation process.

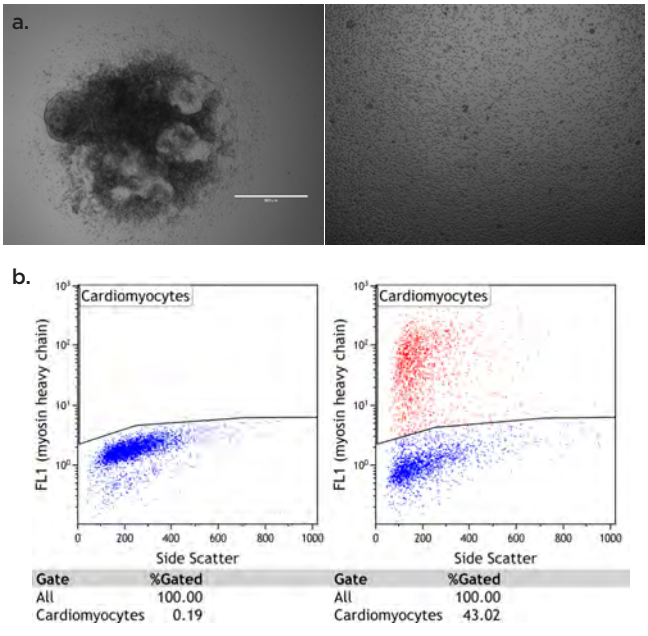

Fig. 4. a. Adhered embryoid body (EB) before (left, bar = 500 µm) and after (right) automated enzymatic cell separation. **b.** Flow cytometry results following automated dissociation and staining for cardiomyocytes (myosin heavy chain positive) of undifferentiated (left) and 8-day EB-differentiated stem cells (right).

Antibody Cocktail Preparation

Flow analysis of complex samples such as blood panels frequently requires the use of numerous multicolor antibody cocktails. Beckman Coulter has developed an automated system that simplifies the preparation of these cocktails while reducing the workload and likelihood of errors in the laboratory. The Biomek 4000 ACPrep Workstation utilizes customized software (Figure 5a) with an intuitive user interface to aid the creation and distribution of antibody cocktails to sample tubes. This system has unique racks (Figure 5b) that hold antibody source vials from a variety of vendors. Flat-bottomed vials are tilted in the racks to maximize antibody recovery, and antibody usage is tracked within the software. In addition, a variety of output formats (tube racks, carousels, plates) are allowed to accommodate samples for a variety of flow cytometers.

a.

b.

Fig. 5. The Biomek 4000 ACPrep Workstation. **a.** The system features an intuitive user interface software that walks the user through the creation of antibody panels, and tracks reagent usage to ensure sufficient volumes. **b.** A variety of custom racks—for input antibody vials and output vials, tubes or plates—are utilized on the system.

Cell Staining

Cell staining is frequently the most time-consuming and repeated aspect of flow cytometry sample preparation. To alleviate this bottleneck, we utilized the Biomek NX^{P} Workstation with an integrated microplate centrifuge to automate the entire fixation, permeabilization, blocking, staining, and washing workflow. Murine embryonic stem cells were differentiated in the process described above, but with varying media additives. Cells were fixed on days 6 through 9 of the differentiation process, and stained for Sox2, Nanog, Nestin and Brachyury to study the time course of differentiation and lineage formation. We utilized a worklist generated in Microsoft® Excel® to identify the antibodies to be added to each well. Not only should this worklist reduce the likelihood of errors in a frequently confusing step, but it should also enable changes to be made more easily between experiments. Figure 6a illustrates that the 3 treatments give very similar expression patterns over time with the exception of a transitory increase in the number of cells expressing Brachyury with treatment 3 (green).

Another way of reducing the complexity of automated flow cytometry sample preparation is to utilize reagents that remove the requirement for centrifugation. The PerFix-nc kit from Beckman Coulter can be used to easily automate the staining protocol on a stand-alone Biomek Workstation. We compared manual and automated fixation, permeabilization, and Oct3/4 staining of murine embryonic stem cells using PerFix-nc. Figure 6b shows that automated staining was highly comparable to manual preparations. In addition, automation of the PerFix-nc method is not limited by the throughput of an integrated centrifuge.

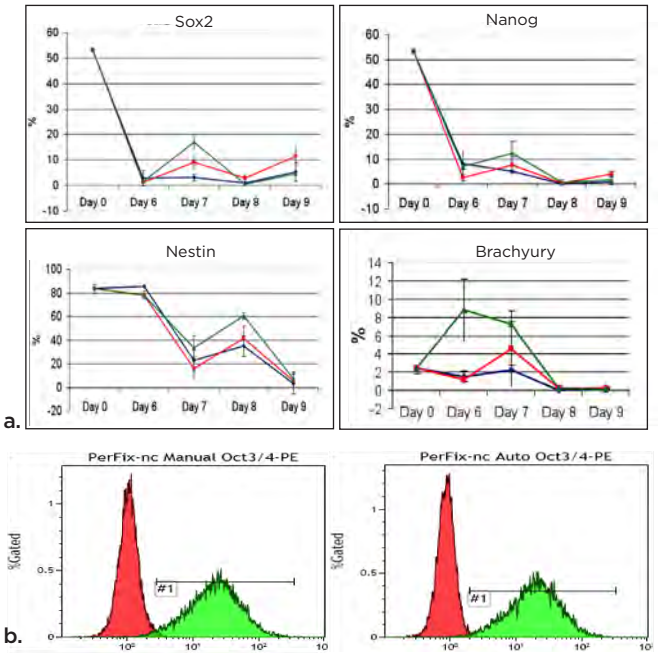

Fig. 6. Automated cell staining of murine embryonic stem cells. **a.** Expression of pluripotency and early lineage markers over a differentiation time course. Cells treated with 3 alternative differentiation protocols targeting cardiomyocyte formation were stained using a Biomek NX^{P} workstation with an integrated microplate centrifuge. Cells differentiated for 6 to 9 days were stained for Sox2, Nanog, Nestin, and Brachyury and were compared to undifferentiated cells (day 0). **b.** Undifferentiated stem cells were fixed and permeabilized with the PerFix-nc kit and stained for Oct3/4 in a manual (left) and automated (right) fashion. No centrifuge was required for this preparation and flow cytometry results show excellent consistency between automated and manual preparations.

Conclusion

We have shown how automation can assist and improve the preparation of samples for single-cell analysis through flow cytometry. The ability of the Biomek Workstation to utilize or integrate with a variety of devices provides maximum flexibility in the workflows that are possible to automate. This ability enabled the sample heating to study the kinetics of cell signaling as well as the automation of the frequent centrifugation steps during cell staining. In addition, the fine liquid handling control that can be achieved on the Biomek Workstation facilitated the generation of cell suspensions from complex three-dimensional adherent cultures. Finally, our user interfaces—such as the one featured on the Biomek 4000 ACPrep Workstation—reduce the automation learning curve, allowing the time-saving and error-reducing benefits of automation to be achieved more rapidly. These are just some examples of how the powerful and flexible automation solutions from Beckman Coulter can help researchers expand and accelerate their flow cytometry research.

Authors

Michael Kowalski, *Staff Applications Scientist*
Li Liu, *Senior Development Scientist*
Amy Yoder, *Staff Development Scientist*
Beckman Coulter Life Sciences, Indianapolis, IN USA

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Life Sciences

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