An Automated, Centrifuge-Free Workflow for Cell Staining and Washing of Flow and Mass Cytometry Samples

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Abstract

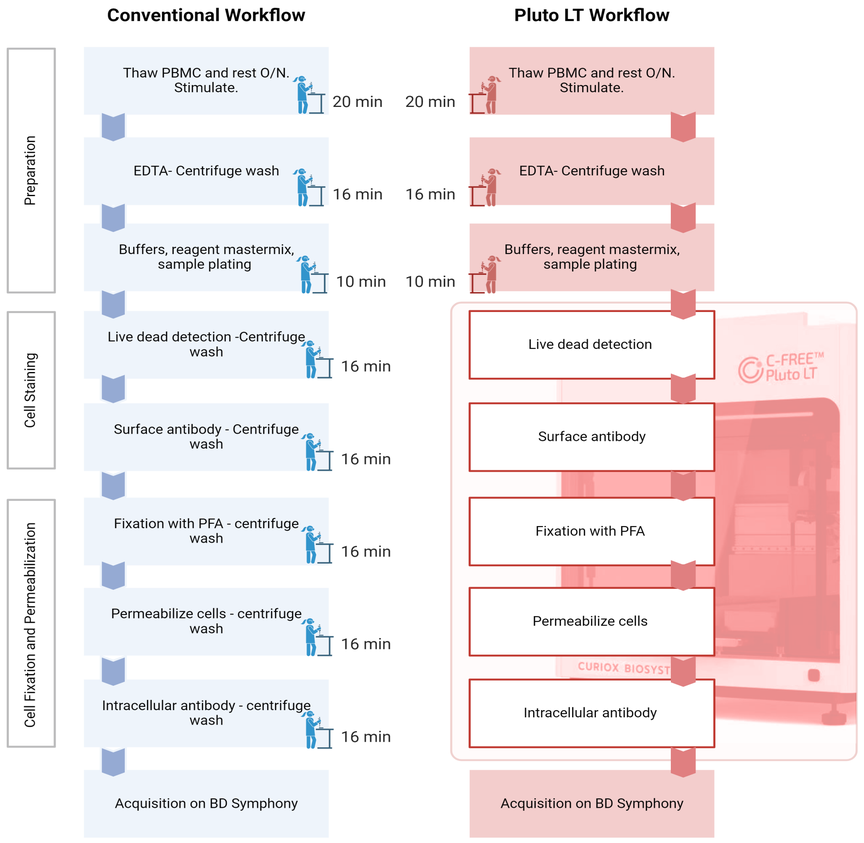
We tested a fully automated, centrifuge-free (C-Free™) system for staining, washing, and processing peripheral blood mononuclear cells (PBMC). Using protocols standardized in the Stanford Human Immune Monitoring Center, flow cytometry and mass cytometry assays that included cell surface and intracellular staining were performed on the C-FREE™ system from Curiox Biosystems. Results were compared to existing manual workflows in the lab. Cell recovery, debris removal, and staining quality, as well as the precision of these parameters, were assessed as points of comparison. The results suggest that C-FREE™ technology can generate highly reproducible data across multiple types of assays, with a fully automated workflow.

Introduction

As flow cytometry becomes a ubiquitous cell-analysis approach in both research and clinical settings, solutions to automate the sample preparation workflow have become the focus of many academic, hospital, and commercial labs. Many solutions to automate the workflow have focused on large, modular liquid handling solutions which require much lab space and can be costly and time-consuming to develop. However, the availability of Centrifuge-Free (C-FREE™) technology offers the potential to streamline sample prep workflows within a smaller footprint and at lower cost. The C-FREE™ technology from Curiox capitalizes on laminar flow principles to gently wash and stain cells in traditional 96-well microplates, without centrifugation and with pre-configured, optimized workflows. In addition to wash steps, the C-Free technology can perform reagent addition, mixing, and incubation steps, resulting in a potentially fully automated workflow. Potential benefits for sample preparation may include better debris removal, improved cell recovery, and gentler cell handling. Logistical benefits could also include greatly reduced technician time, and increased reproducibility compared to manual methods of sample preparation.

Methods

Human PBMCs were isolated by density-gradient centrifugation and cryopreserved, then thawed by a standard protocol (Gupta and Maecker, 2018). After resting overnight at 37°C, PBMCs were either left unstimulated or stimulated with PMA-Ionomycin for 4–6 h along with addition of 10 µg /ml Brefeldin A. At the end of the stimulation period, cells were treated with EDTA for 15 min to remove adherent cells. Cells were then aliquoted to separate workflows for parallel processing and comparison. The Pluto LT workflow was fully automated after loading the automation deck containing reagent and buffers. The conventional workflow was carried out with manual pipetting and a centrifuge. Dead cell staining was done at room temperature for 15 min using LIVE/DEAD® fixable red cell viability dye, followed by cell-surface marker staining with a cocktail of CD3, CD4, CD8, CD20, and CD33 antibodies at room temperature for 30 min. After fixation with 2.8% paraformaldehyde for 10 min at room temperature and permeabilization with Invitrogen FACS Perm buffer for 30 min, intracellular staining with IFNγ was done at room temperature for 30 min. Washes were performed between each step using PBS/FBS and centrifugation for 8 min at 638 x G (787 x G after fixation). Prior to acquisition, viability of cells was measured on a Beckman-Coulter Vi-Cell. Acquisition was performed on a BD Symphony A5 flow cytometer and analyzed using FlowJo software.



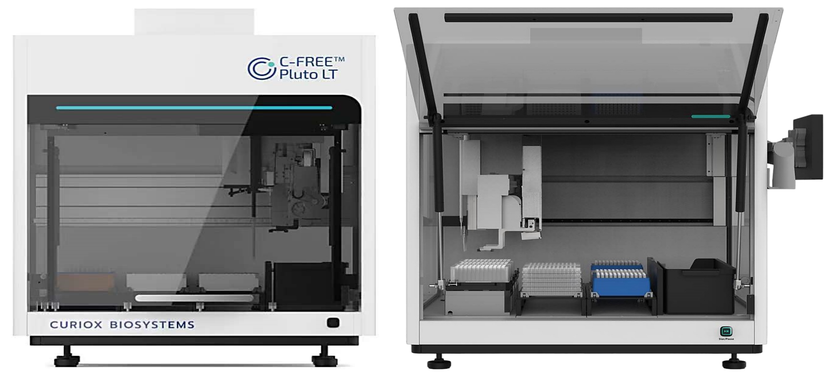


Figure 1: Automated sample preparation using C-Free™ Pluto LT for flow cytometry analysis. Left panel shows comparison of hands-on time for conventional workflow compared to Pluto LT automated workflow. Human peripheral blood mononuclear cells (PBMCs) were processed following a standard centrifuge protocol, from cell thawing to post-stimulation washing. Subsequently, reagents, buffers, and cell samples were organized into designated reservoirs on the Pluto LT for automated handling. The system efficiently executed sample transfers and reagent additions for cell staining. After staining, cells underwent washing, fixation, and permeabilization steps for intracellular marker analysis. The final cell suspension was placed into FACS tubes for data acquisition using a BD Symphony A5 flow cytometer. Right panel illustrates the C-Free™ Pluto LT, showing its external design and the configuration of its internal deck.

Results

Fig.2 Gating Strategy

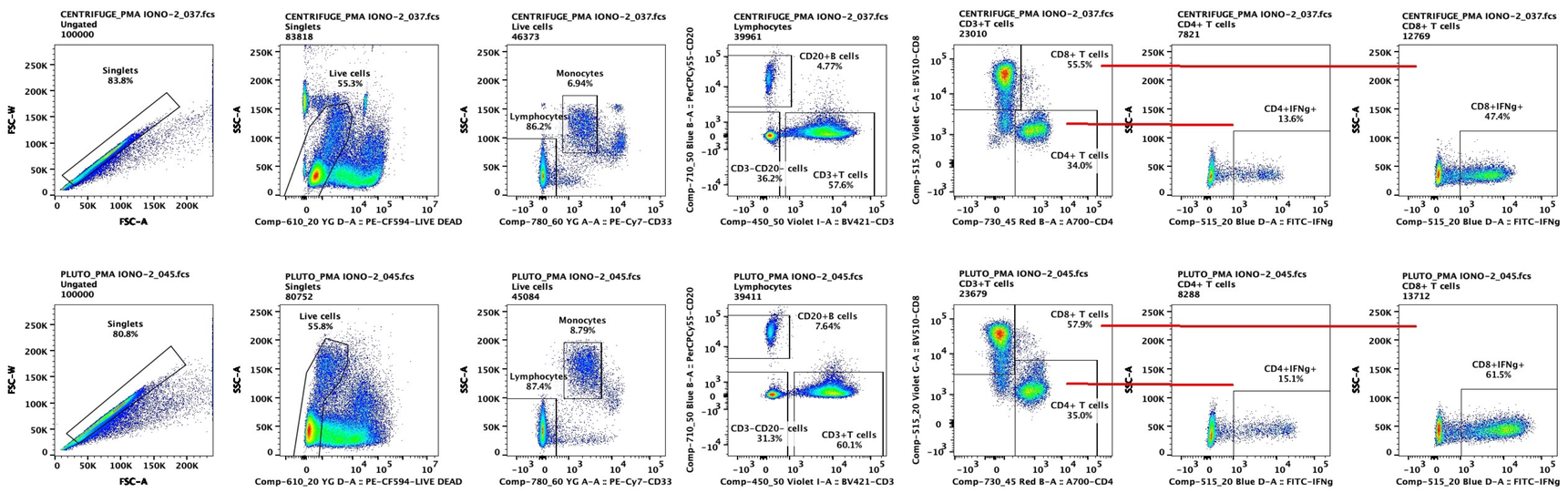
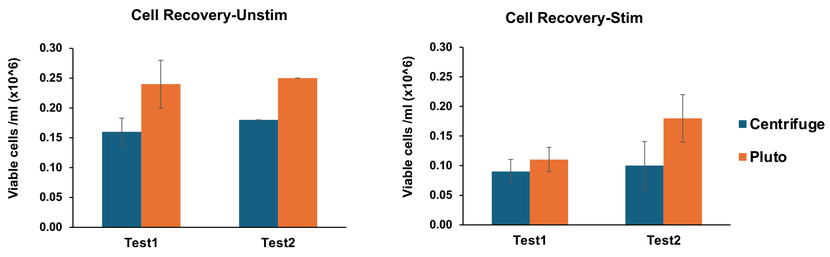
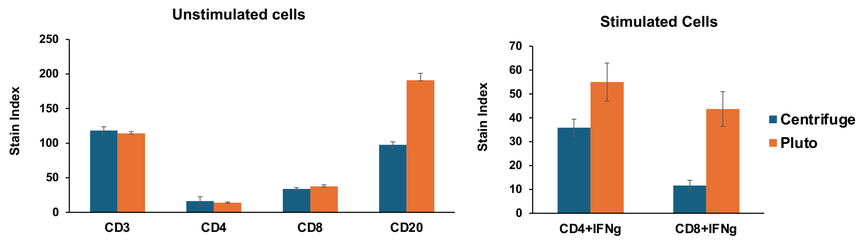


Fig.3 Cell Recovery



- Cell viability was measured on a Beckman-Coulter Vi-Cell prior to acquisition on flow cytometry.
- C-Free™ Pluto LT samples consistently show as much as 50% better cell retention over manual processing using centrifugation.

Fig.4 Stain Index



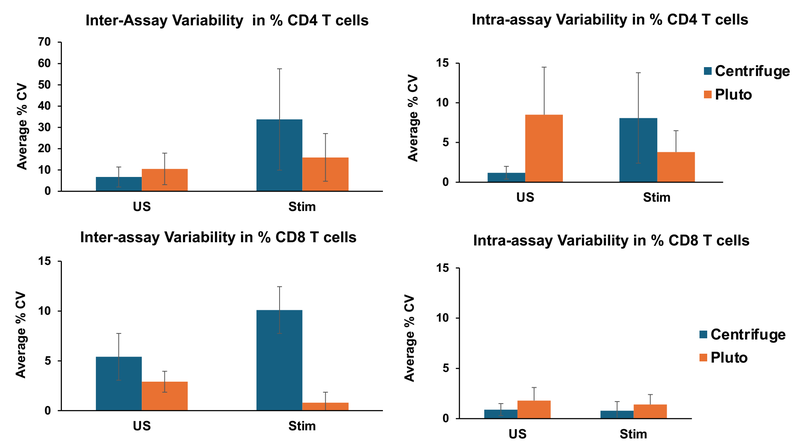
- The stain index is defined as the difference between the mean fluorescence intensity of the positive and negative populations, divided by two times the standard deviation of the negative population.

$$\text{Stain Index} = \frac{MFI_{pos} - MFI_{neg}}{2\sigma_{neg}}$$

MFI = Mean Fluorescence Intensity
σ = Standard Deviation

- Improved staining and greater population separation resulting from higher cell retention with Pluto LT samples compared to manual processing.

Fig.5 Inter and Intra-Assay Variability



- Coefficient of Variation (CV) is a mean-normalized Standard Deviation.
- CV = StdDev ÷Mean × 100. In FlowJo, the CV statistic is displayed in percent.
- Standard error of the coefficient of variation (SE(CV))= CV ÷ √(2n)
- Inter- and intra-assay variability in C-FREE™ Pluto LT samples was consistently below 10% CV, demonstrating high reproducibility and standardization with automated sample preparation.

Conclusions

The C-FREE™ Pluto LT allows walk-away sample preparation that includes staining, washing, and fixation/permeabilization. We found the platform could provide equivalent or better cell recovery, data quality (as measured by stain index), and reproducibility within and between experiments, compared to using centrifugation and manual pipetting.

Discussion

The Curiox C-FREE™ Pluto LT is a compact, economical, benchtop liquid handler that enables walk-away automation of cell staining and cell washing protocols without the need for centrifugation or manual pipetting. It comes with single- and 8-channel pipetting modules, temperature control and accelerated cell settling stations, with optional 21 CFR part 11 compliance.

One of the benefits of the Pluto LT platform is its ability to wash cells without the need for a centrifuge. This is made possible by the C-FREE™ wash method. This patented sequence of aspiration and dispensation cycles is meticulously engineered and embedded within the PlutoLT workstation's software core. Briefly, cells settle to the bottom of a 96-well microplate and are gently washed by the Pluto LT pipette head and tips, removing debris while retaining the cells.

The automated nature of the Pluto LT allows for a 'walkaway' workflow, enabling researchers to focus on other critical tasks without compromising the precision of their experiments. This level of automation minimizes the variability often introduced by manual handling, translating into more consistent and trustworthy results.

The data consistency provided by the Pluto LT platform could be especially pertinent in fields requiring the highest level of precision, including clinical research, diagnostics, and clinical immune monitoring, where the quality of flow cytometry data is paramount.

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