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A Rapid Single Cell Sorting Verification Method Using Plate-Based Image Cytometry

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Abstract

Single cell sorting is commonly used for ensuring monoclonality and producing homogenous target cell populations. Current single cell verification methods involve manually confirming the existence of single cells or colonies in a well using a standard light microscope. However, the manual verification method is time-consuming and highly tedious, which prompts a need for an accurate and rapid detection method for verifying single cell sorting capability. Here, we demonstrate a rapid single cell sorting verification method using the Celigo Image Cytometer. Calcein AM-stained Jurkat cells and fluorescent beads are sorted into 96-well half area microplates using the MoFlo Astrios EQ. Whole well bright field and fluorescent images are acquired and analyzed using the image cytometer in less than 8 min. The proposed single cell verification detection method in multi-well microplates can allow for quick optimization of FACS instruments at flow core laboratories, as well as improvement of downstream biological assays by accurately confirming the presence of single cells in each well. © 2018 International Society for Advancement of Cytometry

Key terms

single cell sorting; image cytometry; fluorescence activated cell sorting (FACS); cell line development; monoclonality; Celigo

Cell sorting is one of the most important methodologies required for research fields such as immunology, oncology, protein/cell engineering, neuroscience, and stem cell research (1–6). Specifically, single cell sorting is commonly used to ensure monoclonality and to produce homogenous cell populations for cell line development (7). In general, after single cell sorting has been performed, the cells are allowed to grow into colonies for a certain amount of time, and subsequently, analyses such as genomic, transcriptomic, or proteomic assays are performed on the cells (7).

Current technologies used for single cell sorting include Flow Cytometry (FC), Magnetic-Activated Cell Sorting (MACS), Laser Capture Microdissection (LCM), microfluidic devices, cell printing, and manual cell picking (8). In order to verify the single cell sorting capability or efficiency of these methods, researchers must allow the single cells to settle, and manually confirm the existence using a standard microscope or verify single colony several days after outgrowth (9). This method typically requires 30–40 min./plate due to the need to maneuver the plate to search for a single cell or colony. The time-consuming and highly tedious nature of the manual verification method necessitated for an accurate and rapid detection method to verify the performance of single cell sorting.

In this work, we demonstrate a rapid single cell sorting verification method using the Celigo Image Cytometer (Nexcelom Bioscience, Lawrence, MA). Fluorescent beads or calcein AM-stained Jurkat cells are sorted into a 96-well half area plate to determine the sorting efficiency of a MoFlo Astrios EQ (Beckman Coulter, Brea, CA). The plate-based image cytometer is used to rapidly capture whole well bright

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field and fluorescent images of the entire 96-well plate and automatically identify the single beads or cells in each well. The imaging and analysis time requires less than 8 min., which is a significant improvement from the manual method. The ability to rapidly detect a single cell in a multi-well microplate is highly important to flow core laboratories for optimizing their fluorescence activated cell sorting (FACS) instruments, as well as to the researchers, for confirming the presence of single cells in each well and improving their downstream biological assays.

Materials and Methods

Jurkat Cell Preparation

Jurkat cells (ATCC, Manassas, VA) were cultured in RPMI-1640 medium supplemented with 1% penicillin/streptomycin and 10% FBS and incubated at 37°C and 5% CO_2. Five milliliters of the cultured Jurkat cells were transferred into a 15 ml conical tube under sterile condition. Five microliters of calcein AM (Nexcelom Bioscience, Lawrence, MA) were pipetted into the Jurkat cell suspension (1,000× stain dilution). The conical tube was mixed and incubated at room temperature in the dark for 45 min. After staining, the Jurkat cells fluorescence was verified using the Cellometer Vision Image Cytometer (10–12) before performing the single cell sorting verification experiment.

MoFlo Astrios EQ

There were 4 major components, aside from alignment, to monitor and adjust on the MoFlo Astrios EQ for ensuring proper operation of the instrument (IntelliSort II, CyClone, deflection plates, and operating pressure) (13). First, the IntelliSort II utilizes the stream camera and software to automatically optimize droplets and determine drop delay without the use of calibration particles. When a sort is in progress, the IntelliSort II monitors the droplet stream for fluctuations in the droplet break-off. In addition, it monitors ambient temperature, fluid temperature, and pressure changes. It can sense a difference in temperature change of ±2°C, and a sheath pressure change of ±1 psi. Changes in these parameters can alter the stream that may decrease the sorting efficiency, where a warning will appear and terminate the sort. Accurate drop delay, satellite break-off, and verticality are vital in single cell deposition. Automated monitoring of these functions maintains an increased level of quality assurance.

The CyClone is the arm on which the plate is moved during the duration of the sort. There are pre-configured sort output definitions that can be chosen, such as 96- or 384-well plates. It allows the operator to manually set the corner locations of a plate and then it can calculate all the drop locations at each well location.

The deflection plates provide the electric field that deflects individually charged droplets into the appropriate receptacles. These plates can be polarized with up to 7,000 Vdc and can be easily removed and cleaned.

The operating pressure differential between the sheath tank and sample can be adjusted. When operating with a 70 μm nozzle, the typical pressure is run at 60 psi. The operator is then able to adjust the differential by 0.1 psi increments up to and including 4.5 psi.

Two MoFlo Astrios EQ were used in this work. The differences between instrument 1 and 2 included the manufacture date and the number of lasers present in the system. Manufacture dates were March 2017 and October 2013, respectively. The number of built-in lasers were 4 and 7, respectively. Charge phase and defanning settings were both set up according to the individual sorter and not copied between the two instruments. Both were equipped with the 70 μm nozzle operating at roughly 60 psi.

Single Cell Sorting Using MoFlo Astrios EQ

Two initial experiments were performed to sort single green fluorescent polystyrene beads (Flow Check Pro, Beckman Coulter, Item No. A69183) directly into dry 96-well half area plates (Greiner Bio-One, Item No. 675090). The purpose was to verify the sorting efficiency of the MoFlo prior to utilizing live cells. The first experiment sorted 10 beads into wells A1, D7, and H12 for 5 plates, which served as focus wells for the Celigo Image Cytometer. After slight assay optimization, the second experiment sorted 100 beads into well D5 for focus.

For the live cells experiment, an 8-channel pipettor was used to first pipette 20 μl of phosphate buffered saline (PBS) or Isoflow sheath fluid (Beckman Coulter) into each well in the 96-well half area plates. Similarly, two experiments were performed where single calcein-positive Jurkat cells were sorted into plates with 1,000 cells in well D5 serving as the focus well using two MoFlo Astrios EQ instruments. Prior to cell sorting, quality calibration (QC) was performed according to manufacturer's recommendations. After extensive troubleshooting, additional steps were added to the traditional QC given by the manufacturer (Supporting Information S1). The single cell sort process then followed conventional strategies (14).

Single Cell Resorting Capability Testing

In addition to the single cell sorting verification experiments, we have examined the resorting capability of the proposed detection method. The Celigo was used to quickly identify wells without single cells and the MoFlo Astrios EQ was used to resort into those wells. The resort experiment was performed where single calcein-positive Jurkat cells were sorted into three plates and resorted two more times into empty wells. The experiment was repeated using the second MoFlo Astrios EQ.

Single Cell Sorting Verification Using the Celigo Image Cytometer

Previously, the Celigo Image Cytometer has been utilized to perform cell-based assays in microplates by capturing and analyzing whole well images (15–17). In this work, the Celigo Image Cytometer was used to rapidly count sorted single cells in Greiner 96-well half area plates in order to determine the sorting efficiency of the FACS. The image cytometric method captured both bright field (BF) and green fluorescent (FL) images for each well, and automatically counted the bright

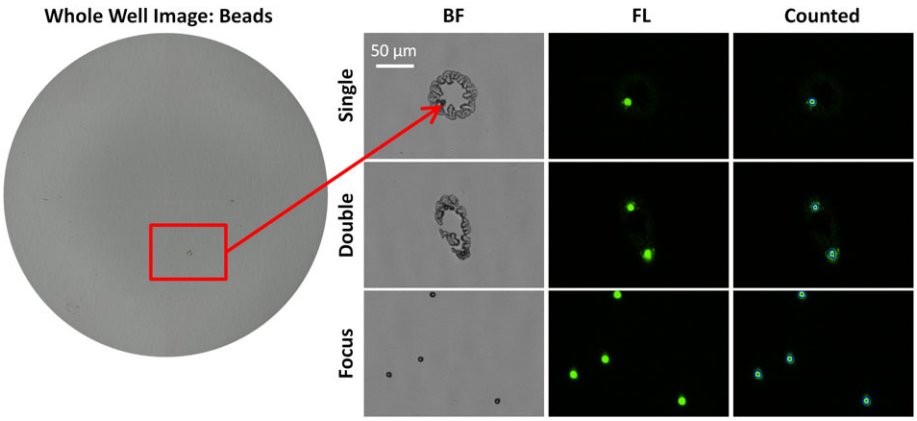

Figure 1. Whole well and zoomed in bright field and fluorescent images of beads. Single beads were directly counted in fluorescent images captured from each well in the whole plate. The counting results are used to calculate the sorting efficiency. Zoomed in images show the counted single, double, and focus beads in fluorescence, as well as dried PBS crystals surrounding the beads in bright field. [Color figure can be viewed at wileyonlinelibrary.com]

green fluorescent beads or Jurkat cells. The scanning and analysis time of 96-well half area plates were less than 8 min.

After sorting the beads or Jurkat cells on the MoFlo Astrios EQ, the prepared plates were transferred to the image cytometer. The software followed five major steps: START, SCAN, ANALYZE, GATE, and RESULTS. In the START tab, Greiner 96-well half area microplate (675090) was selected, and a unique Plate ID was typed in to identify each prepared plate. In the SCAN tab, the Target 1 + 2 Application was selected with bright field and green fluorescent channels, respectively. First, the Target 1 (Bright Field) channel was setup with auto-exposure and hardware-based autofocusing in focus wells (A1, D5, D7, or H12) to set up the focusing mechanism during image acquisition. Next, the Target 2 (Green) channel was setup with an exposure time of 10,000 ms, and approximately +25 µm focus offset from the bright field channel. In the ANALYZE tab, different parameters were adjusted to automatically identify and count beads and calcein-positive cells in the green fluorescent channel. The Intensity Threshold for Target 1 and 2 was set to 255 and 13, respectively, so only the beads or cells are counted in the fluorescent images instead of bright field. In addition, to avoid counting cellular debris, the following Pre-Filtering parameters were used; Cell Area (60–10,000), Cell Intensity Range (0–255), and Minimum Cell Aspect Ratio (0.000). The RESULTS tab was used for visual verification using heat maps and evaluation of sorting efficiency of single beads or cells in each well. The GATE tab was not utilized because it was not necessary for direct single cell counting. A total of 13 plates of beads and 13 plates of cells were imaged and analyzed, where each plate was observed under a standard microscope to confirm the sorted beads or cells in each well.

The counting results from the Celigo software were exported into an Excel file for calculation of sorting efficiency. The percentage of wells with single cells were calculated by adding each well that contained a single cell and dividing by the number of wells that were selected to have one cell sorted.

Results

Initial Method Development Using Single Bead Sorting

In order to develop a method to verify the single cell sorting capability of a FACS instrument, we first sorted single

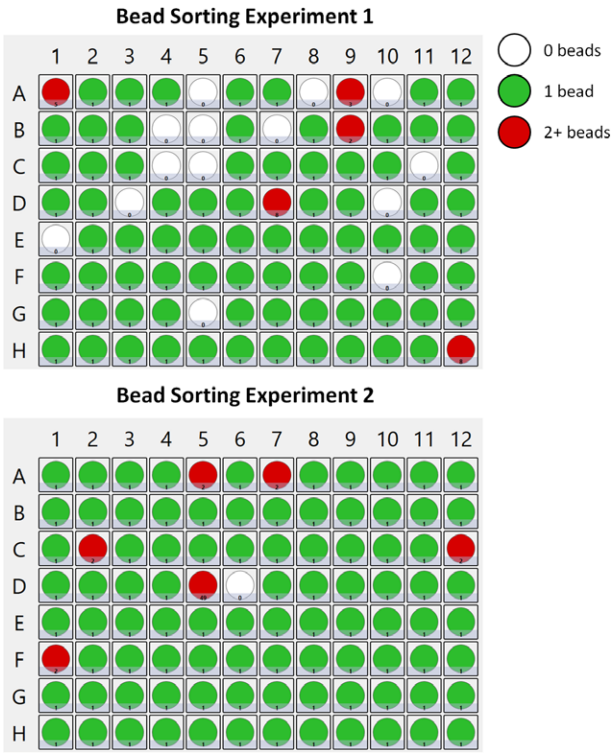

Figure 2. Heat maps of single bead sorting for Experiment 1 and 2 with white, green, and red indicating no beads, 1 bead, and 2 or more beads. The example heat map results showed sorting efficiencies of 82.8% and 93.7%, respectively. The sorting efficiencies were 82.3%, 82.3%, 79.6%, 84.4%, and 88.5% (Mean: 83.4% ± 3.3%) for the first experiment and 93.7%, 90.5%, 92.6%, 94.7%, 89.5%, 85.3%, 88.4%, and 95.8% (Mean: 91.3% ± 3.5%) for the second experiment. [Color figure can be viewed at wileyonlinelibrary.com]

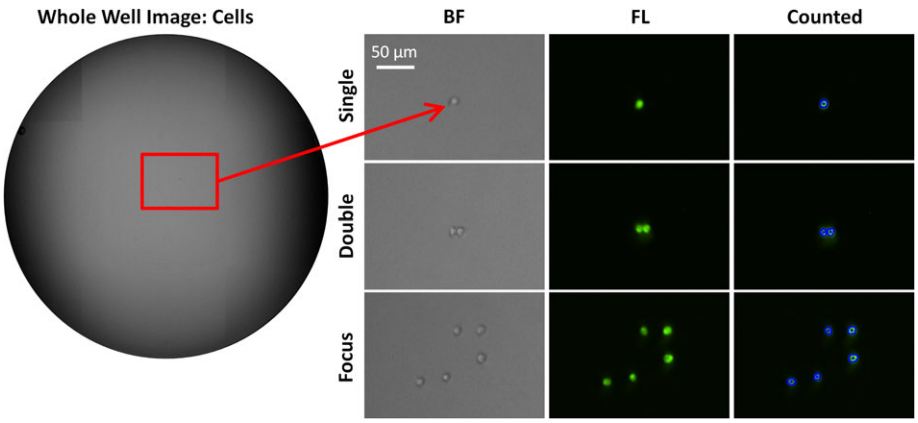

Figure 3. Whole well and zoomed in bright field and fluorescent images of calcein-stained Jurkat cells. Single cells were directly counted in fluorescent images captured from each well in the whole plate. The counting results are used to calculate the sorting efficiency. Zoomed in images show the counted single, double, and focus cells in fluorescence. [Color figure can be viewed at wileyonlinelibrary.com]

beads into dry microplates and calculated the bead sorting efficiencies using MoFlo Astrios EQ instrument 1. Two separate beads sorting experiments were conducted during the initial method development phase. In the first experiment, five plates were imaged and analyzed that resulted in sorting efficiencies of 82.3%, 82.3%, 79.6%, 84.4%, and 88.5% (Mean: 83.4% ± 3.3%). In the second experiment, eight plates were imaged and analyzed that resulted in sorting efficiencies of 93.7%, 90.5%, 92.6%, 94.7%, 89.5%, 85.3%, 88.4%, and 95.8% (Mean: 91.3% ± 3.5%). Most of the wells contained one bead that can be seen in the fluorescent images and a small amount of dried buffer shown in the bright field images (Fig. 1). The wells that contained more than one bead, can also be observed in Figure 1. The differences in sorting efficiencies could be due to the QC process that was performed on the MoFlo Astrios EQ, such as improved cleaning, calibration and manual verification of the drop delay, using different drop envelopes, or change in charge phase. Heat map examples of the bead sorting efficiencies are shown in Figure 2.

Single Cell Detection Method Testing and Verification

To demonstrate the proposed method with a practical application, calcein-stained Jurkat cells were sorted into microplates using two different MoFlo Astrios EQ instruments (Fig. 3). Two experiments were conducted, the first without a rigorous QC process, and the second adopting the QC process described above. In the first experiment, the live cell sorting efficiencies were 64.2% and 66.3% from instrument 1, and 94.7% from instrument 2. In the second experiment, rigorous QC process was conducted, which resulted in efficiencies of 95.8% and 97.9% from instrument 1, as well as 89.5% and 97.9% from instrument 2. This data shows that adherence to a strict QC process can result in a high sorting efficiency for the tested cells. Other factors that may also reduce cell sorting efficiency from instrument 1 (prior to QC) may be attributed by the physical differences between beads and live cells (18), potential cellular damages due to the shear stress in the flow chamber (8), and the need for instrument calibration (18). Heat map examples of the cell sorting efficiencies are shown in Figure 4.

The single cell detection results generated by the Celigo Image Cytometer were directly validated by visual inspection

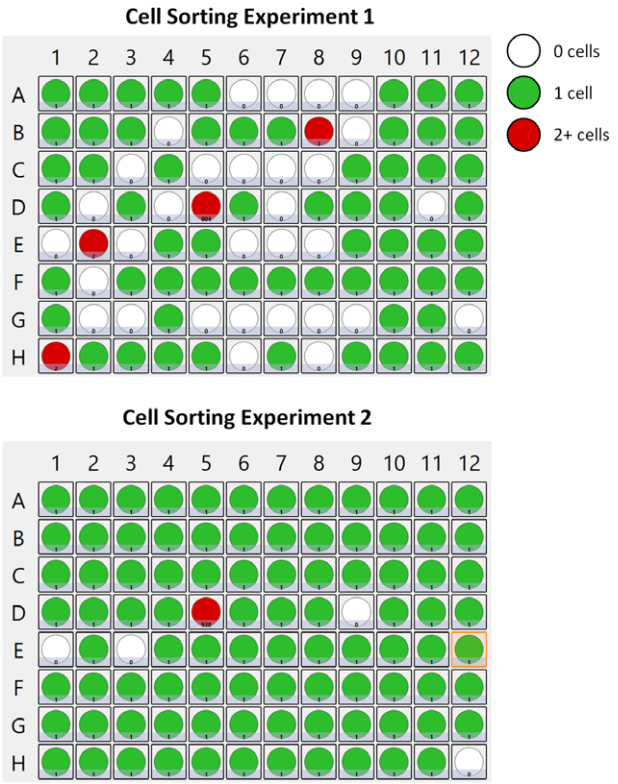

Figure 4. Heat maps of single cell sorting for Experiment 1 and 2 with white, green, and red indicating no cells, 1 cell, and 2 or more cells. The example heat map results showed sorting efficiencies of 64.2% and 95.8%, respectively. The sorting efficiencies were 64.2%, 66.3%, and 94.7% (Mean: 75.1% ± 17.1%) for the first experiment and 95.8%, 97.9%, 89.5%, and 97.9% (Mean: 95.3 ± 3.9) for the second experiment. [Color figure can be viewed at wileyonlinelibrary.com]

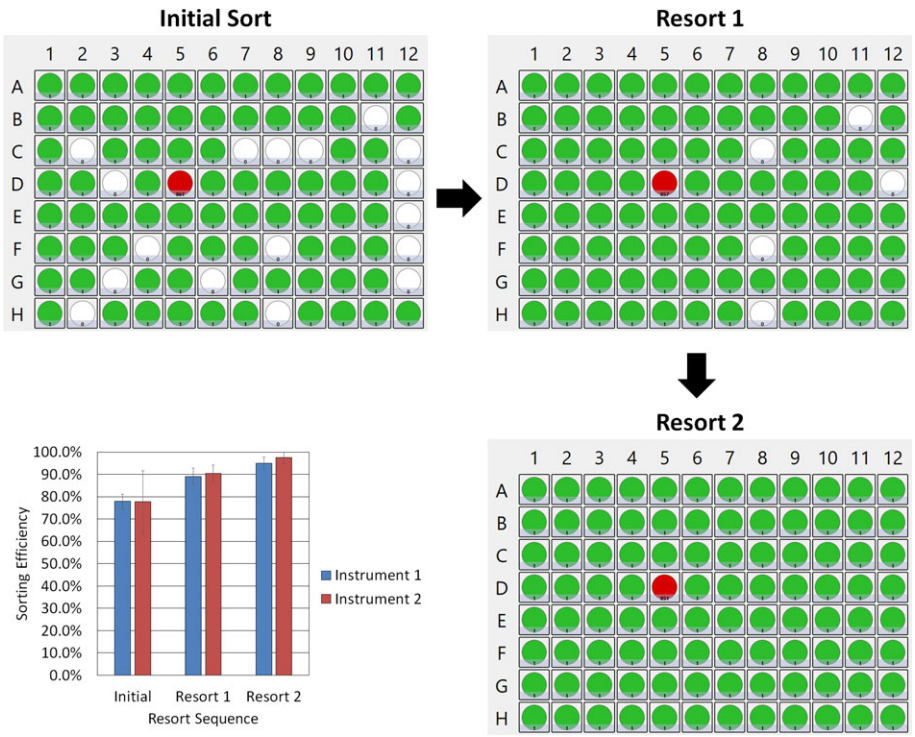

Figure 5. Heat maps of single cell resorting experiment. The example heat map results showed the increase in sorting efficiencies from 82.1 to 94.7, and finally 100%. The mean sorting efficiencies after each resort is plotted in the bar graph showing the similar results between Instrument 1 and 2. [Color figure can be viewed at wileyonlinelibrary.com]

of each plate using a standard microscope. The manual examination of the wells required approximately 30–40 min./plate, which entailed physical maneuvering of the plate to check different fields of view in the well. In comparison, the automated imaging method only required 7–8 min./plate for imaging and analysis, as well as digital record of the sorted plates.

Single Cell Resorting Capability

A total of 6 plates were sorted to investigate the resorting capability of the FACS. For instrument 1, the initial sorting efficiencies for the 3 plates were 74.7%, 81.1%, and 77.9%. The first resort showed improved efficiencies of 92.6%, 89.5%, and 85.3%. The second resort showed final efficiencies of 97.9%, 94.7%, and 92.6%. For instrument 2, the initial sorting efficiencies for the next 3 plates were 64.2%, 76.8%, and 82.1%. The first resort showed improved efficiencies of 87.4%, 89.5%, and 94.7%. The second resort showed final efficiencies of 94.7%, 97.9%, and 100%. Heat map examples and mean values of the sorting efficiency improvements are shown in Figure 5.

Discussion

Single cell sorting is a vital part of flow core laboratories, which is often utilized by researchers for oncological, immunological, and cell line development studies (1,6,7). The current single cell sorting verification method commonly uses a light microscope to manually identify the existence of single cells after settling or a single colony after days of outgrowth (9). The current method is impractical for flow core managers to quickly identify and resolve issues with their FACS instruments. In addition, it is time-consuming and tedious for researchers to increase the number of monoclonal colonies.

In this work, we have demonstrated a rapid and high-throughput single cell detection method using a plate-based image cytometer. The detection method performed fast whole well imaging and counting of sorted cells in 96-well plates. The Celigo was also able to directly count the number of beads or calcein-positive cells in the green fluorescent images. Large, small, and irregular-shaped debris were excluded from the counting results in the software to improve the cell counting accuracy. On the results page, the software was able to automatically display the cell counts using a heat map mode for a quick at-a-glance review of the sorting results. The heat map was generated by setting 0 and 1 as the minimum and maximum cell count, respectively. Any wells containing more than one cell, can be visually inspected directly on the digital image if needed. Once the plate was analyzed, the sorting efficiencies were directly calculated by exporting the counting data into an excel file.

Initially, the MoFlo Astrios EQ was calibrated to sort the beads with high efficiency, however, when sorting the calcein-stained Jurkat cells, the setup and gating procedures for beads were ineffective which is shown in the sorting results. Rigorous QC procedures were performed to ensure proper sorting of cells into the plates. Interestingly, most of the missed wells for beads are wells containing 2 beads (Figs. 1 and 2), whereas missed wells for cells are empty wells (Fig. 4). There were also

some wells containing 2 cells, which were at one location and touching, potentially indicating they were sorted into the well together (Fig. 3). These observations can be due to the physical differences between beads and cells, thus it may be ineffective to utilize beads to verify FACS for single cell sorting.

One significant advantage provided by the image cytometry method for post-sorting verification is that empty wells could be resorted, and wells with multiple cells could be marked and excluded from further use on the day of sorting. As shown in the resorting results, plates can be resorted multiple times to ensure high sorting efficiencies in the plates. Therefore, if FACS sorting efficiencies cannot be increased, multiple resorts may achieve the same desired outcome for researchers.

The current manual single cell verification using a microscope required keeping track of wells containing cells to determine the sorting efficiency of each plate. The demonstrated detection method reduced the single cell verification time from 40 min. to less than 8 min. By using the image cytometry method, flow core managers can quickly diagnose potential sorting issues and attempt QC or improvements to the sorting parameters. Furthermore, researchers can assess the sort results to improve their biology assay workflow. In conclusion, the proposed method can change the calibration methods for FACS instruments, which can significantly improve the performance for their users.

Acknowledgment

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Conflict of Interest

The authors SP, WR, and LLC declare competing financial interests. The research in this manuscript is for reporting on product performance of Nexcelom Bioscience, LLC. The work was to demonstrate a rapid single cell sorting verification method using the Celigo Image Cytometer.

Literature Cited

1. Gillissen MA, Yasuda E, Jong G, Levie SE, Go D, Spits H, PMv H, Hazenberg MD. The modified FACS calcein AM retention assay: A high throughput flow cytometer based method to measure cytotoxicity. J Immunol Methods 2016;434:16–23.
2. Guez-Barber D, Fanous S, Harvey BK, Zhang Y, Lehrmann E, Becker KG, Picciotto MR, Hope BT. FACS purification of immunolabeled cell types from adult rat brain. J Neurosci Methods 2012;203(1):10–18.
3. Iriondo O, Rábano M, Vivanco MM. FACS Sorting Mammary Stem Cells. In: Vivanco MM, editor. Methods in Molecular Biology: Mammary Stem Cells. Volume 1293. New York, NY: Humana Press; 2015; p. 63–72.
4. Mattanovich D, Borth N. Applications of cell sorting in biotechnology. Microb Cell Fact 2006;5(12):12.
5. Tauc HM, Tasdogan A, Pandur P. Isolating intestinal stem cells from adult drosophila midguts by FACS to study stem cell behavior during aging. J Visual Exp 2014; 94: 52223.
6. Xiong B, Ma L, Hu X, Zhang C, Cheng Y. Characterization of side population cells isolated from the colon cancer cell line SW480. Int J Oncol 2014;45(3):1175–1183.
7. Gallagher C, Kelly PS. Selection of high-producing clones using FACS for CHO cell line development. In: Meleady P, editor. Methods in Molecular Biology: Heterologous Protein Production in CHO Cells. Volume 1603. New York, NY: Humana Press; 2017; p. 143–152.
8. Hu P, Zhang W, Xin H, Deng G. Single cell isolation and analysis. Front Cell Dev Biol 2016;4:116.
9. Battye FL, Light A, Tarlinton DM. Single cell sorting and cloning. J Immunol Methods 2000;243(1–2):25–32.
10. Chan LL, Wilkinson AR, Paradis BD, Lai N. Rapid image-based cytometry for comparison of fluorescent viability staining methods. J Fluoresc 2012;22(5):1301–1311.
11. Chan LL, Zhong XM, Pirani A, Lin B. A novel method for kinetic measurements of rare cell proliferation using Cellometer image-based cytometry. J Immunol Methods 2012;377(1–2):8–14.
12. Chan LLY, Laverty DJ, Smith T, Nejad P, Hei H, Gandhi R, Kuksin D, Qiu J. Accurate measurement of peripheral blood mononuclear cell concentration using image cytometry to eliminate RBC-induced counting error. J Immunol Methods 2013;388(1–2): 25–32.
13. Maecker HT, Trotter J. Flow cytometry controls, instrument setup, and the determination of positivity. Cytometry Part A 2006;69A(9):1037–1042.
14. Orfao A, Ruiz-Argüelles A. General concepts about cell sorting techniques. Clin Biochem 1996;29(1):5–9.
15. Chan LL-Y, Smith T, Kumph KA, Kuksin D, Kessel S, Déry O, Cribbes S, Lai N, Qiu J. A high-throughput AO/PI-based cell concentration and viability detection method using the Celigo image cytometry. Cytotechnology 2016;68:2015–2025. https://doi.org/10.1007/s10616-016-0015-x.
16. Cribbes S, Kessel S, McMenemy S, Qiu J, Chan LL-Y. A novel multiparametric drug-scoring method for high-throughput screening of 3d multicellular tumor spheroids using the celigo image cytometer. SLAS Discov 2017;22(5):547–557.
17. Zhang H, Chan LL-Y, Rice W, Kassam N, Longhi MS, Zhao H, Robson SC, Gao W, Wu Y. Novel high-throughput cell-based hybridoma screening methodology using the Celigo Image Cytometer. J Immunol Methods 2017;447:23–30.
18. Montes M, Jaensson EA, Orozco AF, Lewis DE, Corry DB. A general method for bead-enhanced quantitation by flow cytometry. J Immunol Methods 2006;317(1–2): 45–55.