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APPLICATION NOTE

Novel high-throughput image cytometry method for T cell immunophenotyping and viability readouts.

Key features

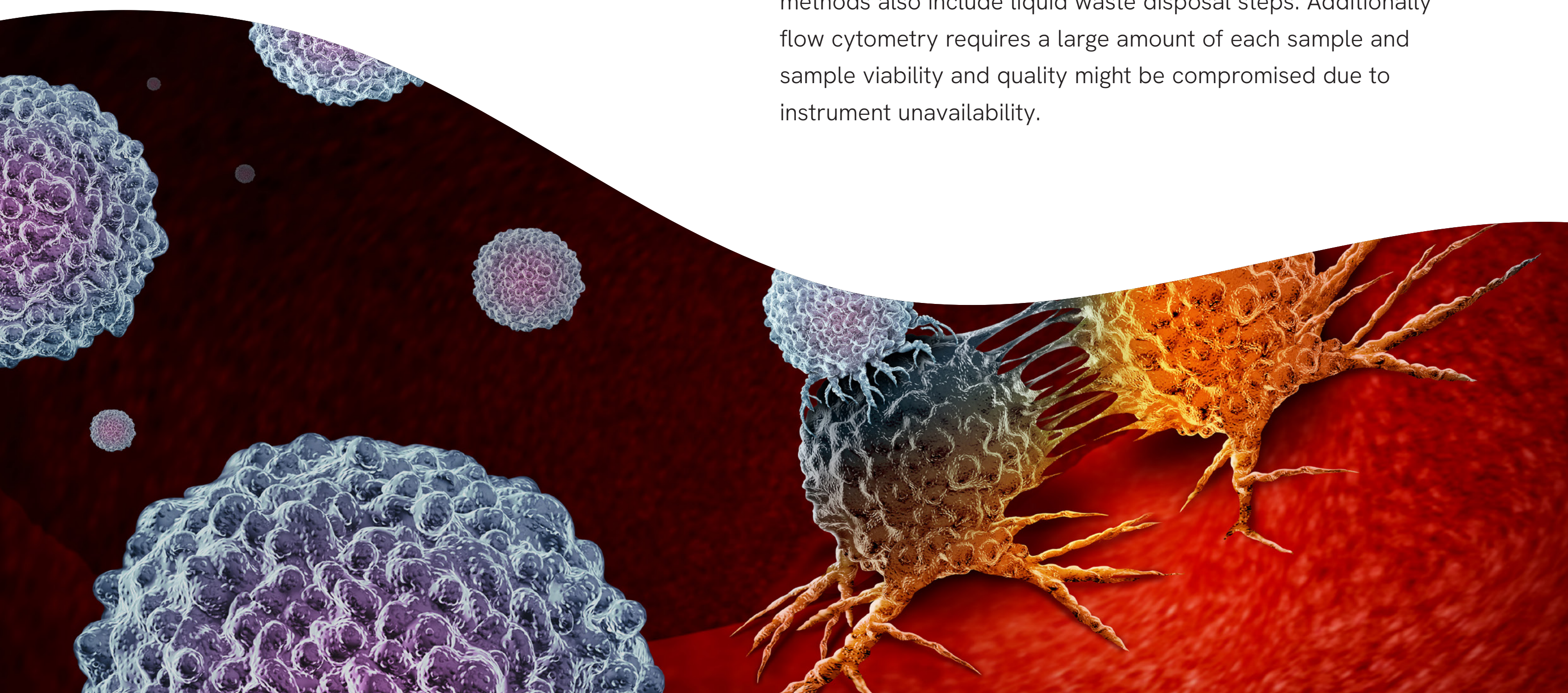
- High-throughput and rapid cell concentration analysis with sample-efficient experimentation and optimized assay kits
- Accurate multiplexed surface marker staining and viability at the benchtop
- Image cytometer with a streamlined workflow and excellent data correlation to a flow cytometer

Introduction

Cellular therapies have gained much attention in the last decade due to their many successes in discovery and clinical research. For cellular therapeutic products, the manufacturing process requires the guidance of chemical manufacturing and controls (CMC) for analytical method development focusing on the critical quality attributes (CQAs). The CQAs consist of, but are not limited to, identity, cell count, purity, potency, relevant biological activity, viability, stability, and maturation profile. One of the major CQAs for cellular therapy products is identity, which is typically characterized by quantitatively determining and distinguishing the antigen receptors on the cells. Immunophenotyping using flow cytometry is the most widely used technique by laboratories particularly focused on immunology, immuno-oncology, cell health/viability, and cell-based product manufacturing such as chimeric antigen receptor (CAR) T cells.

Flow cytometry challenges

Flow cytometry has some distinct disadvantages that can impede research progress,[1,2,3] including, for example, the requirement of an expensive instrument which, for many laboratories, can only be accessed via a core facility. Despite providing valuable information, data acquisition via flow cytometry can be time-consuming, not only due to sample preparation and run-time but also because it requires an expert user and access to a flow cytometry instrument. These methods also include liquid waste disposal steps. Additionally flow cytometry requires a large amount of each sample and sample viability and quality might be compromised due to instrument unavailability.



Alternative method for multi-channel immunophenotyping assays

The Cellaca® PLX image cytometer is a high-throughput cell counting and image cytometry instrument that provides flow-like data by using pre-programmed, simple, multi-channel assays directly at the laboratory bench. It measures cell populations and viability, as well as cell concentration, without the need for a time-consuming flow cytometer run. Each assay requires as little as 15 µL of sample and provides accurate and consistent results without the need for single-stained controls, resulting in faster assay setup and run times.

The Cellaca PLX system produces rapid results: users can obtain cell images, cell counts, size measurements, surface marker analysis, and viability calculations in seconds and use customizable data tables to plot cell population as a histogram, scatter, dot, or contour plots. With output speed of less than one minute per sample, researchers can quickly perform a cell purity check at the bench in order to continue their downstream assays without needing to wait for usage time on a flow cytometer. Other advantages include up to six channels of fluorescent excitation and emission combinations selected from four excitation filters and five emission filters. Another advantage is that the image-based analysis algorithms can be optimized for specific assays and for various complex cell types such as fresh PBMCs, bone marrow, splenocytes, and primary cells. Whether using it as a complementary method for orthogonal data acquisition or as a quick check prior to initiating downstream assays, image cytometry can save researchers precious time and samples. By performing multiplexed assays at the bench, laboratories can reduce their reliance on a busy flow cytometry core facility and streamline data collection for routine assays.

Multiplexed surface marker staining and viability

Staining for surface marker expression and viability in primary human peripheral blood mononuclear cells (PMBCs) was performed to highlight the capabilities of the Cellaca PLX system. One million PBMCs were resuspended in medium. Surface marker-specific antibodies CD3-KIRAVIA Blue 520™[4] and CD4-PE, along with the viability dyes RubyDead and Hoechst were added to the cells to a final volume of 100 µL. The staining mixture was incubated at 4°C for 30 minutes. After incubation, 200 µL of 1 x PBS was added to the cells as a wash, then centrifuged, the supernatant aspirated, and the pellet suspended in 100 µL of medium. Simultaneously, individual surface marker isotype controls with viability dyes were prepared with 1 x 10^6 PBMCs. Cellaca PLX slides were loaded with 15 µL of stained samples and isotype controls, placed into the instrument, and multiple cell images were collected. The resulting data was automatically exported to the flow cytometry software, FCS Express™, where data can be visualized, analyzed, saved, and exported.

In contrast to traditional flow cytometry, the Cellaca PLX image cytometer acquires data for each stained population (Fig. 1). This allows researchers to examine cell morphology in brightfield to visually confirm positive fluorescent staining for CD3 and CD4 markers while assessing the overall sample health and viability.

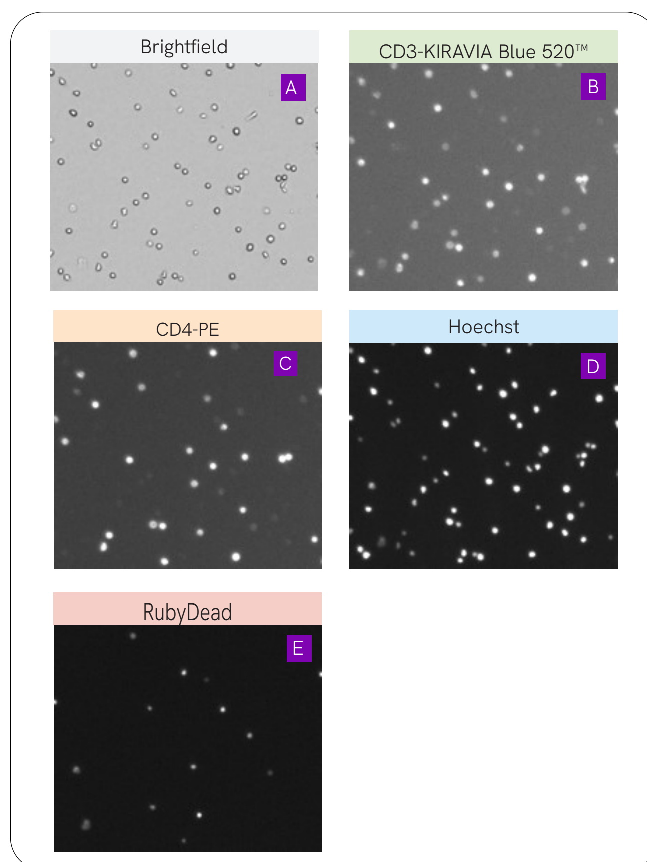


Figure 1. Cellaca PLX acquired images using the CD3/CD4 viability kit. PBMCs in brightfield (A) were stained with mouse anti-human CD3-KIRAVIA Blue 520™ (B) CD4-PE (C), and counterstained with Hoechst (D) total dye and RubyDead (E) viability dye.

Acquired image data is automatically exported to FCS Express™ with pre-designed templates for population analysis. The assay design and execution allow for the concurrent examination of cell viability and surface marker discrimination. Samples are first gated for viability (Fig 2A) providing vital information on sample quality. Primary human samples are often processed for various downstream assays, and the Cellaca PLX system can function as a high-throughput cell counter to determine cell health at multiple checkpoints during sample handling. The versatility of the Cellaca PLX allows a single instrument to be used for measuring routine cell concentration and viability as well as more complex surface marker populations. Here, results show that 79.27% of cells are alive. Of the live cells, 58.77% are CD3-positive, while 30.41% are CD3- and CD4-positive (Fig 2B). The isotype control is overlayed and represented in quadrant 1 as a blank scatter plot.

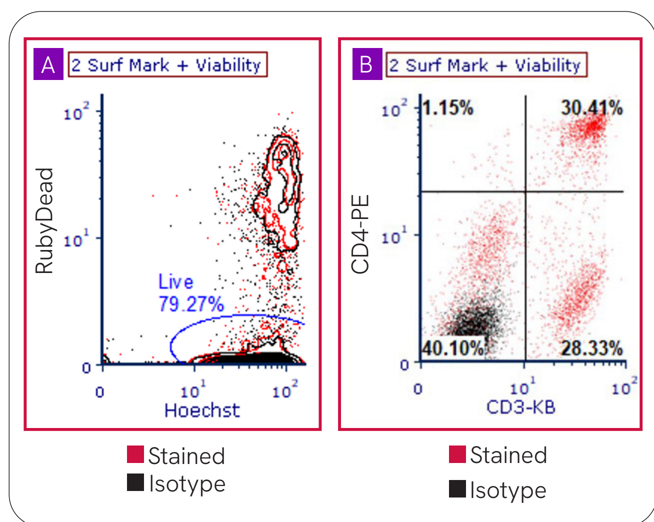


Figure 2. Population analysis was performed using FCS Express™ Flow Cytometry software to determine the percentage of live and dead cells (A, bottom right and top right quadrants, respectively). Gated live cells show CD3-KIRAVIA Blue 520™ positive (CD3-KB+) cells in the bottom right quadrant and CD3-KB+ CD4-PE+ cells located in the top right quadrant (B). Double negative cells show up at the bottom left quadrant (B).

Immunophenotyping comparison between the benchtop Cellaca PLX and flow cytometer instruments

A comparative study was performed with a flow cytometer and the Cellaca PLX image cytometer to examine instrument and assay functions using primary human PBMC samples. Many simple assays requiring quick checks to determine purity or CD-marker breakdown are typically done on a flow cytometer, but we demonstrate here that they can be performed on a simple-to-run image cytometer instead. As mentioned above, there are significant time-saving advantages to performing these assays on the Cellaca PLX system. This study examined the population breakdown of CD3, CD4, and CD8 T cells on the Cellaca PLX and the flow cytometer. Human PBMCs (1 x 10^6) were resuspended in 84 µL medium. Surface marker-specific anti-human CD3-KIRAVIA Blue 520™, CD4-PE, and CD8-APC antibodies and Hoechst counterstain were added to the cells at a final volume of 100 µL. The mixture was incubated at 4°C for 30 minutes. After incubation, 200 µL of 1 x PBS was added to the cells as a wash, cells centrifuged, the supernatant removed, and the pellet was resuspended in 100 µL of medium. Simultaneously, a single isotype control sample with Hoechst was prepared with 1 x 10^6 PBMCs. Cellaca PLX slides were loaded with 15 µL of stained samples and isotype controls, placed into the instrument, and then multiple cell images were collected. The resulting data was automatically exported to FCS Express™. The same PBMCs stained samples were also run on a flow cytometer.

Prior to running samples on the flow cytometer, we prepared additional single color-stained control samples (CD3-KB, CD4-PE, CD8-APC, and Hoechst only) that are required for flow cytometry channel compensation. Since the Cellaca PLX is an LED-driven system and is designed with non-overlapping filters, it does not require any of the traditional flow cytometry controls or adjustments (single color stains, PMT (photomultiplier tube) voltage adjustments, and FM (Fluorescence Minus One)).

Acquired image data for each channel, including brightfield, demonstrate brightly stained PBMCs (Fig 3). Cells of interest can be viewed, identified, and counted. In this assay, only Hoechst-positive nucleated cells are directly exported to the data analysis software, whereas residual RBCs and cell debris are automatically gated out.

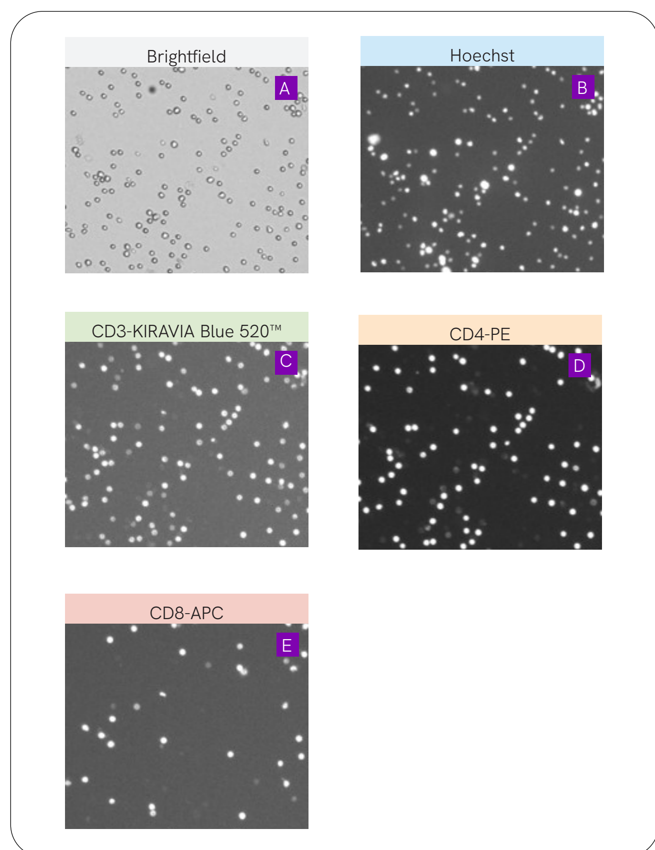


Figure 3. Cellaca PLX acquired images using the CD3/CD4/CD8 total cell kit. Acquired brightfield image (A), counterstained with Hoechst (B) are shown. PBMCs stained with anti-human CD3-KIRAVIA Blue 520™ (C), anti-CD4-PE (D), and anti-CD8 (E) images are displayed.

The Cellaca PLX results were exported to FCS Express™ Flow Cytometry software to determine the breakdown of CD3-positive, CD4-positive, and CD8-positive cells. Initial gates were applied to identify nucleated (Hoechst+) PBMCs and CD3-positive populations (Fig 4A). Of the 71.6% of CD3-positive cells, 63.6% are CD4-positive and 24.1% are CD8-positive (Fig 4B).

In the Cellaca PLX image cytometer, gating is performed after image acquisition on Hoechst-positive cells, allowing for immediate visual verification of counted cells prior to export to data analysis software for subsequent surface marker characterization. In contrast, a flow cytometer exports bulk data and the end-user is required to first identify their cell population using forward and side scatter plots (Fig 5). Additional gating may be applied to further isolate single cells within the sample. Results from a flow cytometry instrument are then gated for CD3-positive and Hoechst-positive cells and further broken down into CD4-positive and CD8-positive populations, similar to the Cellaca PLX system.

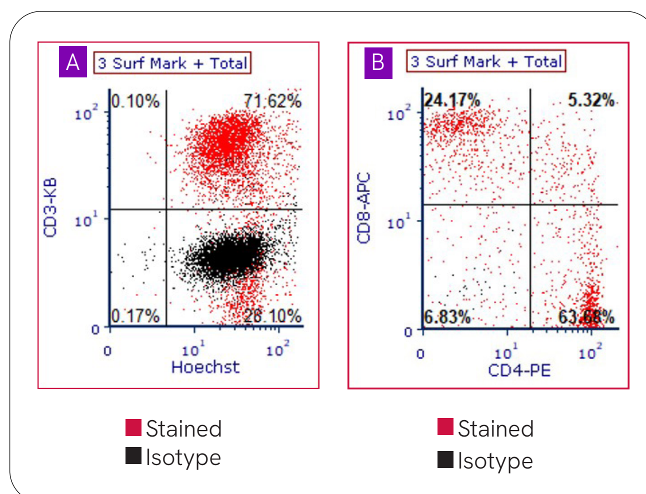


Figure 4. Data from Cellaca PLX is exported to FCS Express™ Flow Cytometry software for population analysis. CD3-KIRAVIA Blue 520™ PBMCs that are Hoechst-positive are shown in (A). Positively-gated CD3 PBMCs allow for identification of CD4-PE and CD8-APC positive cells (B).

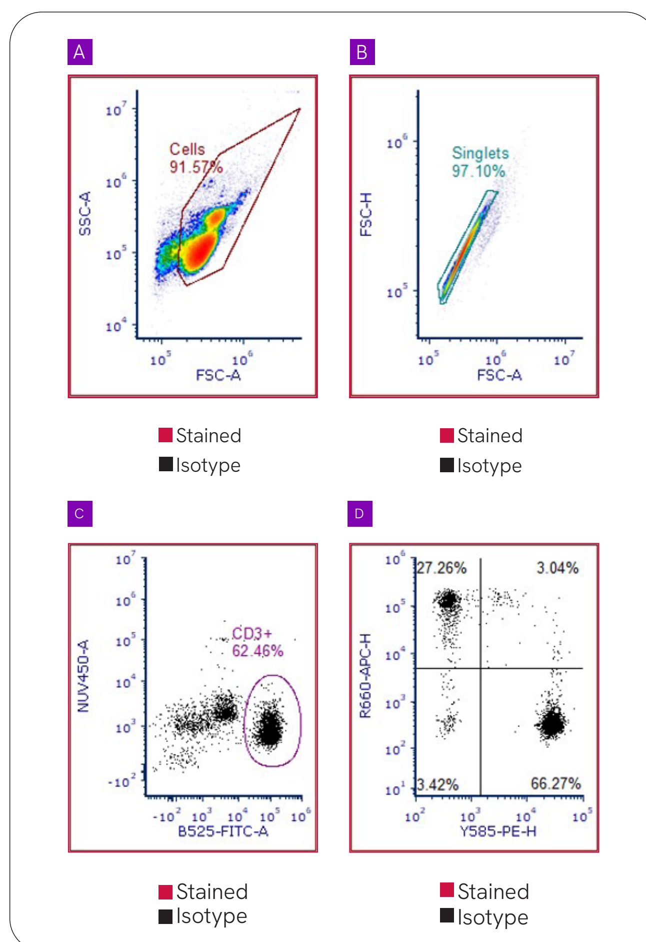


Figure 5. Data from the flow cytometer was exported to FCS Express™ software for population analysis. Forward and side scatter is performed to identity an initial population of interest (A). Single PBMCs are further gated (B). CD3-KIRAVIA Blue 520™ PBMCs that are Hoechst-positive are shown in (C). Gated CD3-positive cells are plotted for CD4-PE and CD8-APC (D).

Table 1: Data from the Cellaca PLX system and flow cytometer show correlating percentages of analyzed surface markers.

	Flow cytometer	Cellaca PLX
CD3+	62.46 % (Singlets)	71.62 % (Hoechst +)
CD4+ (of CD3+)	66.27 %	63.68 %
CD8+ (of CD3+)	27.26 %	24.17 %

Gated CD3-positive cells had a 9% difference between the Cellaca PLX and flow cytometry. Subsequent gating on CD3-positive cells showed that there is only a 3% difference for both CD4-positive and CD8-positive populations between the two instruments. The close correlation between the Cellaca PLX system and flow cytometry highlights the quality of the data gathered from an image cytometer versus a flow cytometer. Furthermore, it strengthens the concept that image cytometers can produce data comparable to flow and can do so more efficiently.

Conclusions

The Cellaca PLX image cytometer is an all-in-one system capable of performing cell counting, primary cell viability, and cell-based assays with accurate and consistent results. To continue with downstream assays promptly, researchers at the bench can quickly assess the viability and bulk percentages of CD3, CD4, and CD8-surface markers. Software, hardware, and assay reagent kits have been optimized to allow for an efficient process of assessing multiple markers simultaneously. The correlation between the Cellaca PLX system and flow cytometry is precise. It allows researchers to confidently perform multiplexing assays at the bench, reducing the reliance on a busy flow cytometry core facility, and the expertise of super users to analyze results.

References

1. Couvillion *et al.*, 2019.
2. Alvarez-Barrientos *et al.*, 2000.
3. Davey & Kell, 1996.
4. KIRAVIA Blue 520™ is a trademark of Sony. This product is subject to proprietary rights of Sony and is made and sold under license from Sony Corporation.

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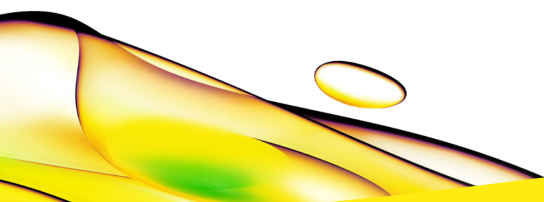

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