Application Note 20

Cyto-Mine® Chroma: Single Cell Analysis and Isolation using Multiplexing in Picodroplets

Jitender Bisht, Elena Shvets, Maryam Ahmadi, and Richard Hammond

Introduction

Multiplexing has transformed biological research and development by enabling the analysis of multiple targets or parameters within a single experiment. This innovative approach offers numerous benefits, including reduced sample requirements, increased throughput, enhanced assay accuracy, cost efficiency, and a more comprehensive understanding of complex biological systems (1-2). As this technology advances, researchers are increasingly utilizing combined assays to gain deeper insights into intricate biological processes and to drive innovation in healthcare and biotechnology (3-8). Nonetheless, multiplexing presents challenges such as the need for specialized assays and equipment, sophisticated data analysis and software tools, and the risk of cross-reactivity leading to false positives (1-2).



sphere bio
work small, think big.

Sphere Bio proprietary picodroplet technology (9) facilitates automated, high-throughput screening and selection of single cells based on surface markers or secreted molecules. The first-generation Cyto-Mine® performs high-throughput single-cell analysis using a fluorescent assay powered by a single excitation laser and two detection channels. Expanding the number of excitation lasers and detection channels would enable fluorescence multiplexing, enhancing assay specificity and enabling more targeted selection and high-throughput screening.

To address this need, we have introduced the Cyto-Mine® Chroma, the next-generation Cyto-Mine® instrument, equipped with multiple lasers and detectors for automated single-cell analysis and isolation with advanced multiplexing capabilities. Here we demonstrate the potential of spectral multiplexing within droplets, highlighting its advantages for applications such as antibody discovery (AbD) and cell line development (CLD).

To validate the performance and capabilities of the Cyto-Mine® Chroma, we designed multiplexed biological assays utilizing simultaneous analysis of multi-colour parameters. Here we show that Cyto-Mine® Chroma is capable of identifying both secreted and cellular markers in fluorescence- based multiplexed assays. Cells were identified and isolated based on their productivity (IgG secretion) as well as antigen specificity (antibody-antigen binding), while maintaining accuracy. In-line with AbD workflows, we demonstrate that the Cyto-Mine® Chroma is capable of highly accurate detection, analysis and isolation of rare, antibody-secreting, single cells from a heterogenous starting population.

Aims and Objectives

- To demonstrate the detection and isolation of subpopulations of cells based on a fluorescent cell label, and their secretion of IgG measured by Förster Resonance Energy Transfer (FRET) signal.
- To demonstrate isolation of rare antibody-secreting cells based on the binding of a secreted antibody to a surface- expressed antigen in a multiplexed assay.

Methods

Cyto-Mine® Chroma

Cyto-Mine® Chroma is a single cell analysis system that leverages picodroplet technology to functionally analyze encapsulated single cells. Briefly, Cyto-Mine® Chroma uses microfluidics to encapsulate single cells in picodroplets and transport picodroplets through a series of channels on a Cyto-Cartridge®. Cells are first encapsulated in picodroplets, incubated, and then transported to the analysis/sorting area of the Cyto-Cartridge®. Analysis of picodroplets is conducted using 4 independently controllable lasers and 4 PMT detectors with excitation at 405, 488, 561, and 638 nm and detection at 446/34, 520/15, 605/20, 678/30 nm. Design of the optic channel generates a precise laser line which, can profile picodroplet contents to characterize localized, diffused, or contrasting fluorescent signals. Fluorescent signal detection is visualized in the Cyto-Mine® Instrument Control software, and picodroplets can be sorted by users. Notably, different modes are used for optimal detection of either localized signal (surface-binding or cell staining, detected by fluorescence peak) or dispersed fluorescence (FRET signal, detected by fluorescence average). Sorted picodroplets are then transported through the Cyto-Cartridge® to the dispensing area. Fluorescent analysis is conducted again using the same 4 lasers and detectors, and data is visualized on the Cyto-Mine® Instrument Control software. Specific populations of picodroplets can be selected for dispensing. Selected picodroplets are then individually dispensed into a microtiter plate and the instrument captures 5 images of each dispensed picodroplet for monoclonality assurance.

Cell staining

Three different cell dyes were used for staining: eBioscience™ Cell Proliferation Dye eFluor™ 450, CellTracker™ Orange CMRA (CT- Orange), eBioscience™ Cell Proliferation Dye eFluor™ 670. These dyes were obtained from Thermo Fisher Scientific (Leicestershire, UK). Briefly, the dyes were reconstituted in DMSO to a stock concentration of 10 mM, 2.5 mM and 5 mM, respectively. The dyes were diluted in basic media (in the absence of FBS) to a final concentration and each dye incubated at 37°C with $1x10^6$ of pre-washed cells. The staining was stopped by diluting the reaction with 10x volumes of cold complete media and centrifugation. The cells were then resuspended in 1 mL of

encapsulation media Complete culture media, 16% Opti-prep (Merck Life Sciences, Dorset, UK), 10% PF68 (Thermo Fisher) at the desired cell concentration.

FRET assay detection of pre-stained cells

Two different hybridoma cell lines, each at 0.5x10^6 cells/mL were stained with either eBioscience™ Cell Proliferation Dye eFluor™ 450 (eFL450) or eBioscience™ Cell Proliferation Dye eFluor™ 670 (eFL670). Stained cells were then mixed with FRET probes (Donor: anti-mouse IgG Dylight488 (DL488), Acceptor: anti-mouse IgG Dylight594 (DL594)) before being loaded into Cyto-Mine® Chroma. Cells were encapsulated and the picodroplets incubated at 37°C for 2 hours in Cyto-Mine® Chroma, to allow antibody secretion. Picodroplets were gated during sorting for cellular dye and/or FRET signal. Picodroplets were dispensed into 96-well plates and scanned for the presence and fluorescence of cells on NYONE® scanner (Synentec GmbH, Germany).

Surface antigen-binding of recombinant and secreted antibodies

A binding assay was performed to detect the binding of antibodies to their specific target antigen expressed on the surface of cells, as described in Figure 1.

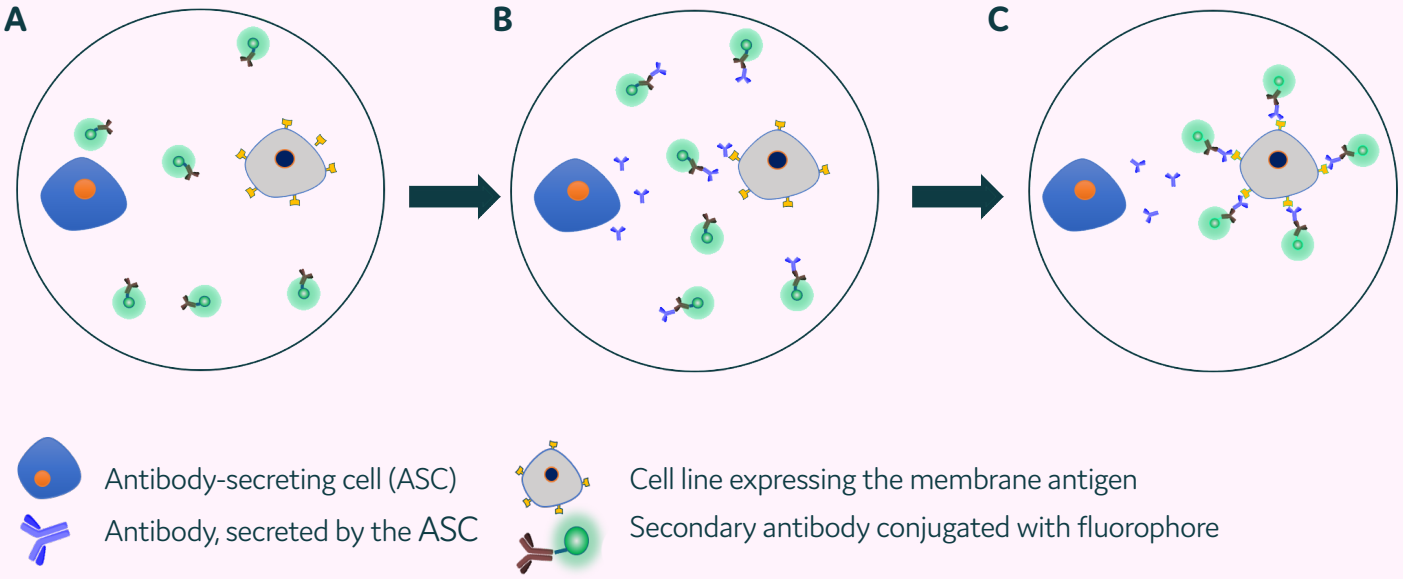


Figure 1. Schematic representation of surface-binding assay, for secreted antibodies: A) Antibody-secreting cells are encapsulated together with target cells expressing antigen on the surface, and detection antibody. B) Upon secretion, primary antibody is recognized by detection antibody and also binds the antigen on the surface of the cell. C) Binding of antibody complex on surface of the target cell results in translocation of some of the detection antibody from homogenously dispersed appearance into cell-surface localization, exhibited as an increase in green fluorescence on Cyto-Mine® scatter plot.

Accordingly, two types of cells were stained and prepared for co-encapsulation in pico-droplets: the target A431 cells (over-expressing EGFR) were stained with eBioscience™ Cell Proliferation Dye eFluor™ 670, and hybridoma cells (secreting anti-human EGFR antibodies; ECCAC) were stained with eBioscience™ Cell Proliferation Dye eFluor™ 450. Next, $3x10^6$ target cells were mixed with antibody secreting cells ($0.01x10^6$/mL - $0.3x10^6$/mL) in 1 mL of encapsulation media (Complete culture media, 16% Opti-prep, 10% PF68) together with anti-mouse DL488-conjugated antibodies (Abcam). Where stated, the binding of Cetuximab (Absolute Antibody) to eFluor™ 670 – stained target cells was detected using anti-human DL488-conjugated antibody (Abcam). For spike-in experiments, a different hybridoma cell-line, secreting anti-human TNF-α antibodies (ECCAC) was stained with Cell Tracker Orange CMRA, and $0.3x10^6$ cells (for 1:1 ratio) or $1x10^6$ cells (for 1:100 ratio) were mixed with the sample. The sample was then loaded onto the Cyto-Mine® Chroma where cells were encapsulated into picodroplets and incubated for 2 hours at 37°C. Positive droplets were sorted and dispensed into 96-well plates and analyzed on the NYONE® scanner for validation.

Validation of cell isolation using microscopy

NYONE® scanner was used to validate correct detection and isolation of the desired populations of picodroplets, whereby all dispensed 96-well plates were scanned at 4 different wavelengths to detect the fluorescence of the dispensed cells. The settings of NYONE® scanner used are summarized below:

Channel	Fluorochrome Detected	Excitation	Emission
1	Cell Proliferation Dye eFluor™ 450 (eFL450)	UV	Blue
2	Dylight 488 (DL488)	Blue	Green
3	CellTracker™ Orange CMRA (CT- Orange)	Lime	RedLP
4	Cell Proliferation Dye eFluor™ 670 (eFL670)	Red	FarRed
5	N/A	BF	Brightfield

Statistical analysis

Statistical analysis was performed using images of dispensed picodroplets captured by NYONE® scanner. All assays were repeated at least twice and two plates set up per assay. For analysis of secreting cells (Figure 2) the following formula was used:

$$\text{Isolation accuracy (\%)} = \frac{\text{wells with eFluor450}^{+}\text{ cells}}{\text{Total No. cell occupied wells}} \times 100$$

For surface binding assay (Figure 4) the analysis was based on:

$$\text{Isolation accuracy (\%)} = \frac{\text{No. wells with [(eFluor450}^{+}\text{ cells) AND (eFluor670}^{+}\text{ cells stained with DL488)]}}{\text{Total No. cell occupied wells}} \times 100$$

OR for surface-binding assay with spiking-in (Figure 5) the analysis was based on:

$$\text{Isolation accuracy (\%)} = \frac{\text{No. wells with [(eFluor450}^{+}\text{ cells) AND (eFluor670}^{+}\text{ cells stained with DL488) NOT (CT Orange}^{+}\text{ cells)]}}{\text{Total No. cell occupied wells}} \times 100$$

Results and Discussions

Isolation of rare antibody secreting cells from a mixed cell population requires accuracy in detection, sorting and dispensing. Antibody secreting cells may be isolated based on the presence of secreted IgG, or the specific binding of secreted IgG to its antigen. To evaluate the ability of Cyto-Mine® Chroma in successfully isolating these cells, we determined:

- Isolation of single cells when droplets were sorted using single or sequential gating. In this assessment both secretion and cell staining were used to successfully gate on the population of interest.
- Accuracy in detecting antibody binding to its surface-expressed antigen. Using a recombinant antibody, we show the detection specificity of antibody binding to target cells.
- Accuracy in isolation of rare cells of interest by multiplexing antigen-binding detection with labelling of ASC and target cells. We show that when two different antibody secreting cells (ASC) are present in the same culture, it is possible to isolate the cells of interest with high accuracy based on the binding of the secreted antibody to its target cell.

Detection and isolation of cells based on antibody secretion and cell-based staining

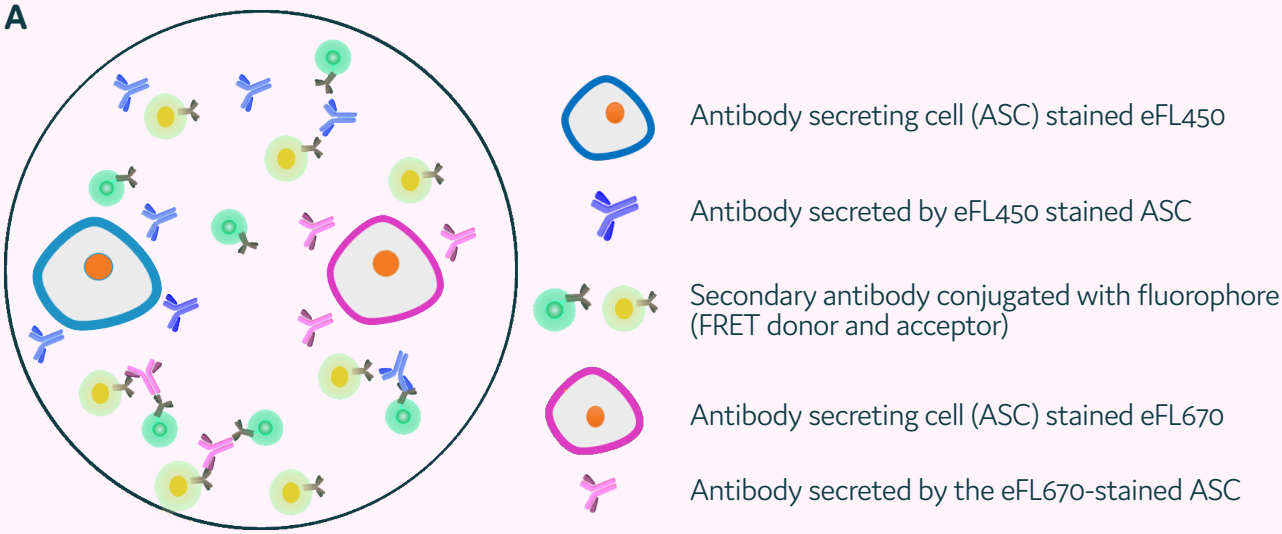
To determine the isolation accuracy of ASC in Cyto-Mine® Chroma, based on the presence of secreted antibody, we used mouse IgG1- secreting hybridoma cells together with FRET probes (Figure 2A). The transfer of energy from Donor probe to Acceptor probe in the presence of their target molecule (mouse IgG) will result in an increase in Acceptor fluorescence signal and reduced Donor signal (10). As shown in Figure 2A, the cells were stained with either eFluor™ 450 or eFluor™ 670 cellular markers and mixed with mouse-specific FRET probes (Donor labelled with DL488 and Acceptor with DL594).

The resulting population of cells was loaded onto Cyto-Mine® Chroma and IgG secretion was detected using the FRET signal. As shown in Figure 2B, hybridoma cells stained with either eFluor™ 450 or eFluor™ 670 were detectable and easily differentiated. Following a 2- hour incubation to allow IgG secretion, the FRET-positive population, recognized by a shift in Donor (reduced) and Acceptor (increased) fluorescence signal, was identifiable (Figure 2C).

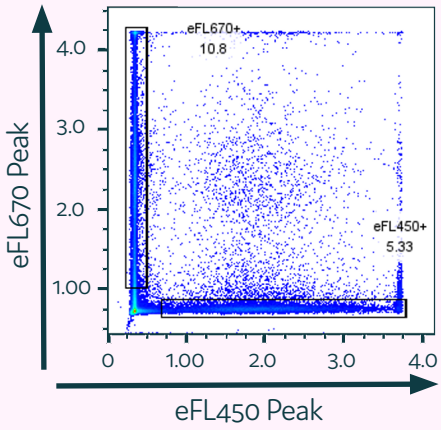
A single gate on the $FRET^+$ population during sorting, resulted in droplets containing either eFluor™ 450- stained, eFluor™ 670- stained or a combination of the two stained cells being dispensed, as expected (Figure 2D). To show that sequential gating could be used to isolate a specific ASC population, droplets were initially gated for FRET signal followed by gating on the eFluor™ 450 stained cells (Figure 2E). The combination of these selection criteria, resulted in isolation of a single population of cells, the eFluor™ 450 stained cells only (Figure 2F).

Single droplets were dispensed into 96-well plates and the fluorescence signal of the isolated cells was validated by fluorescence microscopy. As shown in Figure 2G, droplets gated solely on FRET positive population (Figure 2C), showed an expected distribution of either eFluor™ 450, eFluor™ 670 stained cells, or both (for polyclonal droplets) (Figure 2G and Figure 2I). Droplets sequentially gated for FRET and eFluor™ 450 however (Figure 2E), contained eFluor™ 450 stained cells only (100%, Figure 2H and Figure 2I). These data demonstrate the accuracy of isolating cell populations using single or sequential gating during sorting (Figure 2I).

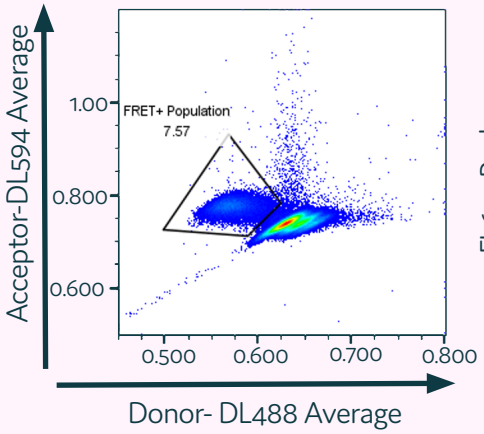
A



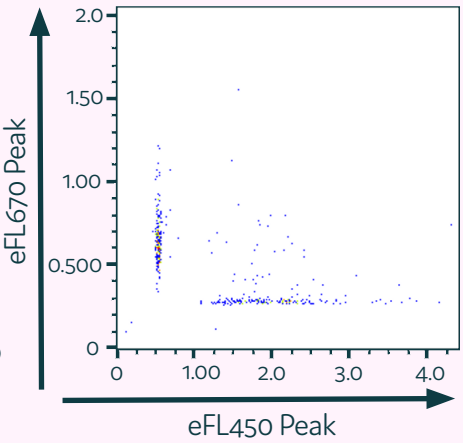
B Unsorted Population



C

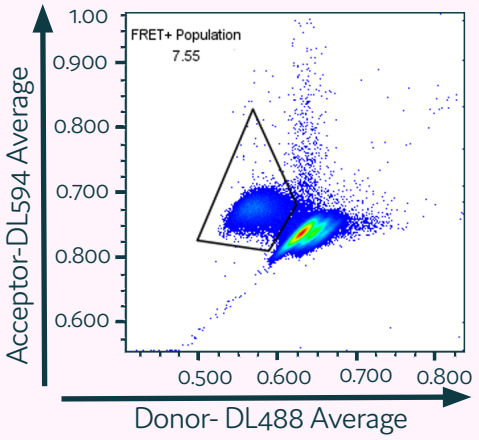


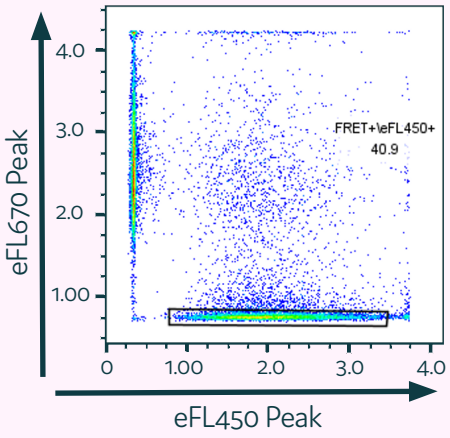
D Dispensed Population



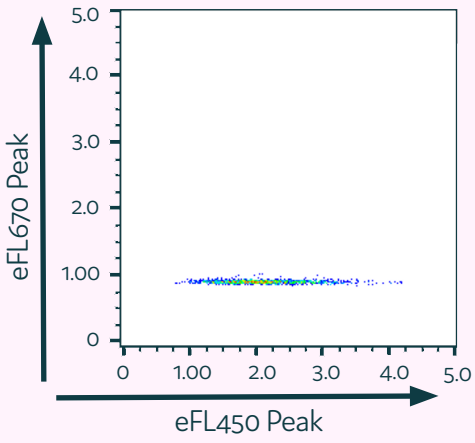
Gating at Sorting

E 1st Gate: FRET+ 2nd Gating: eFL450 labelled

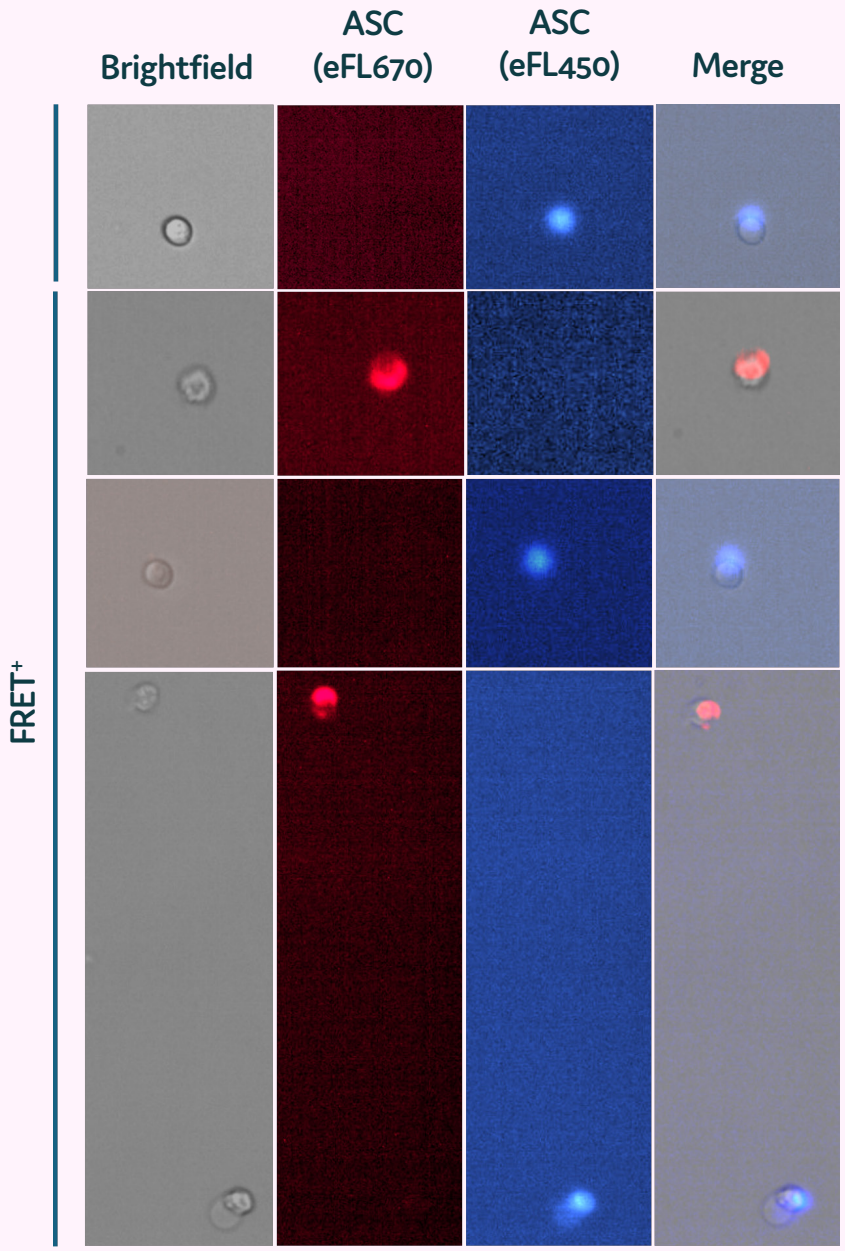




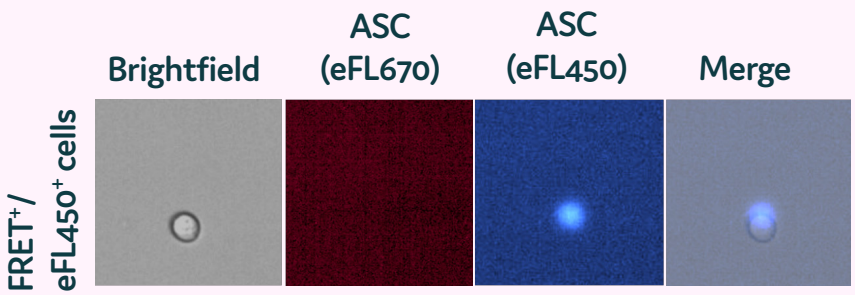
F Dispensed Population



G



H



I

Sorted Population	Cell Detected
FRET+	eFluor™ 450+ only (38%), eFluor™ 670+ only (54%), eFluor™ 450+ and eFluor™ 670+ (8%)
FRET+ /eFluor™ 450+	eFluor™ 450+ only (100%)

Figure 2. Isolation of subpopulation of secreting cells on Cyto-Mine® Chroma. A hybridoma cell line was stained with either eFluor™ 450 or eFluor™ 670 and mixed with Mouse FRET probes. A) Schematic representation of experimental design. B) Detection of different stained cell populations before sorting. C) Cells were gated for FRET signal only during sorting. D) Cells dispensed based on the gating strategy, were analyzed for the presence of eFluor™ 450 and eFluor™ 670 labelled cells. E) Cells were first gated for FRET signal (left panel) and then on eFluor™450 signal sorting (right panel). F) Cells dispensed based on the gating strategy were analyzed for the presence of eFluor™ 450 and eFluor™ 670 labelled cells. G and H) Fluorescence microscopy analysis of dispensed cells from 2D or 2F. I) Statistical analysis of isolation accuracy of FRET$^+$ or FRET$^+$/ eFluor™450 populations.

Detection of antibody binding to target cells in Cyto-Mine® Chroma

Next, we wished to determine whether the binding of antibody to its antigen, expressed on the surface of a target cell, can be accurately detected in Cyto-Mine® Chroma. To this end the target cells (A431 cell line), expressing EGFR, were stained with eFluor™ 670 and mixed with Cetuximab (anti-EGFR hIgG) and detection antibody (DL488- labelled anti-hIgG antibody). Droplets were gated for dual expression of eFluor™ 670 (target cells) as well as the increase in DL488 peak signal. Data show that in the absence of Cetuximab, the background binding of the detection antibody remains low (Figure 3A). The presence of Cetuximab, however shifts the DL488 signal in 60% of total droplets (Figure 3B, equivalent to 80% of droplets containing cells).

The data shown here demonstrates Cyto-Mine® Chroma is able to detect antibody binding to its surface antigen on a target cell.

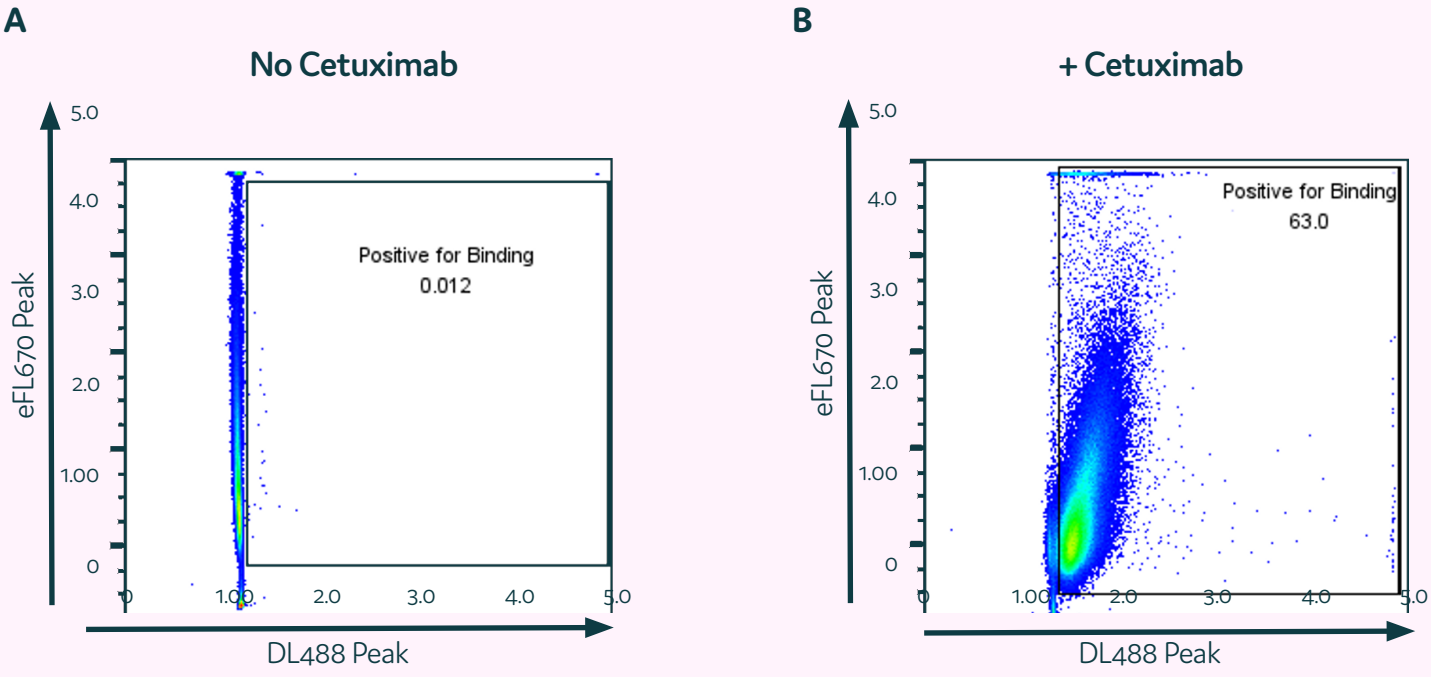


Figure 3. Detection of antibody binding to its target surface antigen. A431 target cell line was labelled with eFlour670™ (eFL670) and mixed with Cetuximab and DL488 labelled anti-hIgG antibody. The mixture was loaded onto Cyto-Mine® Chroma. Droplets were gated during sorting for eFL670 and increased DL488 peak. Dot plots show data accumulated during sorting of droplets in Cyto-Mine® Chroma in the absence (A) or presence (B) of Cetuximab.

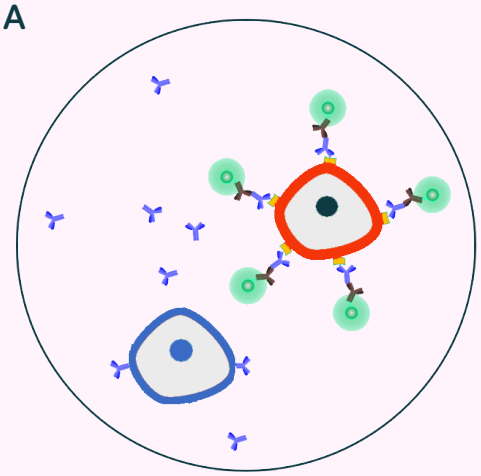
Detection and isolation of ASC based on antigen specificity, in the presence of target cells

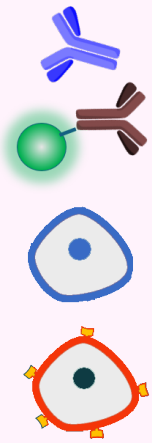
To determine whether presence of ASC could be detected in droplets based on binding of secreted antibody to its surface- expressed antigen, we used a hybridoma cell line that secrets anti-human EGFR mouse antibody. To demonstrate antibody binding to its target antigen, the hybridoma cells were stained with eFluor™ 450 and mixed with eFluor™ 670- stained A431 target cells (Figure 4A). Using the mouse- specific FRET probes, we confirmed that more than 50% of hybridoma cells secreted detectable levels of antibody following a 2- hour incubation in Cyto-Mine® Chroma (Figure 4B). Notably, staining hybridoma cells with eFluor™ 450 did not impair their secretion capability, as they exhibited a similar FRET profile to their unstained counterparts (data not shown).

The binding of the secreted antibody to the target cells was detected using an anti-mouse IgG-DL488 antibody. The hybridoma cells, target cells, and detection antibody were loaded into Cyto-Mine® Chroma® and incubated for 2 hours to allow secretion and binding of the antibody to its target antigen. Following the incubation, the droplet population was gated during sorting for the presence of hybridoma cells (eFluor™ 450+) and increase in detection antibody peak signal (DL488). A second gating step identified droplets containing target cells (eFluor™ 670+, Figure 4C). Using this sequential gating strategy, single droplets were isolated and dispensed into 96-well plates, and the accuracy of isolation was validated with fluorescence microscopy. Representative images from a well are shown in Figure 4D.

As expected, the dispensed droplet showed the presence of secreting cells, target cells and DL488 signal. It is worth noting that the DL488 signal is only seen on the target cells, to which the secreted anti-EGFR antibody would specifically bind (Figure 4D). These data clearly demonstrate Cyto-Mine® Chroma's ability to isolate secreting cells of interest by accurately detecting antigen-specific binding of the secreted antibody.

A





Primary antibody (secreted by ASC)

Secondary antibody conjugated with DL488

ASC labelled with eFluor450™

Target A431 cell line expressing EGFR labelled with eFluor670™

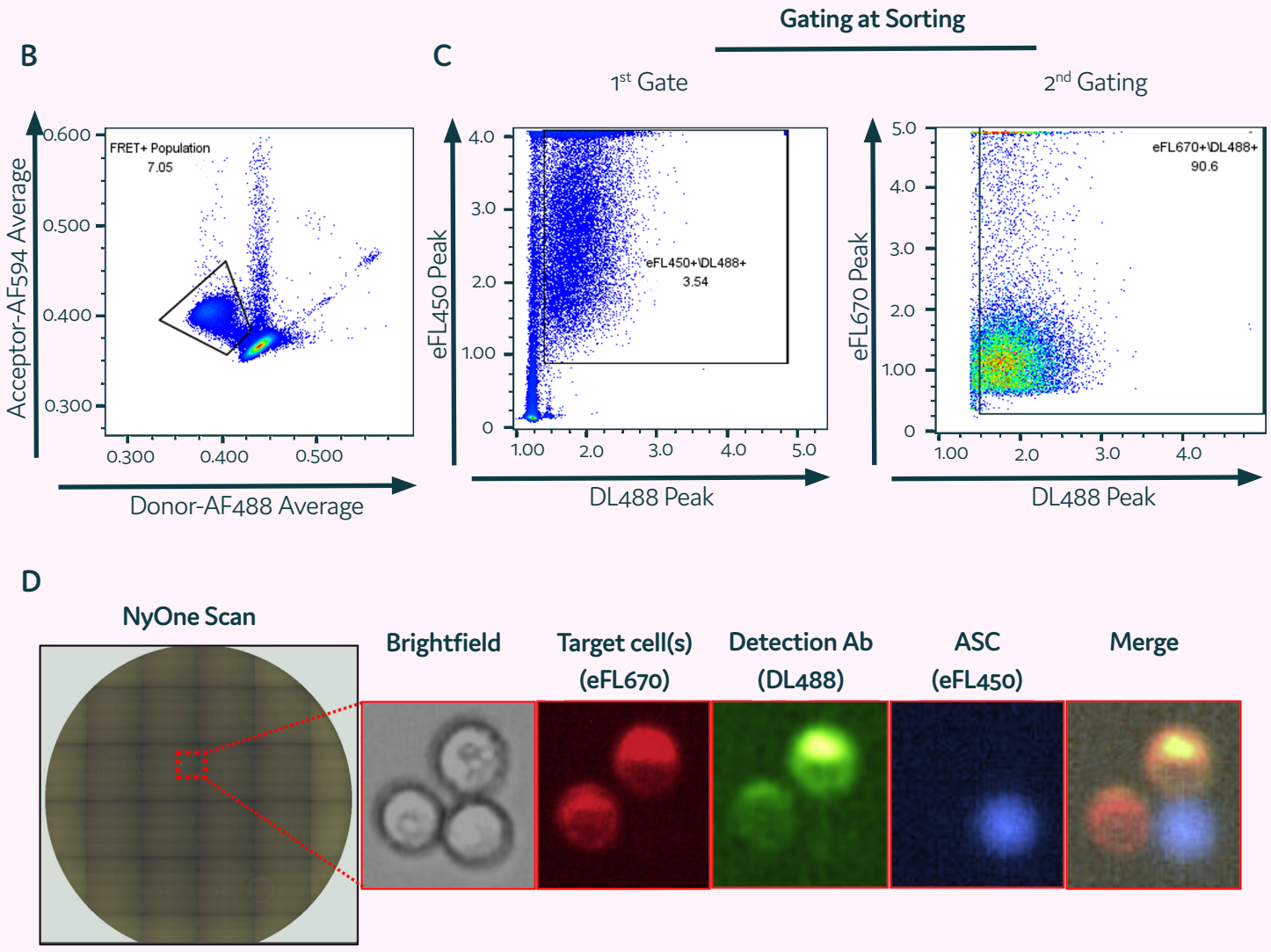


Figure 4. Detection and isolation of ASC based on antigen specificity, in the presence of target cells.
A) Schematic representation of surface binding assay design. B) FRET assay showing secretion of anti-EGFR ASC. C) Detection and gating strategy of 4 different colors in surface-binding assay. D) Microscopy validation of Cyto-Mine Chroma-based cells isolation.

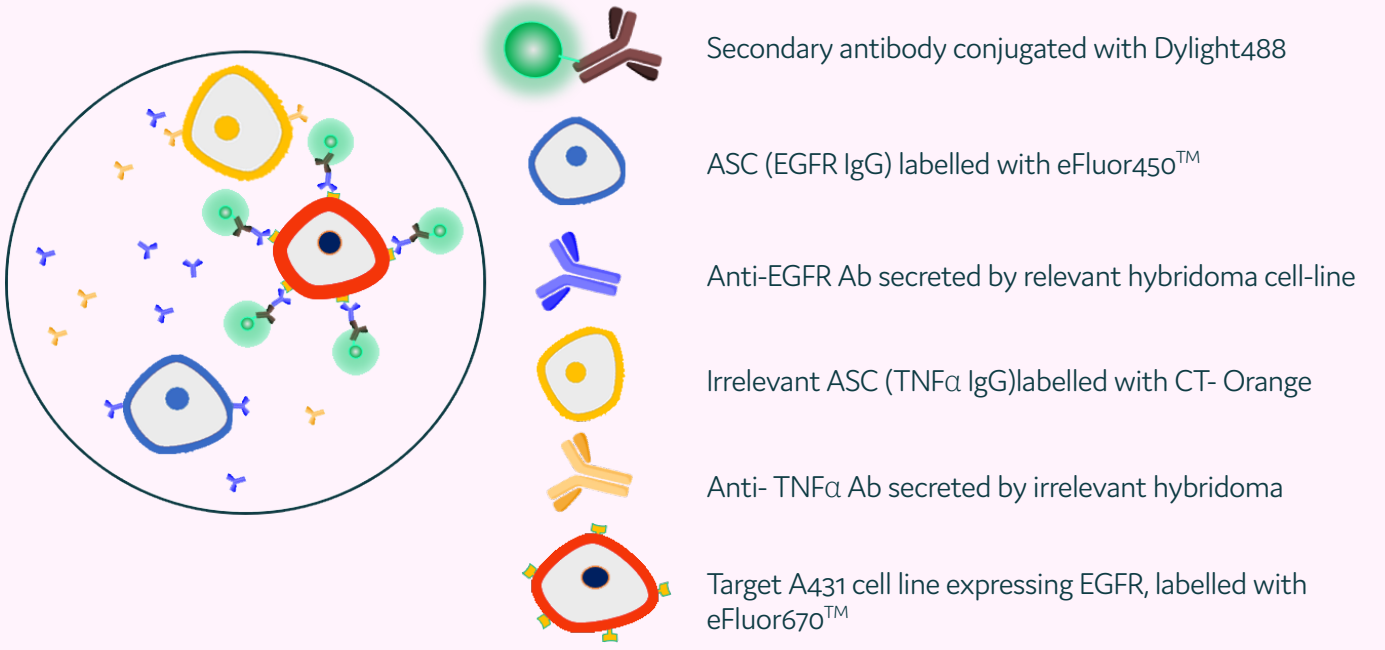
Detection and isolation of ASC based on antigen- specificity in AbD related workflows

To determine the performance of the system in an AbD- related workflow, we set up an assay, whereby a small number of secreting cells of interest are present within a larger population of irrelevant secreting cells. To this end we mixed the anti-EGFR antibody secreting hybridoma cells (stained with eFluor™ 450) with an anti-TNF-α Ab secreting hybridoma cell line (stained with CT- Orange) (Figure 5A). A431 target cells were stained with eFluor™ 670 as before and surface binding of the secreted antibody to its specific antigen was detected in Cyto-Mine® Chroma as described in Figure 4. The two types of secreting cells were mixed in two different ratios, either in equal amounts (ratio 1:1, Figure 5B), or at 1:100 (anti-EGFR : anti-TNF-α secreting cells, Figure 5C). The latter imitates an AbD workflow, where the ASC of interest are rare.

As depicted in Figure 5B and Figure 5C, two gating strategies were applied sequentially. First gating ensured the inclusion of picodroplets containing target cells (eFluor™ 670^{+}) as well as an increase in detection antibody binding (DL488^{+}). The second gating ensured the presence of the anti-EGFR antibody secreting cells (eFluor™ 450^{+}) but excluded the irrelevant secreting cells (CT- Orange^{+}). As expected, the 1:100 dilution of the relevant secreting cells resulted in a lower number of positive binding events compared to the 1:1 dilution (4% vs 0.4%, first gating, Figure 5B and Figure 5C). However, the inclusion of a second gating, led to a similar enrichment of the relevant secreting cells (Figure 5B and Figure 5C). Importantly, the isolation accuracy, determined by presence of the expected combination of cells and fluorescent signal (antigen binding of the secreted antibodies), remained high even in the presence of increased numbers of irrelevant antibody-secreting cells (Table 1).

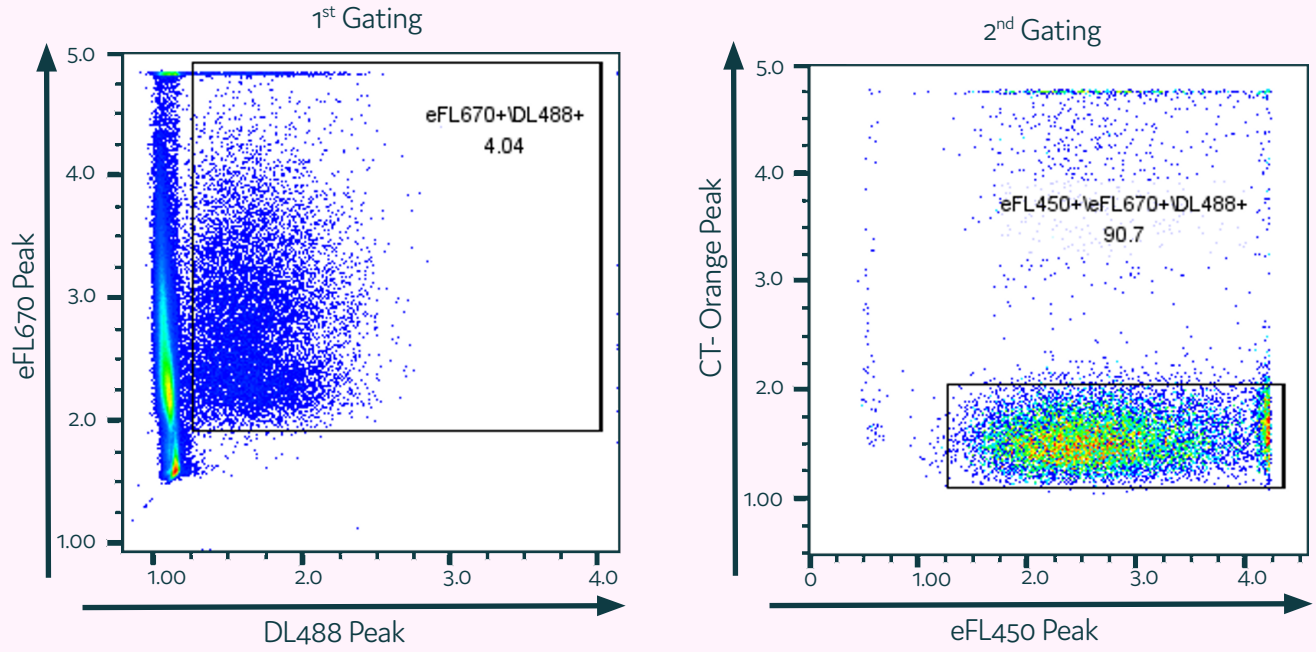
As demonstrated in Figure 5B and Figure 5C, following the first gating, a population of droplets were positive for both CT- Orange stained cells, as well as the eFluor™ 450^{+} cells. To ascertain that these droplets were indeed positive for the relevant antibody secreting cells (eFluor™ 450 stained cells) and would therefore account for the positive binding of the detection antibody, we further analyzed the sorting data. Indeed, further analyses of droplets positive for both the CT- Orange stained cells as well as detection antibody also showed the presence of eFluor™450 positive cells (Figure 5D; 99.2%). Microscopy data of this population following dispensing, further confirmed the co-encapsulation of relevant and irrelevant ASC together with DL488- labelled target cells (Figure 5E).

A



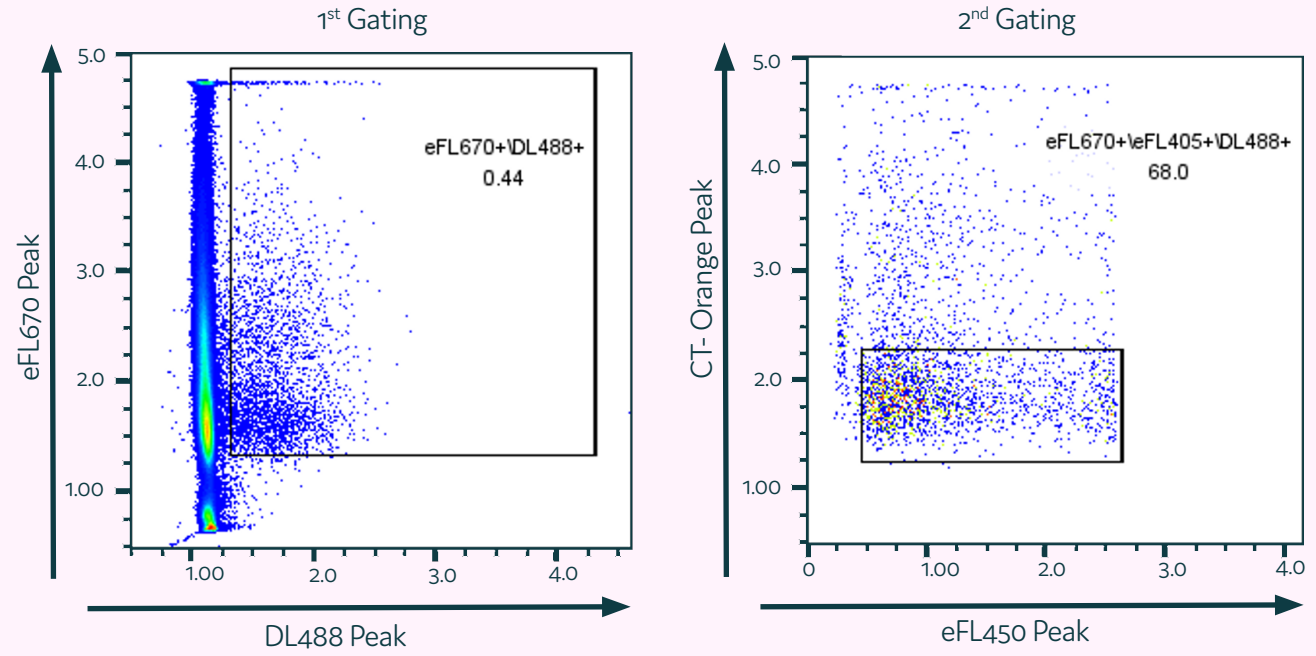
B

Cells ratio 1:1



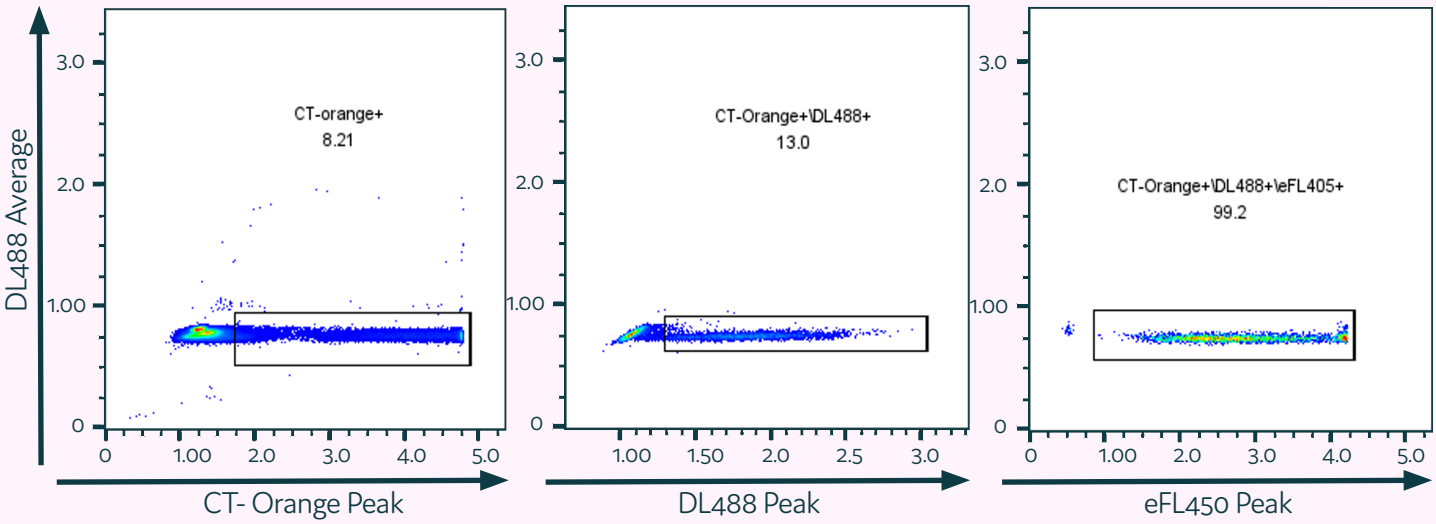
C

Cells ratio 1:100



D

Utilizing Cyto-Mine® Chroma in cell isolation, based on surface-binding of secreted antibodies



E

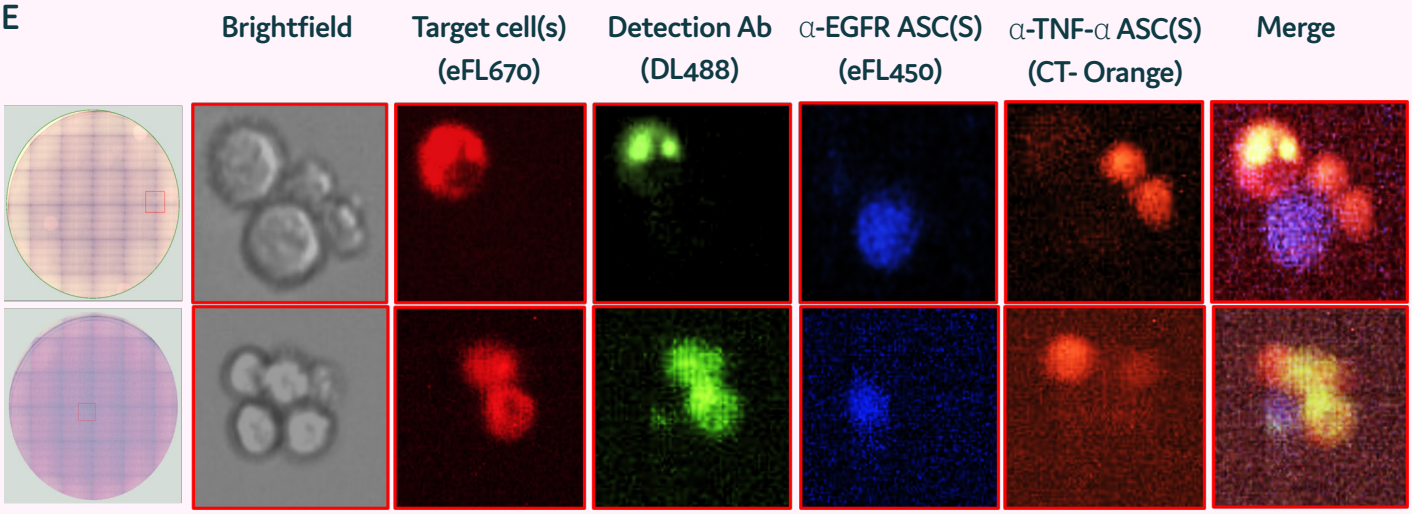


Figure 5. Detection and isolation of antigen- specific ASC in the presence of irrelevant antibody secreting cells on Cyto-Mine® Chroma. A) Schematic representation of surface binding assay. B) Gating strategy for sorting of anti-EGFR secreting cells from sample with ratio 1:1 of EGFR/TNFα cells. C) Gating strategy for sorting of anti-EGFR secreting cells from sample with ratio 1:100 of EGFR/TNFα cells. D) FlowJo analysis of binding events in the population of irrelevant secreting cells (anti-TNFα, stained CT- Orange). E) Microscopy validation of cells dispensed following 4-color surface binding secretion assay.

Ratio anti-EGFR : anti-TNFα Ab secreting cells	Isolation accuracy
1:0	100%
1:1	97.1%
1:100	96.85%

Table 1. Isolation accuracy of antibody secreting cells, based on surface- antigen binding assay. Droplets from experiments conducted using different ratios of anti-EGFR vs anti-TNFα antibody secreting cells (Figures 4 and Figure 5) were dispensed into 96-well plates (2 plates/experiment). Isolation accuracy was calculated as specified in the Methods section. Mean value of 2-3 experiments is presented.

Conclusion

The data presented here demonstrate the Cyto-Mine® Chroma system's multiplexing ability to accurately detect and isolate cells of interest using picodroplets. We show that this multi-laser instrument is capable of sequential gating, accurate sorting and dispensing based on fluorescent signal detected from encapsulated cells or secreted antibody. Moreover, we demonstrate that in more complex secretion assays, where relevant cells are only a fraction of the total cell population, it is possible to accurately isolate droplets containing rare cells of interest based on antibody-antigen binding.

Detection accuracy:

The platform reliably detected localized (antibody-antigen binding) as well as dispersed (IgG secretion, FRET signal) fluorescence. When combined with fluorescently stained cells, the platform accurately distinguished between different cell types based on their specific staining.

Sorting accuracy of gated population:

The platform demonstrated remarkable sorting accuracy and enrichment of target populations following single or sequential gating.

Multiplexing capability:

Cyto-Mine® Chroma demonstrates multiplexing capability by assessing and isolating rare secreting cells based on simultaneous detection of 4 different cellular markers.

References

1. Paul J. Mills and Christine T. Peterson, 2016. Multiplexing and Beyond in Biobehavioral Research.
2. Junfang Chen and Emanuel Schwarz, 2017. Opportunities and Challenges of Multiplex Assays: A Machine Learning Perspective.
3. Chen J. *et al.*, 2017. The Utility of Multiplex Assays for Identification of Proteomic Signatures in Psychiatry.
4. Edwards B.S. *et al.*, 2007. High-throughput cytotoxicity screening by propidium iodide staining.
5. PO Krutzik and GP Nolan, 2006. Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signalling profiling.
6. Robert A.B. *et al.*, 2021. Development and Validation of a Multiplex, Bead-based Assay to Detect Antibodies Directed Against SARS-CoV-2 Proteins.
7. Edwards B.S. *et al.*, 2007. High-throughput flow cytometry for drug discovery.
8. Brouzes E. *et al.*, 2009. Droplet Microfluidic Technology for Single-Cell High-Throughput Screening.
9. Dimitris Josephides *et al.*, 2019. Cyto-Mine®: An Integrated, Picodroplet System for High-Throughput Single-Cell Analysis, Sorting, Dispensing, and Monoclonality Assurance.

Contact Us

Interested in how our technology including Cyto-Mine® Chroma can help transform your workflows? Simply email us at sales@fluidic.com and we'll get back to you!

Notes

Notes

sphere bio

work small, think big

sales@fluidic.com
spherebio.com

The trademarks used herein are the property of Fluidic Sciences Ltd or their respective owners.

For research and development purposes only.
Product specifications subject to change without notice.
Content ©Fluidic Sciences Ltd.

Fluidic Sciences