Cyto-Mine® as a reliable platform for Antibody Discovery (AbD) workflows

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Introduction

Discovery and development of antibodies able to combat numerous diseases via different molecular mechanisms are a key and rapidly growing field of drug discovery, offering effective and precise therapeutic and diagnostic solution (1). Despite numerous methods aiming to detect and isolate desired targets, this field still presents with considerable challenges. Thus, methods and tools focusing on detection and isolation of rare single cells are constantly evolving to deliver the best solution for this challenge (2-5). In the current study we aimed to replicate a real-life scenario of AbD workflow, where a small subpopulation of single cells secreting antibodies of interest is found within a large population of other antibody-secreting cells. We then used Cyto-Mine®, the first fully automated integrated platform based on picodroplet technology (6), for identification, isolation and subcloning of these rare antigen (Ag)-specific single cells. Furthermore, we evaluated the use of Cyto-Mine® in subcloning to enrich small populations of antigen-specific cells, thus demonstrating its application in all types of AbD workflows.



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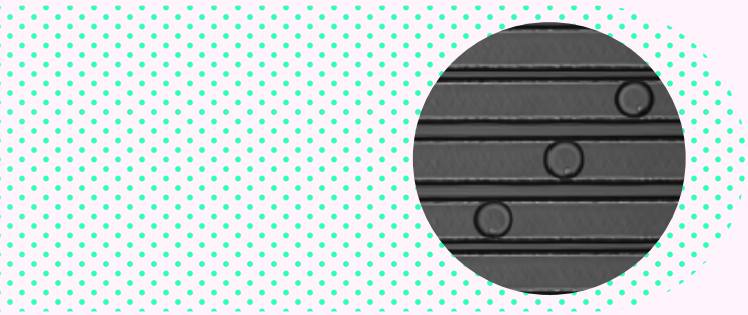
Methods

Ag-specific Fluorescence Resonance Energy Transfer (FRET) assay (Sphere Bio Application Note 4) was previously developed to provide specific and sensitive detection of cells secreting antibodies against a single antigen (in this case anti-TNFα). This method directly evaluates secretion by using FRET, whereby the FRET donor (AF488 labelled TNFα) is paired with a FRET acceptor (AF594 labelled anti-mouse IgG). The two FRET probes will bind and form a three-molecule complex only in the presence of anti-TNFα specific mouse IgG, providing sensitive and highly specific detection of secretion at single cell level.

Spike-in experiment refers to the experiment aiming to mimic AbD campaign where a very small population of cells secreting antibody of interest is present in a much larger population of cells secreting other, irrelevant antibodies. In this set of experiments, we used a small decreasing number of cells secreting anti-TNFα specific antibody (target cell line) mixed with a large, fixed number of other irrelevant hybridoma, shown to secrete similar levels of a different mouse IgG (control cell line).



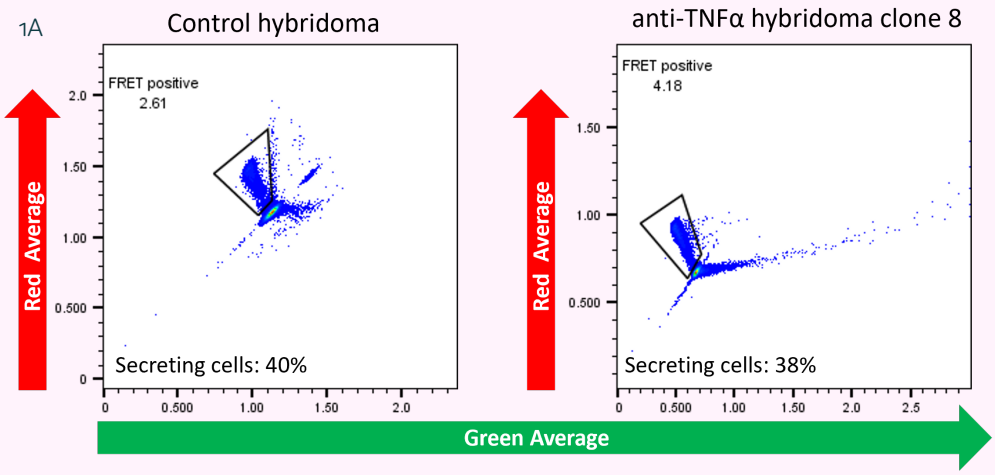
Results and Discussion

Detecting rare population of secreting cells

The sensitivity of Cyto-Mine® in detecting rare cell populations was determined using a spike-in experiment. First, we evaluated the secretion rate of the two hybridoma cell-lines (anti-TNFα secreting- target and irrelevant control cells) and confirmed that both had similar proportion of IgG- secreting cells, as judged based on the FRET positive signal and the number of cells input to the experiment (Figure 1A;

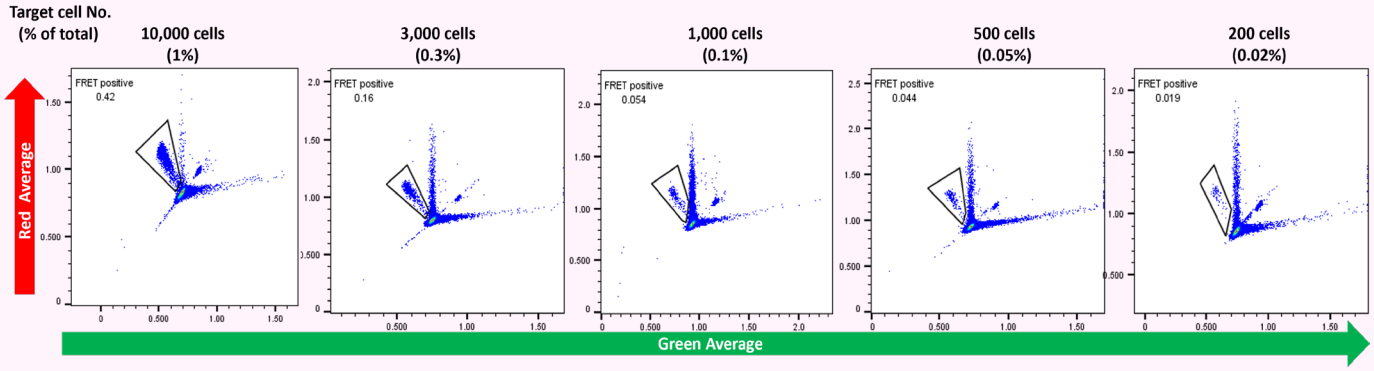
Control hybridoma: 40%; anti-TNFα secreting hybridoma: 38%). Next, target cells were fluorescently labelled with CellTracker Deep Red, undetectable by Cyto-Mine® (data not shown). Labelled secreting target cells were mixed at decreasing numbers with 1 x 10^6 control cells. Successful labelling (Figure 1B, left, unmixed population) and detection of these cells following mixing with control unlabelled cells was determined using Fluorescent Microscope (Figure 1B, right). Figure 1C shows scatterplots of decreasing number of secreting target cells detected on Cyto-Mine® using Ag- specific FRET assay. Data demonstrates clear reduction in percentage of FRET-positive gated population, correlating with expected target cell number. These rare hits were sorted and dispensed, using Cyto-Mine® platform, into 96-well plates.

The plates were then imaged using NYONE plate imager to detect CellTracker Deep Red- labelled target cells. Figure 1D shows two examples of dispensed droplets, both containing one labelled cell, illustrating successful isolation of rare secreting cells based on Ag-specific FRET signal. All experiments were analysed for the presence of labelled cells (Figure 1E) in dispensed plates and statistical analysis revealed a very high level of accuracy (95-100%), demonstrating Cyto-Mine® and FRET assay as robust and selective tools in detection, selection, and isolation of AbD targets. Using images provided by Cyto-Mine® during dispensing, it is possible to identify wells which do not contain single cells (such as Example 2 in Figure 1D), thus ensuring polyclonal wells

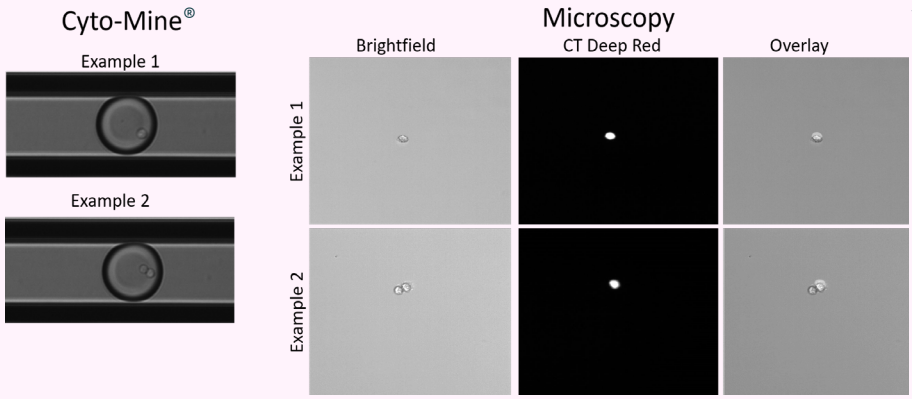


are not selected for downstream processing if required.

1C



1D



1E

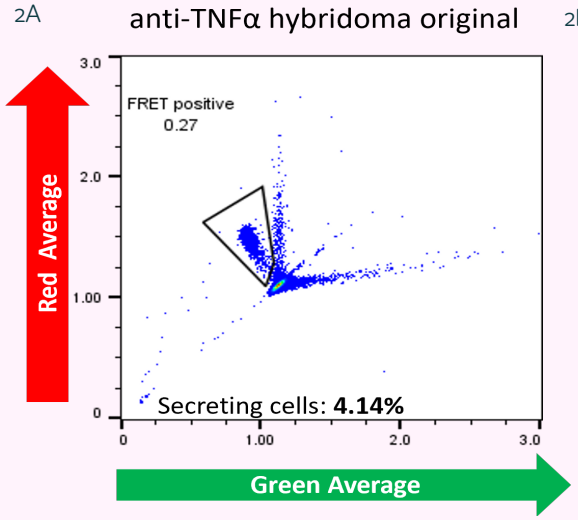
Exp#	Target cell number	Positive cells (%)	Isolation accuracy (%)
1	10000	1	97.92
2	3000	0.3	100
3	1000	0.1	96.88
4	500	0.05	100
5	200	0.02	95.83

Figure 1: Cyto-Mine® is a powerful tool in detection and isolation of rare secreting cells. A. Cyto-Mine® scatter plots show secretion of IgG by irrelevant hybridoma cell-line (left) detected by general IgG FRET assay. No signal was detected with TNFα-specific FRET assay (not shown). Secretion rate of TNFα- specific cell line (right) was detected by using Ag specific FRET assay. Similar secretion rate was detected with general mouse IgG FRET assay (data not shown). The proportion of secreting cells was calculated by using Poisson distribution formula for 0.15e6 and 0.25e6 input cells, respectively. B. Cells secreting Ag- specific IgG were pre-stained with CellTracker Deep Red (left) and then spiked in $1x10^6$ irrelevant, unstained cells (right). C. Cyto-Mine® scatterplots of decreasing number of Ag-specific secreting cells (number indicated in top of scatterplot) spiked into fixed number ($1x10^6$) of irrelevant secreting cells. D. Cells dispensed from Cyto-Mine® (left 2 examples) into 96-well plates, were imaged in plates for presence of CellTracker Deep Red staining (right). E. Table summarizing conducted experiments and proportion of wells containing at least one pre-stained cell out of total dispensed wells (Isolation Accuracy). CT: CellTracker Deep Red. Statistical analysis was done on 3 dispensed plates with 1000-10000 input range, while for 200-500 input cells all detected positive events were sorted and dispensed.

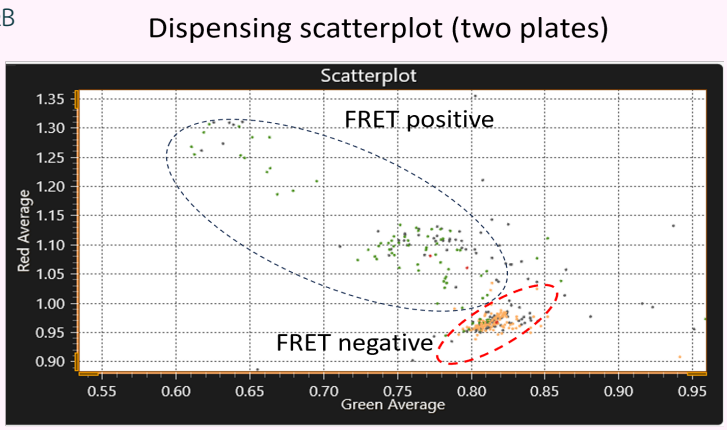
Subcloning/enrichment of rare populations

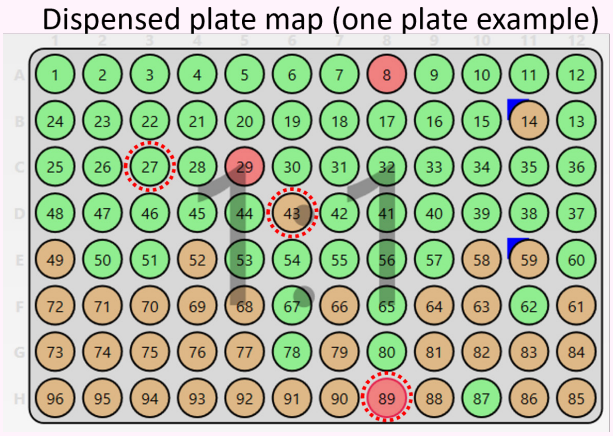
So far, we have demonstrated AbD workflow that imitates experiments performed on rare secreting cells, to mimic use of primary cells, where the isolated target is usually dispensed into PCR plates for downstream analysis. However, AbD workflow can also involve generation of immortalised B cells (myeloma-B cell fusion) followed by isolation, subcloning and outgrowth of high performing clones. To this end we used a hybridoma cell line with reduced recurrence of antibody secreting cells (4.14%; Figure 2A). Cyto-Mine® in combination with Ag- specific FRET assay was used to detect and isolate droplets containing FRET- positive cells. FRET-negative population, which are indicative of cells not secreting Ag-specific antibody or empty droplets, were used as control and sorted together with FRET- positive droplets. Dispensing scatterplot and plate map show that only FRET- positive population of droplets contained cells (Figure 2B), demonstrating specificity of the FRET assay. Next, dispensed cells were cultured for two weeks to allow outgrowth, resulting in 46.25% of single cells outgrowth (Figure 2C).

2A



2B





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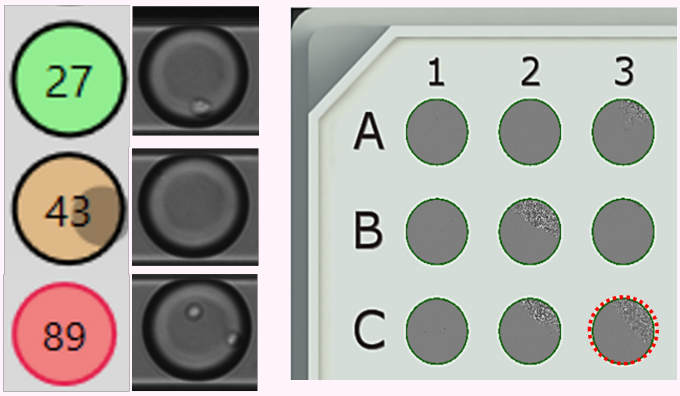
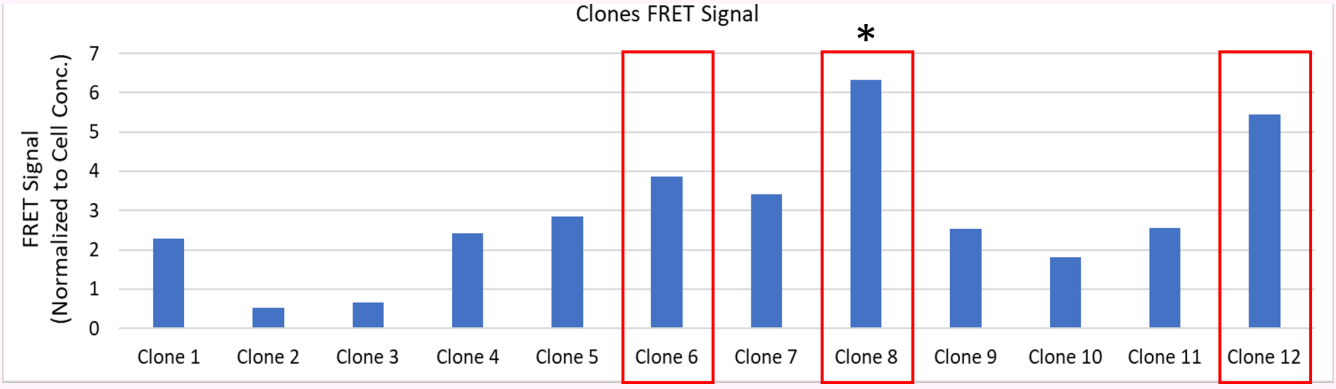


Twenty-four clones were chosen for upscaling from 96- well plate into 24- well plate, based on their growth. Further upscaling was performed for the top 12 clones and titre was determined using plate reader-based FRET assay. Figure 2D demonstrates normalised data (titre normalised to cell confluency) for the chosen clones, with those showing highest level of production further highlighted (red box). The enrichment of the culture of highest producing clone, Clone 8, was further verified using Cyto-Mine®. The data from Cyto-Mine® indicates that subcloning approach resulted in a 13-fold increase in secreting population (Figure 2E, from 4.14% to 55.8%), thus emphasizing the relevance and specificity of Cyto-Mine® in hybridoma-based AbD application.

2D



2E

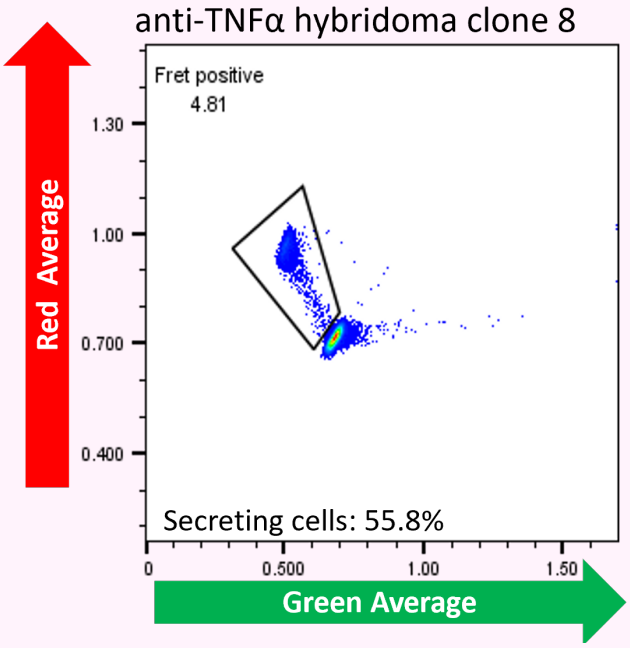


Figure 2: Subcloning of Hybridoma cell-line to obtain high-secreting cellular model. A. Shows secretion rate of Ag- specific cell-line detected on Cyto-Mine® by using Ag- specific FRET assay (<5% secreting population). Similar secretion rate was detected with general mouse IgG FRET assay (data not shown). Proportion of secreting cells was calculated by using the Poisson distribution formula for 0.15e6 input cells. B. Gated droplets (FRET- positive) were sorted and dispensed into 96-well plate. Plate map based on Cyto-Mine® imaging analysis depicts green wells: monoclonal droplets (1 cell only), red wells: polyclonal droplets (>1 cell), yellow wells: empty droplets. The colours are coordinated with the scatter plot (left), where FRET positive population is encircled in black shape and FRET negative is encircled in red shape. Example images are shown on the right. C. Dispensed single cells were cultured to grow into colonies. Twenty-four colonies originating from monoclonal droplets (single cells outgrowth) were transferred to 24-well plate for subsequent growth and analysis. Exemplar monoclonal colony is encircled by red frame. D. Supernatant from twelve colonies were analysed for secretion using Ag- specific FRET assay together and BMG Labtech Clariostar Plus plate reader. Example of best producing clone is highlighted by an asterisk. E. Cyto-Mine® analysis of chosen clone with Ag- specific FRET assay. Proportion of secreting cells was calculated by using Poisson distribution formula for 0.15e6 input cells.

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