Application Note 14

Picodroplet Re-injection: Cyto-Mine® as a flexible platform for a wide range of applications

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Introduction

Cyto-Mine®, the world's first fully automated platform using picodroplet technology with droplet sorting and dispensing capabilities, successfully demonstrated clone selection and monoclonality assurance in several high-throughput single-cell analysis applications (Josephides, D et al., 2020).

Here, we investigate the expansion of Cyto-Mine® applications beyond established Antibody Discovery (AbD) and Cell Line Development (CLD) workflows. We explore the concept of re-injection, wherein externally generated droplets are loaded into the Cyto-Cartridge® for further detection, sorting and dispensing rather than generating the droplets using the cartridge (Figure 1).

Standard Cyto-Mine® Run	Reinjection Cyto-Mine® Run
Aqueous Sample Preparation	Aqueous Sample Preparation
	External Droplet Generation
	External Droplet Incubation
Load Aqueous Sample into Cyto-Cartridge	Load Emulsion Sample into Cyto-Cartridge
Cyto-Mine® Droplet Generation	Emulsion Loading
Incubation	
Sorting	Sorting
Dispensing	Dispensing



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work small, think big.

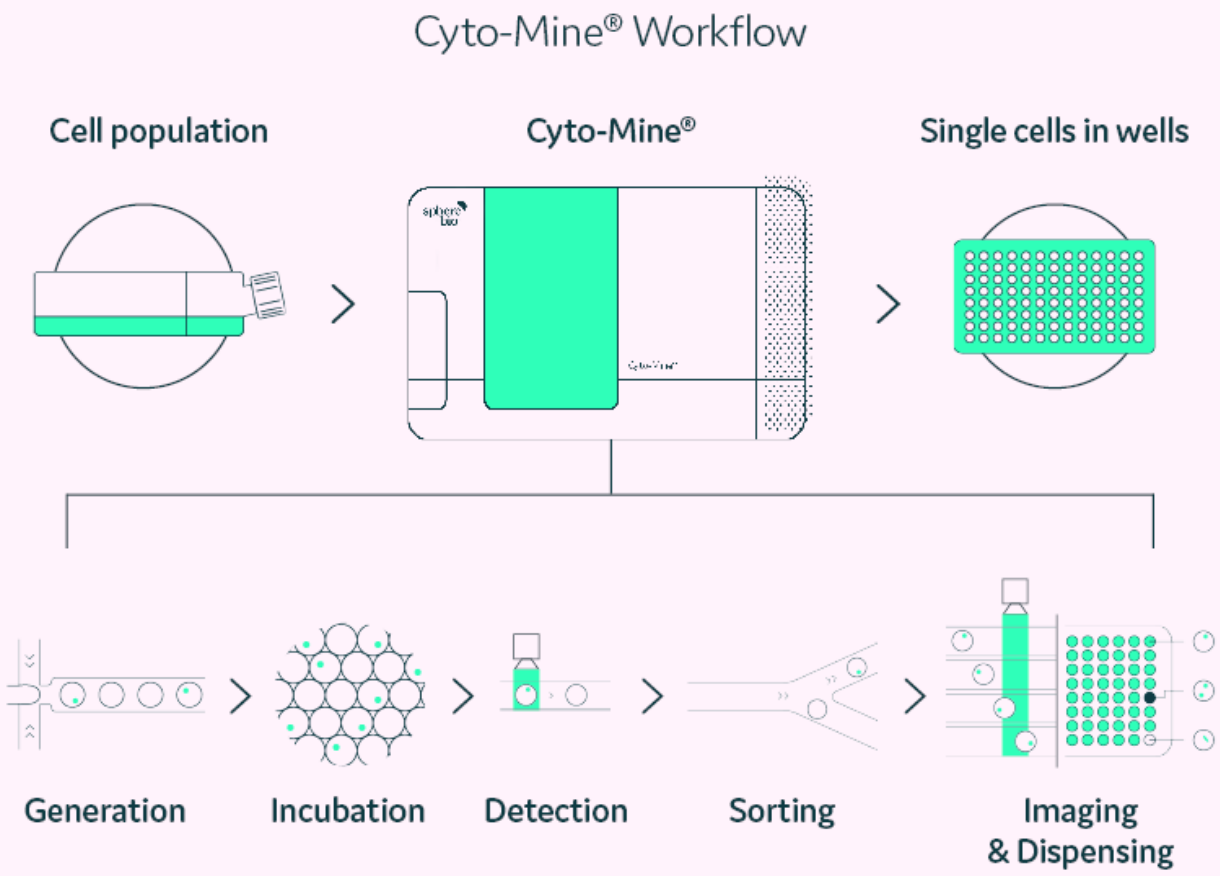

Figure 1. A Cyto-Mine® workflow. Integration of single cell screening, sorting, isolation, and verification using a fully integrated microfluidic process.

We examine how droplets containing cells can be generated offline, incubated for extended periods, and then assess the integrity of these droplets when re-introduced to Cyto-Mine® for productivity assessment and selection. This novel application broadens Cyto-Mine®'s capabilities to encompass more complex workflows with prolonged incubation times, incorporating mixed pools of droplets, or post-encapsulation droplet manipulation. Some examples of potential applications benefiting from re-injection include:

- Applications requiring long incubation times followed by clonal selection, such as:
    - Productivity assessment of slow secreting cells.
    - Cytotoxicity and mode of action assays – requiring long incubation time of co-encapsulated secreting cells and target/reporter cells.
    - Transfection or transduction of single cells in droplets prior to selection.

- Applications requiring complex set-up of droplet contents with varying reagent types or concentrations:
    - Quantitative and semi-quantitative analysis of secreted proteins using Cyto-Mine® - generating standard curves using externally generated droplets containing titration of protein of interest.
    - Performance evaluation of Cyto-Mine® – using pools of droplets containing different concentrations of fluorescent proteins to assess measurement limit of detection and sensitivity.
    - Assay development for Cyto-Mine® – for example testing different concentrations of assay reagents to assess performance.
- Applications requiring significant manipulation during droplet generation:
    - Drug candidate selection – using picodroplet fusion techniques to inject different types, concentrations, or combination of drugs with encapsulated cells to determine effects such as cytotoxicity with viability assays.
    - Mode of action/functional assessment of encapsulated cells – controlled co-encapsulation of secreting cells with antigen expressing cells to determine the mode of action.

To demonstrate that re-injection is compatible with a Cyto-Mine® workflow, we verified the stability of the droplets following off-line generation and extended incubation outside the Cyto-Mine®. We show here that these droplets remain stable throughout the Cyto-Mine® workflow. Moreover, our findings indicate that cross-contamination of encapsulated proteins, between droplets co-incubated off-line, remains low. These results indicate that Cyto-Mine® can deliver an extended range of applications by supporting more complex workflows where droplets are generated separately for subsequent fully automated assessment, selection and dispensing.

Materials and Methods

Sample preparation

Detection of re-injected droplets in Cyto-Mine®

For initial testing and optimization experiments, Encapsulation Media containing IgG specific FRET probes (2 µg/mL of Goat anti Mouse IgG–AF488 and 20 µg/mL of Goat anti-Mouse–AF594) was used to ensure detection of droplets within Cyto-Mine®. Droplet size and stability as well as protein leakage were examined.

FRET Validation Assay in droplets

To further assess droplet stability, a FRET validation assay was conducted using a recombinant Mouse IgG antibody. The recombinant antibody was titrated (2.50 µg/mL – 0.31 µg/mL, 2- fold dilution, 4– point titration) in the presence of anti-mouse IgG FRET probes (2 µg/mL of Goat anti Mouse IgG–AF488 and 20 µg/mL of Goat anti-Mouse–AF594). The titrated Mouse IgG antibody (4 concentrations) and Control (no antibody) were encapsulated separately using the Pico-Capture® platform to generate 5 different droplet populations, as discussed in Off-line Droplet Generation below. All droplets were collected into the same Eppendorf tube to minimize manual handling, reducing risk of droplet breakage. The Eppendorf tube was then placed in a static incubator, 6% CO_2, 37°C for up to 48 hours prior to placing into Cyto-Mine®.

Off-line droplet generation

Droplets were generated off-line using an external picodroplet generation platform (Pico-Capture®) with Droplet Generation Biochips (Sphere Bio, C103). Cyto-Surf® B was used as the carrier oil to ensure identical droplet composition as the Cyto-Mine® platform.

Off-line generated droplets were 450 pL and 95 µm in size, with variation maintained within a standard deviation of 30 pL / 2 µm. This range of droplet sizes aligns with the droplets generated on the Cyto-Cartridge® during a standard run and was used to minimize the likelihood of any issues with subsequent sorting and dispensing in the Cyto-Cartridge® after reinjection.

Droplet stability

Stability of droplets during off-line incubation

To assess stability of off-line generated droplets, 10 µL of emulsion was sampled at different timepoints (0-hours, 24-hours and 48-hours). This emulsion was loaded onto a C-Chip disposable haemocytometer (NanoEnTek Ref: 631-1098) and examined under a microscope (EVOS M5000 Imaging System), using x10 magnification objective, to determine droplet stability.

Stability of droplets during Cyto-Mine® workflow

Off-line generated droplets were re-injected into Cyto-Cartridge® and subjected to sorting and dispensing. Data generated during Cyto-Mine® run was extracted and analysed by FlowJo (v10.10.0) to determine stability of the droplets throughout the workflow. Stability was determined by assessing 'intra-droplet width' for each droplet passing through the channel. Intra-droplet width is defined as the time delta for each droplet as the fluorescence signal rises and falls and thus gives a measure of the individual droplet size within the Cyto-Cartridge®. Each droplet is numbered – called 'sample number' – so the intra-droplet width can be plotted as a function of sample number and the overall size stability assessed as shown in Figure 4. For each condition, a fixed given percentage of the population was selected (>99%) and the range of intra-droplet width calculated to give a direct comparison of size variation between conditions.

Re-injection of droplets into Cyto-Mine®

For re-injection of off-line generated droplets into Cyto-Mine®, a number of configuration parameters need to be set at Pre-Experimental Configuration on the Cyto-Mine® Studio Suite software. Specifically, the "Generation Type" is changed from 'Regulate Size' to 'Constant Pressure' during Experimental Configuration set up. While the 'Regulated Size' setting ensures consistent generation of Picodroplets with uniform size during a standard Cyto-Mine® run, 'Constant Pressure' enables consistent pressure within the sample loading chamber, allowing off-line generated droplets to pass through the generation junction at a stable rate.

About 1,100 µL of emulsion was carefully loaded into Cyto-Cartridge® to ensure minimal breaking of generated droplets. During these experiments, "Approximate Number of Generated Picodroplets" was set to '1 Million'. Standard Run and Re-injection Run settings can be seen in Table 1.

	Standard Run	Re-injection Run
Generation Type	Regulated size	Constant
Approximate Number of Generated Picodroplets	1, 1.5 or 2 million	1, 1.5 or 2 million
Incubation time [min]	Depending on assay	0

Table 1. Experimental Configuration Settings for Standard Run and Re-injection Run.

During sorting, the scatterplot axes were set to 'Green Average' versus 'Red Average' and a narrow gate was defined going through the middle of the main populations of detected droplets as shown in Figure 6. This gating strategy ensures the same number of droplets were selected from each population (in this case, 15,000 droplets) and deliberately aims to isolate consistently-sized droplets for dispensing, as average fluorescent signal is a function of droplet size. Finally, sorted droplets were assessed for droplet stability (as described in Droplet Stability above) and signal level consistency during the standard Cyto-Mine® dispensing process.

Re-injection of droplets into Pico-Mine®

Droplets were carefully loaded into a 1 mL syringe and run on the Pico-Mine® platform using a Pico-Sort™ Re-injection/Sorting BioChip (Sphere Bio, C202). Scatterplot was set to 'PMT1 Average' versus 'PMT2 Average'.

Results and Discussion

Droplet stability following off-line incubation

First, we evaluated whether off-line generated droplets are stable for prolonged period of times to support the re-injection application in Cyto-Mine®. To this end, droplets were generated to mimic those produced in Cyto-Mine® workflow in size and composition. Off-line generated droplets were incubated for up to 48 hours at 37°C, 6% CO_2, and were monitored under microscope to determine their stability. As shown in Figure 2, visually inspected droplets maintained consistent size and integrity, with minimal droplet fusion observed at all timepoints, confirming their compatibility with applications requiring longer incubations.

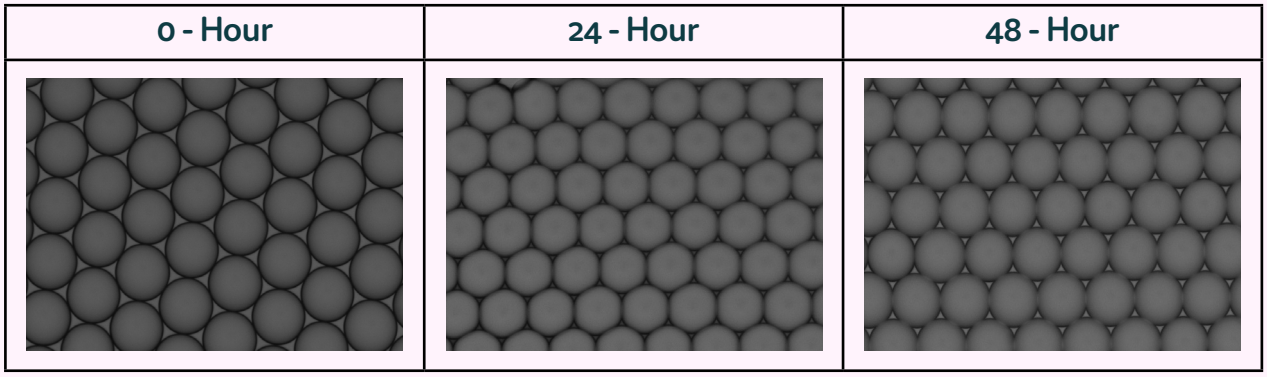

Figure 2. Off-line generated droplets are stable for prolonged periods of time . Droplets were generated as described in Methods section and incubated at 37°C, 5% CO_2 for up to 48h. Microscopy images were taken at 0, 24 and 48 hour time points.

Cyto-Mine® workflow using off-line generated droplets

So far, we have shown that droplets generated outside Cyto-Mine® are stable and comply with requirements for Cyto-Mine® workflow. To further assess the suitability of these in Cyto-Mine® we next loaded the emulsion into the sample loading chamber of a Cyto-Cartridge® and ran on the Cyto-Mine® (Figure 3).

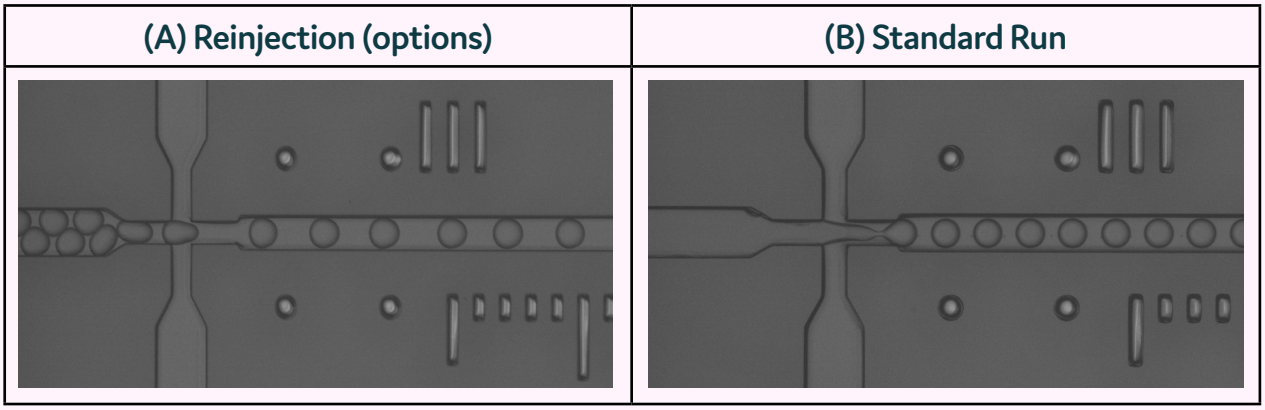

Figure 3. Reinjection of pre-generated droplets into Cyto-Mine®. (A) Reinjection of droplets (B) Standard droplet generation in Cyto-Mine® is shown. Droplets were either generated off-line by Pico-Capture® (A, Reinjection) or in Cyto-Mine® (B, Standard generation). Pre-generated droplets were loaded onto Cyto-Cartridge® and are visible in Cyto-Mine® before the Generation Junction. These are spaced out by the flow of oil through the Junction. In the Standard Cyto-mine® run, the droplets are generated downstream the Junction.

The standard downstream workflow of detection, sorting and dispensing was followed. During the re-injection Cyto-Mine® run, at sorting, 99.3% of the droplet population lie within an intra-droplet width range variation of 0.121 at 24 hours incubation and an increased range variation of 0.355 at 48 hours incubation. At dispensing 99.7% of the droplet population had an intra-droplet width range variation of 1.2 for both 24 and 48 hour incubation. This compares with an intra-droplet width range of 0.093 at sorting and 1.3 for dispensing in a standard Cyto-Mine® run.

This indicates there is more variation of droplet size at sorting using reinjection, but they remain within acceptable limits throughout the run, and tight gating eliminates this variation for dispensing. These are all within acceptable size and stability parameters throughout the Cyto-Mine® Runs, (Figure 4). Note, both 24-hour and 48-hour off-line generated droplets were generated and dispensed into the same Eppendorf, during removal of sample for the 24-hour Cyto-Mine® re-injection run it is possible this resulted in minor increase in droplet fusion for the 48 hour measurement.

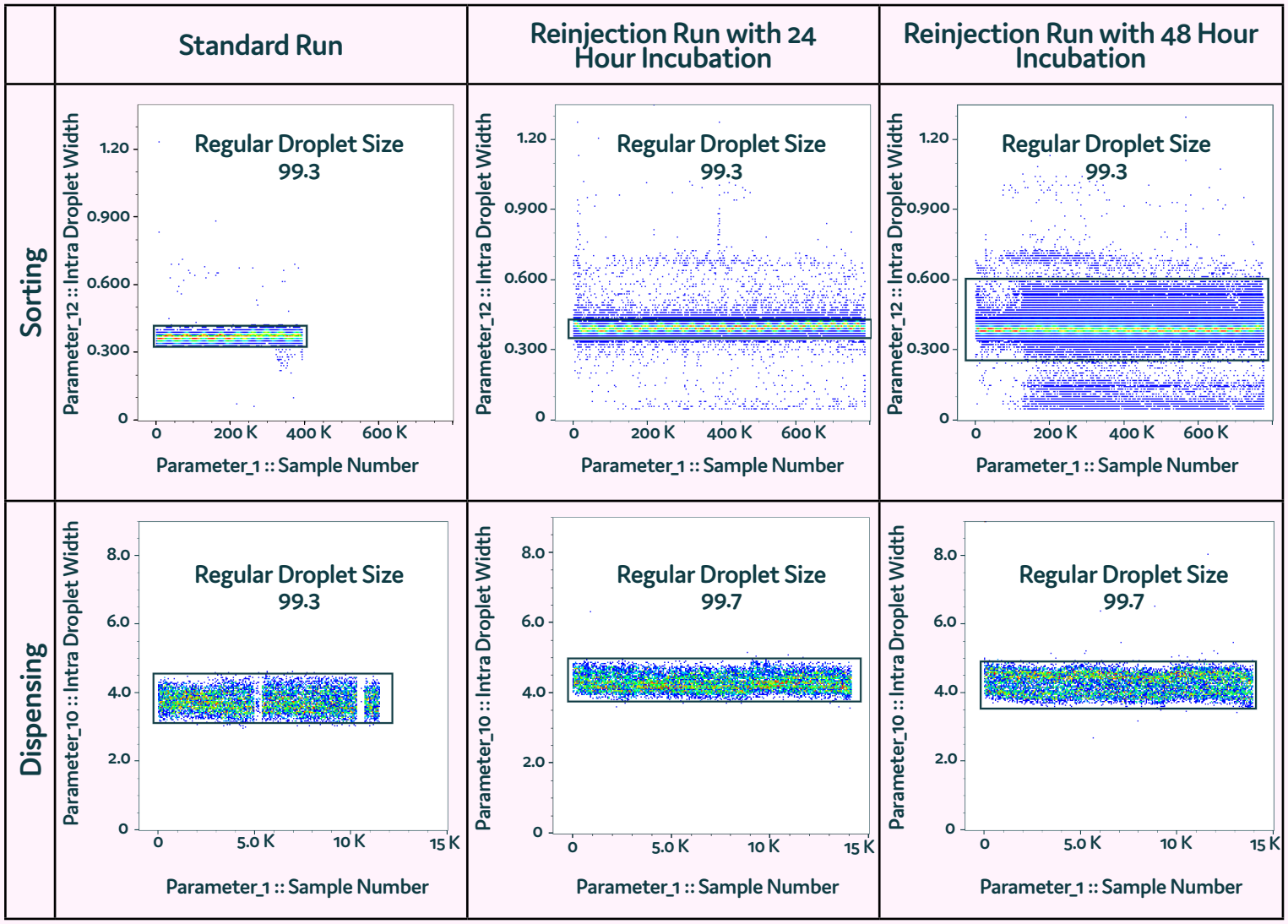

Figure 4. Droplet Stability throughout Cyto-Mine® workflow. Droplets from Standard or Reinjection Cyto-Mine® experiments were analyzed by FlowJo 10.10.0 Software for droplets stability (measured as droplet size vs sample number).

Assay development on Cyto-Mine® – an example of re-injection application

Detection of secreted protein by FRET assay is one of the main technological advances of picodroplet technology. FRET assay, primarily developed in plate reader requires further optimization and validation in picodroplets. This process involves encapsulation of fixed amount of FRET probes with increasing concentrations of target antibody in different subpopulations of picodroplets. These populations are then combined (SF app note 1 and 2 and Method section) and re-injected into microfluidics platform for detection and evaluation of assay properties. Here we adapted re-injection of off-line generated droplets into Cyto-Mine® to determine applicability of the new workflow to FRET assay development.

We determined the stability of fluorescent probes/protein within the off-line generated droplets, as well as droplet integrity, following 24- and 48-hours incubation. Using Cyto-Mine®, five distinct FRET populations of droplets were detected, correctly corresponding to four Mouse IgG concentrations and the Control sample (Figure 5, 24 hours). Increasing incubation time of droplets to 48 hours before re-injecting into Cyto-Mine®, did not affect the integrity of droplets (Figure 5, 48 hours).

This clear separation of FRET signal demonstrates that stability of the FRET probes was maintained throughout the experiment, further indicating that no cross-contamination of target IgG occurred. As a comparison, Figure 5 shows a similar assay run with the same process of droplet generation and then re-injection of droplets on the Pico-Mine® platform. Though different concentrations of Mouse IgG were used compared to the Cyto-Mine® re-injection, it is clear that they show distinct population of droplets, similar to that seen on the Cyto-Mine®, demonstrating compatibility of Cyto-Mine® re-injection in assay development.

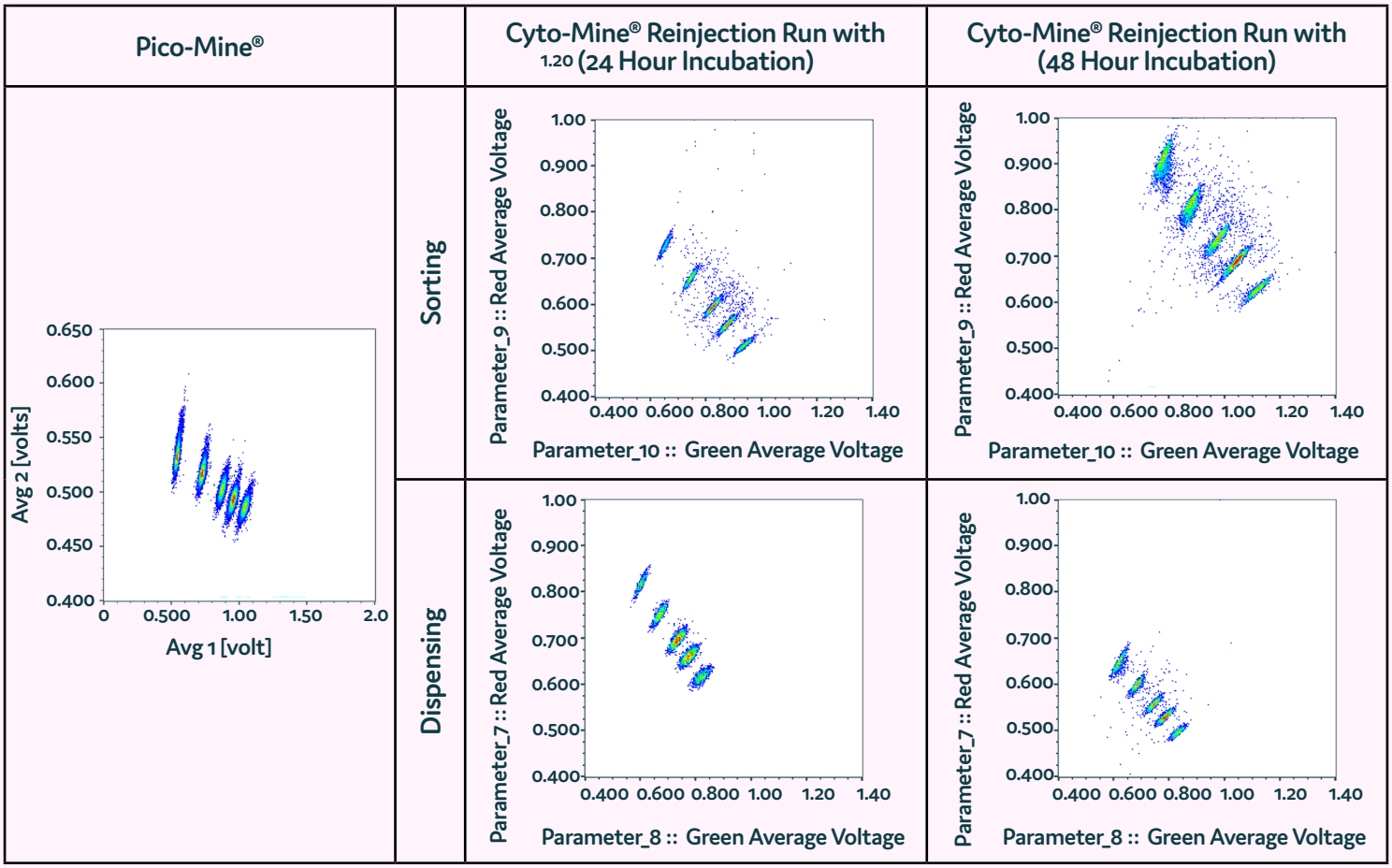

Figure 5. FRET assay development on Cyto-Mine®. Scatterplot FlowJo Analysis of FRET validation assay re-injected in Pico-Mine® (one time point) or Cyto-Mine® (two time points) and detected on both platforms. Five populations were detected during sorting and dispensing steps of Cyto-Mine® re-injection workflow.

Pico-Mine® Mouse IgG Concentrations: 0 µg/mL (Control); 0 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL and 20 µg/mL.

Cyto-Mine® reinjection Mouse IgG Concentrations: 0 µg/mL (Control), 0.3125 µg/mL, 0.625 mg/mL, 1.25 µg/mL and 2.5 µg/mL. It can be noted that the location on the scatterplots are different, this comes from gain settings used for each Cyto-Mine® reinjection run.

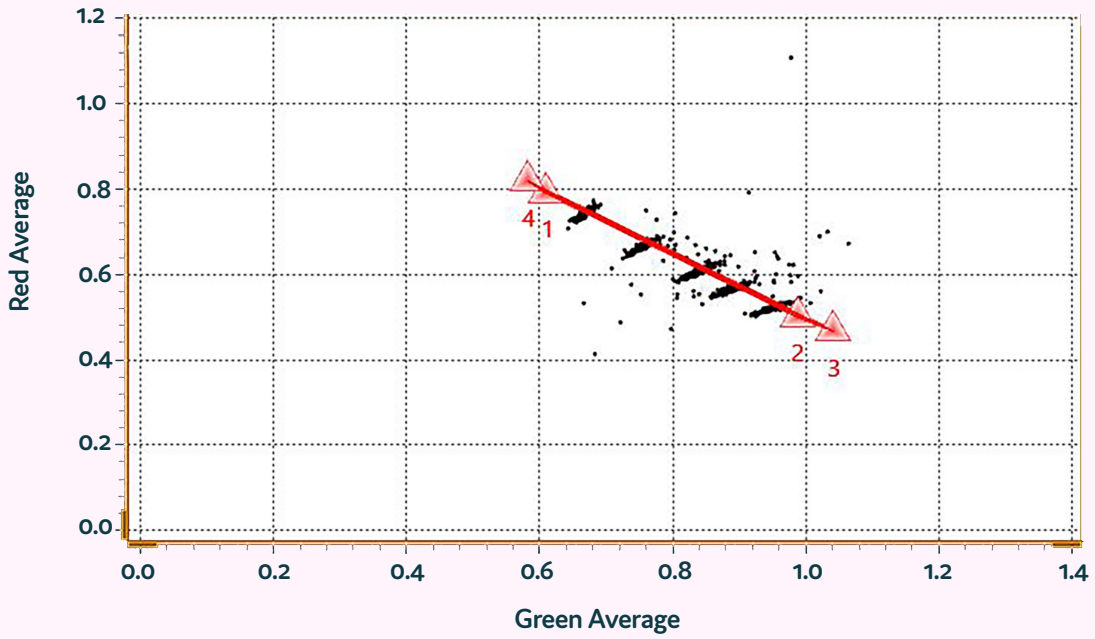

Figure 6. Scatterplot showing gating of different IgG FRET populations during FRET assay development reinjection run.

Conclusion

These experiments demonstrate the power of re-injection workflow on the Cyto-Mine® platform which allows for expanded range of Cyto-Mine® application. We took two workflows - long incubation and assay development - as examples of applications requiring re-injection and demonstrated that droplet stability and integrity is maintained throughout the entire workflow, reaching characteristics comparable to standard Cyto-Mine® or Pico-Mine® experiments.

References

1. Josephides, D *et al.* Cyto-Mine®: An Integrated, Picodroplet System for High-Throughput Single-Cell Analysis, Sorting, Dispensing, and Monoclonality Assurance. SLAS Technol. 2020 Apr;25(2):177-189.

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Notes

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