TECHNICAL NOTE

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Improved double-emulsion sorting using purpose-built software functionality for large particles

Summary

- Double-emulsion droplets can encapsulate live cells for analysis and sorting via flow cytometry.
- With the new software for Large Particle Sorting on the Sony MA900 Cell Sorter, we achieve over 90% sort recovery of double-emulsion droplets.
- The high sorting recovery and purity is maintained even when the double-emulsion droplets contain live immune cells.

Introduction

Conventional cell analysis by flow cytometry of surface markers and intracellular molecules fails to detect important biomarkers such as secreted proteins. To understand the full functionality and phenotype of cells, reliable methods for confining and analyzing single cells are required. Droplet microfluidics enables encapsulation of cells in micro-containers. In contrast to other types of oil droplets, double-emulsion droplets (water - oil - water) are compatible with standard flow cytometers and droplets can be analyzed and sorted by flow cytometry.

With a technology accessible to non-expert users in microfluidics, the Xdrop instrument encapsulates biological material in double-emulsion droplets [1]. The DE50 double-emulsion droplets with a inner aqueous volume of 65 pl are designed to encapsulate a single or more mammalian cells. Xdrop, together with the dedicated DE50 Cartridge, generates >1 million DE50 droplets in just 5 minutes, making it the optimal choice for functional high-throughput screening.

When sorting large particles (>25µm) with a standard cell sorter, the recovery rate is generally low. Even with manual adjustments, dramatic improvements in recovery are rarely observed despite the time invested. By using the new Large Particle Sorting Option feature, the recovery rate typically exceeds 90% without the need for manual adjustments. With the Large Particle Sorting Option in Sony MA900 Cell Sorter and SH800 Cell Sorter, users can now sort large particles such as spheroids, hydrogel microcarriers, and double-emulsion droplets.

Encapsulation of beads in DE50 droplets

To demonstrate the sorting capabilities of the Sony MA900 Cell Sorter with the Large Particle Sorting Option, both red and green 6 µm fluorescent beads (Alignflow™, Thermo Fisher) were mixed in RPMI medium (Gibco) and encapsulated in DE50 double-emulsion droplets. As shown in Figure 5, DE50 droplets are optimized to hold living cells but solid fluorescent beads are well suited for verifying flow cytometer settings. The beads were diluted before encapsulation to ensure that the double-emulsion droplets only contained one bead. Figure 1 shows DE50 droplets containing either red, green, or no beads at two different magnifications. The encapsulation of beads and cells in double-emulsion droplets follows the Poisson-distribution. After double-emulsion droplet production with the Xdrop and the dedicated DE50 Cartridge, the DE50 droplets were resuspended in Xdrop DE flow buffer before loading on the flow cytometer.

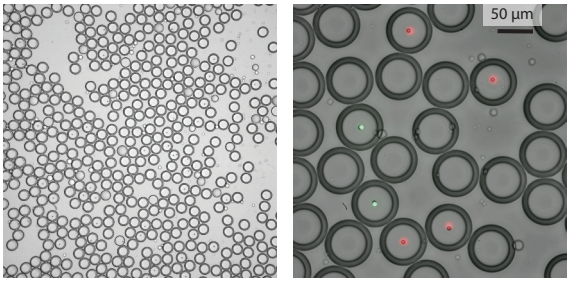


Figure 1. Double-emulsion droplets (DE50) containing fluorescent beads. Left: Bright field microscope image at 10X magnification of DE50 droplets produced on Xdrop with the DE50 Cartridge. Right: Pseudo colored overlay of bright field and fluorescent images of DE50 droplets containing either green or red 6 µm fluorescent beads. 40X magnification.

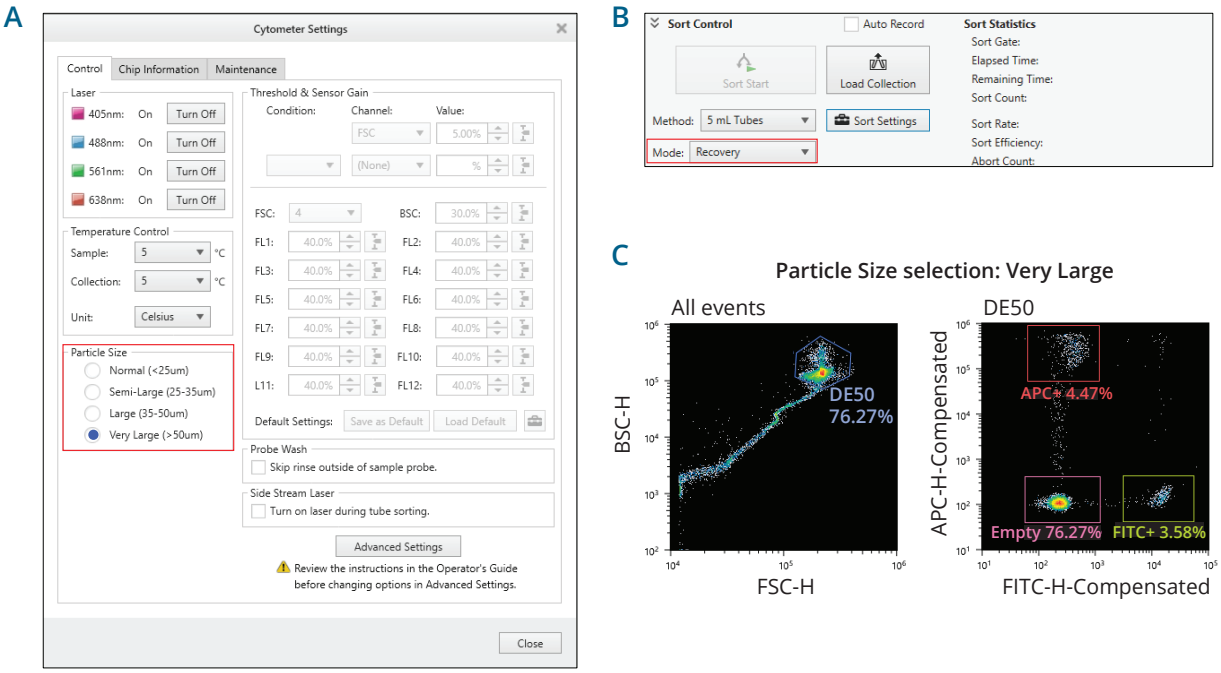


Figure 2. Sorting large double-emulsion droplets on Sony MA900 Cell Sorter with Large Particle Sorting Option software. A. The software presents the option to select between four particle sizes. B. "Recovery" sort mode can be selected to increase the recovery of large particles. C. Left: Scatter plot of all events from a DE50 production batch. The DE50 double-emulsion droplets can clearly be distinguished on a FSC-H vs. BSC-H plot. Right: From the selected DE50 population, we gated the green (FITC+) and the red (APC+) DE50 droplets.

Setting up double-emulsion sorting on Sony MA900 Cell Sorter with the Large Particle Sorting Option

Flow cytometry analysis of DE50 droplets is performed with the same considerations as for other large particles. The DE50 droplets have an outer diameter of 75 µm but they can be sorted with a 130-µm sorting chip nozzle due to droplet flexibility and low likelihood of aggregation. With an average event rate of 300-400 events/second with the 130-µm sorting chip nozzle, about 400.000 DE50 droplets can be analyzed per hour.

Initially, set the sample pressure to maximum to draw the droplets into the channels of the flow cytometer[2]. When the droplets appear on the screen, decrease the sample pressure to an event rate is about 300 events/second. Under the cytometer settings, select the "Very Large" particle size (Figure 2A). This will focus the DE50 population and advance the gating precision. To sort with high recovery of large particles, select the "Recovery" sort mode (Figure 2B).

The entire droplet production collected from the DE50 Cartridge will have a scatter profile like the one shown in Figure 2C. The profile may vary depending on factors such as sample pressure and sample buffer, as well as size and composition of the encapsulated material. DE50 droplets containing fluorescent labeled particles can be separated based on color. In this example, the encapsulated material is APC+ and FITC+ beads, and the two population of DE50 droplets are clearly seen on the plot (Figure 2C, left).

High purity and recovery sorting of large double-emulsion droplets

By using "Recovery" mode, we sorted 50 double-emulsion droplets containing beads onto microscope slides. We repeated this 20 times and analyzed the slides by microscopy to assess recovery and purity. We sorted either droplets with green beads (FITC) or red beads (APC). The purity of the sorting was assessed by counting red (APC) beads on the slides of droplets sorted for green (FITC) beads and vice versa. The purity of sorting was >95% for both colors. As shown in Figure 2C, the proportion of droplets containing either a green or a red bead was 3.58% and 4.47% respectively of all DE50 droplets.

Figure 3 shows a comparison between the recovery of sorted DE50 droplets using different settings for sorting.

When no adjustments were made to optimize the sorting of DE50 droplets, the recovery was about 5%. When the drop delay was manually adjusted, the recovery was improved to about 50%. However, when we sorted 50 DE50 droplets using the Particle Size Selection function and the "Recovery" sort mode, the recovery rate was >96%. This shows the accuracy of sorting obtainable with the Large Particle Sorting Option on the Sony MA900 Cell Sorter.

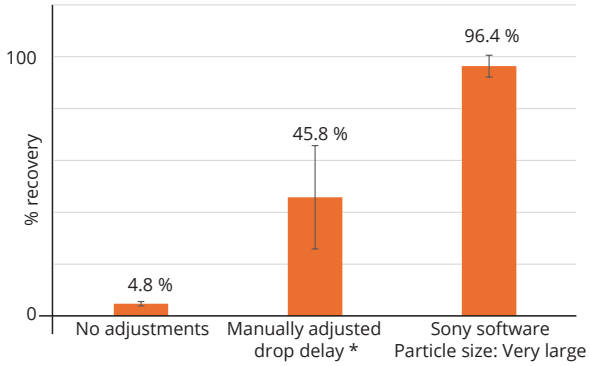


Figure 3. The recovery of sorted DE50 droplets varies significantly with different approaches. DE50 droplets with fluorescent beads were sorted onto a microscope slide. 'No adjustment' signifies that droplets were sorted without manual adjustments and without Large Particle Sorting Option. For the second condition, DE50 droplets were sorted after setting the drop delay manually. Lastly, DE50 droplets were sorted by using the "Very Large" option of the Particle Size Selection function and "Recovery" sorting mode. The user is not required to set the drop delay manually with this software upgrade. * Sorted on a Sony SH800 Cell Sorter, all others on Sony MA900 Cell Sorter.

Single droplet 96-well sorting

We investigated the precision of sorting single DE50 droplets into 96-well plates. DE50 droplets containing red or green fluorescent beads were loaded on a MA900 Cell Sorter. We sorted single green (FITC) DE50 droplets into a flat-bottom 96-well plate. The results of the sorting were recorded by counting fluorescent beads in the wells under an inverted microscope. The encapsulated fluorescent beads allowed for direct and accurate analysis of sort recovery and purity.

We sorted single droplets into 20 wells in triplicate. Single-DE50 droplet sorting was highly accurate, resulting in a single droplet sorted in 88% of the wells (Figure 4). We observed only one well containing two beads; no wells contained more than two beads. All the recovered beads were green (FITC), demonstrating a purity of 100% of double-emulsion plate sorting.

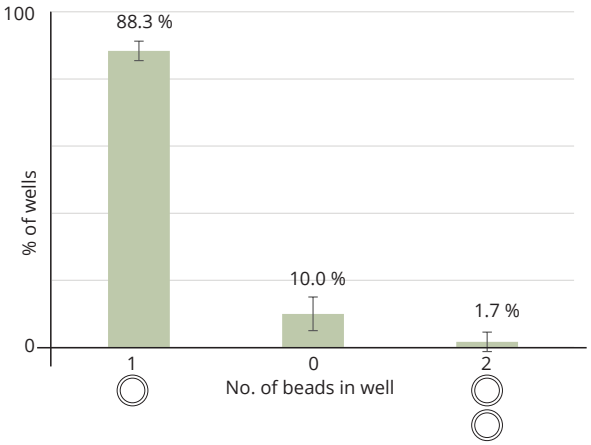


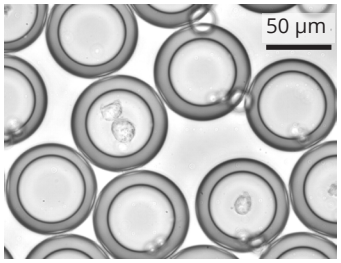
Figure 4. Single droplet sorting in 96-well plates. Results from three replicates of plate sorting of single DE50 droplets encapsulating a 6 µm FITC-labeled beads. The bar chart shows the percentage of wells with 1, 0, or 2 droplets. No wells contained more than 2 beads. Error bars represent standard deviations between 3 plates (triplicates).

Evaluating cytotoxicity in droplets

DE50 droplets are ideal for encapsulating single or multiple living cells. This compartmentalization enables detailed analysis of interactions between different cell types and precise characterization of their functional behavior[3, 4]. We encapsulated two types of live immune cells in DE50 droplets; Natural Killer (NK) cells and K562 target cells in a 1:3 ratio (Figure 5A). Before encapsulation, the cells were stained with CellTrace™ Violet (NK) and CellTrace™ Far Red (K562) to identify the different droplet populations. Figure 5B shows the flow cytometry plot of DE50 droplets containing cells, analyzed using two fluorescence channels (FITC and APC).

The distinct populations of DE50 droplets containing NK cells only (lower right), K562 cells only (upper left), empty DE50 droplets (lower left), or co-encapsulated cells (upper right) were easily identified (Figure 5B). The populations were sorted on the MA900 Cell Sorter with the Large Particle Size Selection function and Recovery sort mode. To evaluate the sorting recovery and purity, we sorted 100 DE50 droplets from each population onto glass slides and analyzed them by microscopy. The sort recovery was 93-97% for the ingle-color droplets and 75% for the droplets with co-encapsulated cells (Figure 5B, red text). All sorted samples consistently showed 100% sort purity.

A



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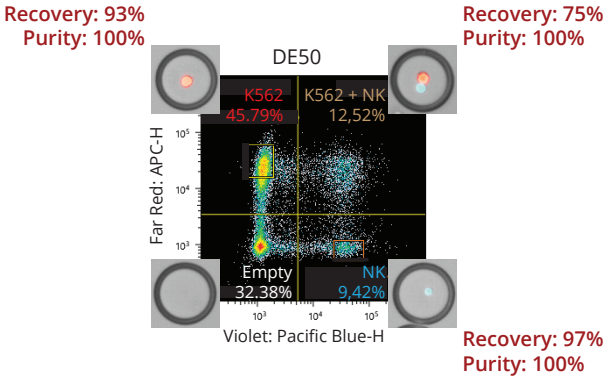

Figure 5. A. Bright field microscopy image of double-emulsion DE50 droplets encapsulating living mammalian cells (co-encapsulated K562 and NK cells). B. Flow cytometry analysis and sorting of DE50 droplets containing two cell types. The K562 target cells are stained with CellTrace™ Far Red (APC) and the NK effector cells with CellTrace™ Violet (Pacific Blue). The "Very Large" option of the Particle Size Selection function and Recovery mode of the MA900 Cell Sorter was used for sorting. The sort recovery and purity for each gate is indicated in red.

Conclusion

The Large Particle Sorting Option in the MA900 Cell Sorter delivers highly efficient and precise sorting of large double-emulsion droplets. Combined with the user-friendly Xdrop work flow, it enables isolation of specific cells based on their functional characteristics revealed by Xdrop single-cell assays[3, 4]. This powerful integration of throughput and accuracy in handling large double-emulsion droplets paves the way for a wide range of advanced and targeted downstream applications, from sequencing to cell culture and more.

For more information about Xdrop products and this technical note, contact Samplix at samplix.com/contact.

SX-001155-TN, ver. 01

References

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3. Anne-Christine Kiel Rasmussen, Thomas Morgan Hulen, David Leander Petersen, et al. Immuno-Oncology and Technology (2024), Volume 24, 100738. doi: https://doi.org/10.1016/j.iotech.2024.100738
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