A QUICK AND EFFICIENT DOUBLE EMULSION GENERATION METHOD FOR FLOW CYTOMETRY DROPLET SORTING

INTRODUCTION

Analyzing cellular behavior at a single-cell level is critical to better understand the heterogeneity of cellular responses such as protein secretion or enzymatic activity. Flow cytometry sorting based on FACS (Fluorescence-Activated Cell Sorting) is a convenient tool for selecting among a complex population of cells, those which display a specific fluorescence signal, indicating the production of a molecule of interest, or the expression of a specific phenotype. However, the FACS method alone has two major limitations: secreted molecules are dissolved in the media and are not detected on the cell's surface unless a binding protocol is performed, and the secretion of the cells influence each other. The analysis then becomes single-cell on a bulk assay of interacting cells. The encapsulation of single cells efficiently overcomes these issues, ensuring the full single-cell feature of the assay as well as confining the secreted molecules in a small volume, making them detectable.

Droplet-based microfluidics emerged in the 2000's as a powerful tool to generate very monodisperse emulsions. The principle is to flow two immiscible phases in microchannels and have them meet at a T-junction or flow-focusing so that the continuous phase squeezes the dispersed phase into droplets [1]. As a result, the generated simple emulsion droplets are monodispersed to a low size (pL to µL droplets), and the encapsulation rate can be controlled.

Conventional emulsions made using microfluidic devices are water-in-oil (W/O) droplets or single emulsions. If such droplets are commonly used to perform single-cell encapsulations, they are not suited to flow cytometry droplet sorting. Indeed, flow cytometry droplet sorting requires the particles to be suspended in an aqueous phase to efficiently sort the particles of interest by applying an electric field [2]. To avoid these limitations, the W/O droplet can be encapsulated in a 2nd aqueous layer to form a water-in-oil-in-water (W/O/W) droplet, also named double emulsion, which is compatible with flow cytometry droplet sorting (figure 1).

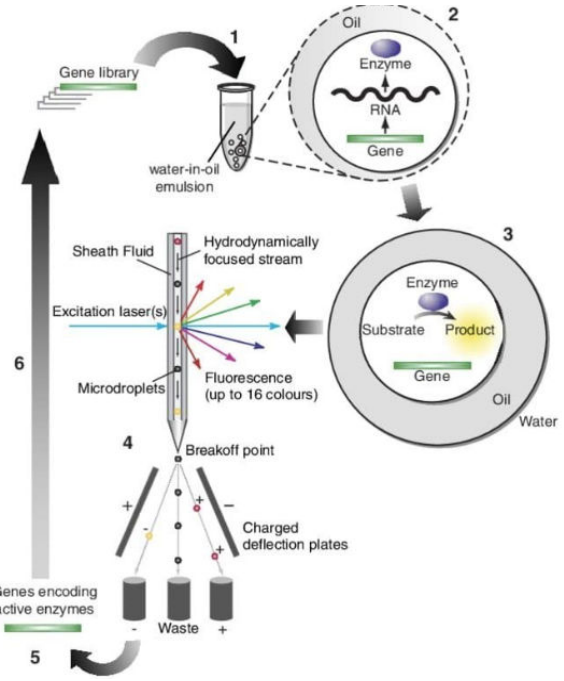

Figure 1: Principle of flow cytometry water-in-oil-in-water droplet sorting based on the presence of specific enzyme [3].

In this paper, we describe the encapsulation of a fluorescent strain of Escherichia coli bacteria in w/o/w double emulsions and flow cytometry droplet sorting using a flow cytometry cell sorter (also commonly known as FACS). A double emulsion platform allows the bacteria to be compartmentalized within a protective shell and ensures compatibility with the sorting device. The platform generates double emulsions in one step, scaling the production of common two-step double emulsion generation methods by up to ten times. Several hundred microliters of emulsions can be produced in 30 minutes, and multiple conditions can be tested within a single day, compared to a week with traditional methods. In addition, the platform integrates fluidic, pneumatic and optic solutions, and does not require complex fabrication steps nor surface treatment. This application note was written in collaboration with Delphine Lestrade (head of the flow cytometry platform at Toulouse White Biotechnology, TWB) and Sophie Lajus (researcher at the Toulouse Biotechnology Institute, TBI).

TESTIMONIAL

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TWB sets up and conducts R&D projects in the field of industrial biotechnology in collaboration with public laboratories and industrial players, supports the development of start-ups by offering them accommodation in its premises in a state-of-the-art scientific and technological environment, and encourages the emergence of breakthrough innovations. By bringing together researchers, entrepreneurs, funders, institutions and industry, TWB integrates and leverages all the skills and expertise and creates synergies while simplifying the contractual relationship. This unique model accelerates the innovation process necessary for the creation of an eco-responsible industry.

At TWB, the Cytometry Platform is responsible for developing and defining new protocols for high-throughput cytometry assays for the identification and characterization of microorganisms."

Delphine Lestrade, Toulouse White Biotechnology (TWB)

MATERIALS & METHODS

1. Materials

Droplet generator

The Raydrop® is a microfluidic device designed by Secoya Technologies to allow for the easy production of single or double emulsions of any kind (W/O, O/W, W/O/W and O/W/O) with no surface treatment required. The chip is based on the alignment of two capillaries placed inside a closed and pressurized chamber. The continuous fluid, contained in the chamber, pinches the dispersed phase (which comes out of the injection capillary through a 3D-printed nozzle) to squeeze it into monodisperse droplets, which are then collected in the extraction capillary. In the case of a double emulsion device, a double emulsion nozzle allows a shell phase to form a protecting layer around the core phase. The continuous phase pinches this 2-layers droplet, resulting in the formation of a core-shell double emulsion (figure 2). In this application note, the Raydrop® used is a 30-70-45 model, enabling the production of DE droplets with a size ranging between 20 and 40µm, compatible with fluorescence-activated cell sorting applications.

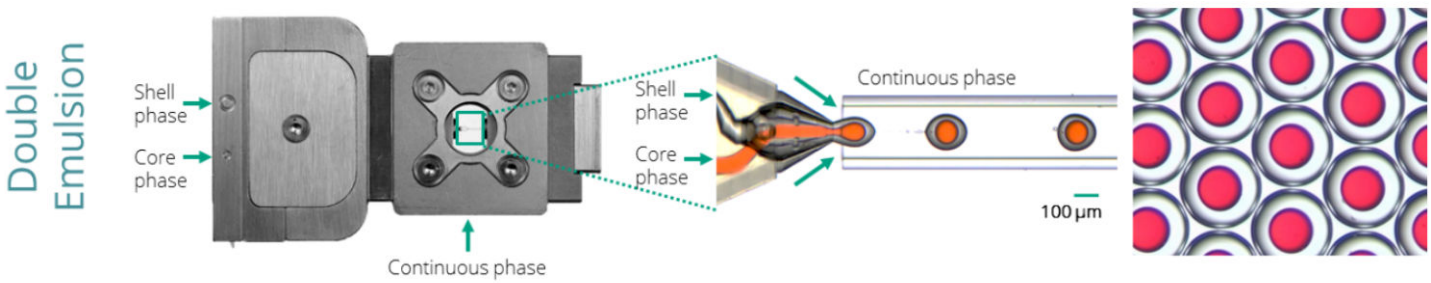


Figure 2: Structure of the Raydrop® [4].

Microfluidic flow control

Fluigent's flow control technology was used to generate highly-monodisperse double emulsions in a controlled environment, which is essential for an efficient droplet FACS sorting. Three Flow EZ 7bar were used for the injection of each phase and connected to microfluidic flow sensors: a flow unit L for the continuous phase, a S for the shell phase and a M for the core phase. The applied pressure and flowrates were controlled and monitored using the Oxygen software.

Double emulsion platform with injection loop

To avoid a complex microfluidic setup and allow for an easy microfluidic droplet generation, Secoya technology developed a platform integrating Fluigent flow controllers and sensors along with valving systems to switch between solutions and inject between delivery sites (Raydrop® microfluidic -device or waste). An optical system is integrated on the platform: a 10X objective, a LED light source, and a camera enable the live visualization of the droplet generation, and a micromanipulation system allows the displacement of the Raydrop® along all three axes (X, Y and Z). In addition to the platform, an injection loop made of Fluigent's L switch and Switch EZ is integrated in the setup for the easy injection of a low and precise volume of biological samples in the core phase. This loop, placed between the platform and the Raydrop®, avoids the circulation of biological samples in all the fluidic line of the core phase and avoids the need for large volumes of samples. In this application note, we used a loop volume of 200µL that can be adapted if needed.

This platform ensures the production of double emulsions in a one-step process at a high frequency (>1kHz), as opposed to traditional microfluidic methods, which rely on two-step mechanisms due to wettability needs and that are incompatible with high-throughput processing.

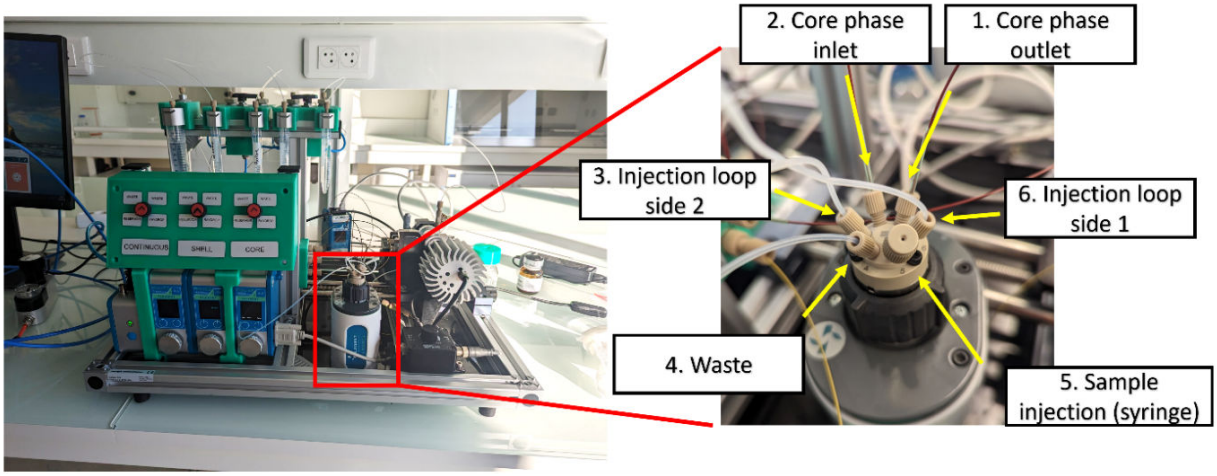
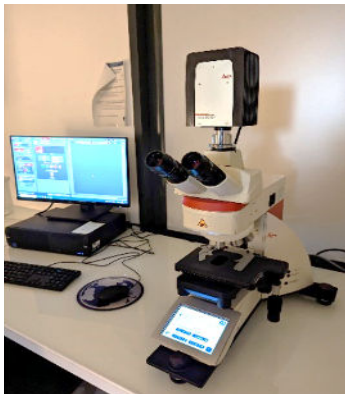


Figure 3: Double emulsion platform with injection loop.

DOUBLE EMULSION GENERATION FOR FACS

Fluorescence microscope

A Leica DM6 M upright microscope, equipped with a Leica DFC7000-T camera and controlled with Leica LAS X software, located at TBI on the Quantitative and functional ecology platform, is used to observe the produced double emulsion and evaluate the bacterial fluorescence.



Flow Cytometry device

A MoFlo Astrios EQ Cell Sorter manufactured by Beckman Coulter and located at TWB's flow cytometry platform was used for the cytometry analysis of DE droplets and the sorting based on the presence of fluorescence in the DE droplets.

2. Reagents

Continuous phase: MilliQ water + 2% V/V Tween20

Shell phase: Dsurf 2% in HFE 7500 oil

Priming phase: MilliQ water for system priming and flow stabilization

Injection phase: LB culture medium with ~108/mL E. coli expressing the Green Fluorescent Protein (GFP) in the injection loop for bacterial encapsulation

Collecting phase: MilliQ water + 2% V/V Tween20 + 150mM NaCl

3. Methods: Encapsulation of E. coli strain in double emulsions

- Fill the reservoirs with the appropriate priming phase, shell phase, and continuous phase
- Place the L-switch on position 2 (figure 4)
- Fill the Raydrop's chamber with the continuous phase by applying a high flow-rate pressure, then switch to a flow-rate control and set a value of 120µL/min

- Create a single emulsion of shell by increasing the pressure until reaching stable droplets in a jetting mode
- Increase the priming phase's flowrate until you see a double emulsion is formed
- Adjust the droplet size by playing with the flowrates of the continuous flow and the shell thickness by adjusting the shell and priming flowrates
- Let the system stabilize for 30 minutes to ensure the elimination of any residue that could hide in the system and disturb your flow (partial or full clogging) when released
- Using a syringe with a 22g blunt-end needle and an adapted connector, fill the loop by injecting your injection phase in port 5 until it emerges from the tubing connected to port 4.
- Switch the L-switch to position 1
- After 50 seconds, the bacteria arrive at the nozzle and get encapsulated. Collect the droplets in a tube filled with 1mL collecting phase. After 20 minutes, stop collecting (with a core phase flowrate of 10µL/min, it takes 20min to collect all the content of the 200 µL injection loop, whereas it would take at least a day with conventional 2-steps methods).

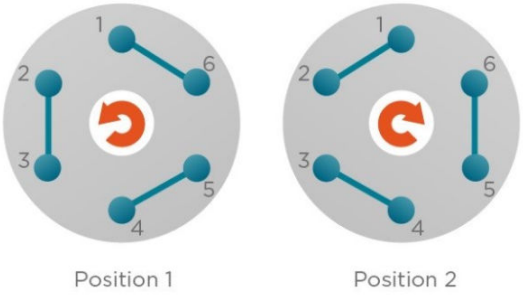


Figure 4: Position 1 and position 2 of the L-switch.

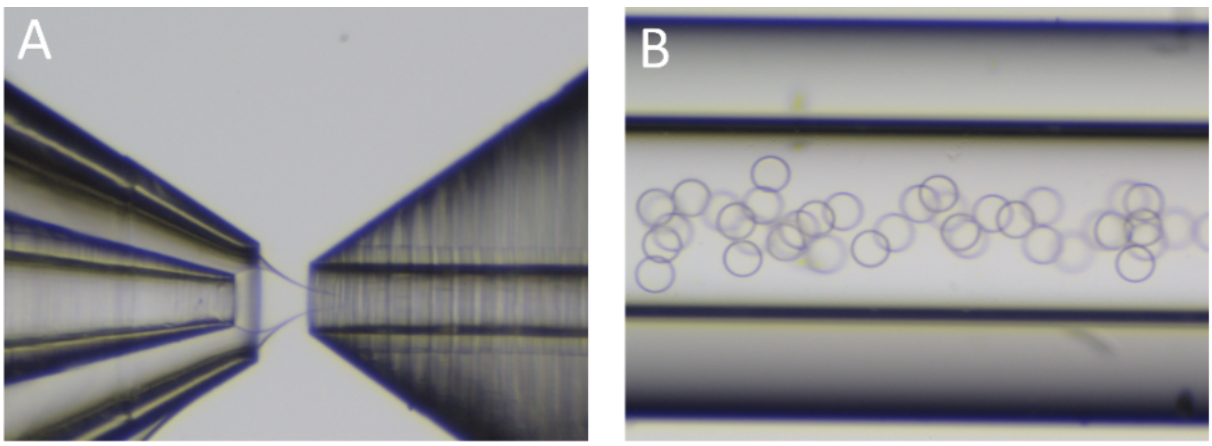


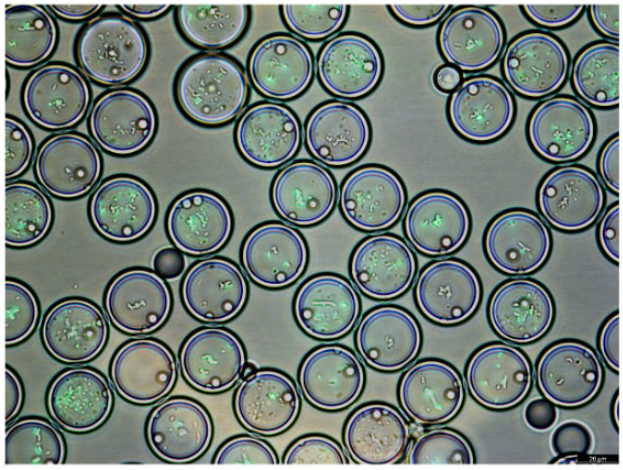
Figure 5: Microscopic observation of the double emulsion (DE) formation at (A) nozzle and (B) extraction capillary.

RESULTS

1. Encapsulation of E. coli in double emulsions

After encapsulation of the bacteria in the double emulsion, brightfield and GFP acquisition was performed to characterize the droplets. A measurement of the core diameter was done using image J: a mean value of 26µm was found on 30 droplets, with a coefficient of variation of 1.65%, expressing excellent monodispersity. A small drop of oil/surfactant was observed inside each droplet, which could not be seen during encapsulation, and did not disturb the microscopic and flow cytometry analysis. This oil/surfactant droplet can be avoided by adjusting the flowrates during DE production. Similarly to the core phase diameter, the mean global DE's diameter was evaluated using image J and found to be 34µm with a coefficient of variation of 2%.

A



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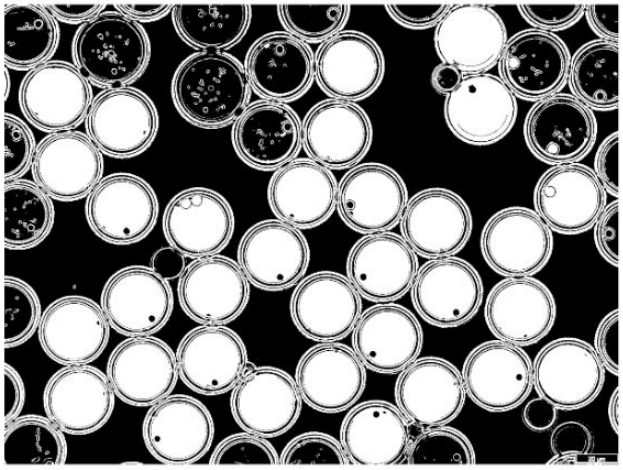

C

Mean core diameter (µm)	26.00
Standard deviation	0.43
CV (%)	1.65

Figure 6: Microscopic observation and analysis of the DE containing E. coli strain expressing GFP with (A) Bright Field (BF) + fluorescent microscopic image stack, (B) the core area finding using image J and (C) the mean core diameter value with the standard deviation; scalebar = 20µm.

We notice on the microscopy image of the DE that most droplets show a fluorescence signal. Some of them do not have fluorescence, which is because of 2 reasons: 1) the droplet does not contain any bacteria or 2) the encapsulated bacteria do not express detectable fluorescence. The presence of the two populations of droplets is necessary to verify the efficiency of the fluorescence-activated cell sorting experiment.

2. Flow cytometry droplet sorting

The DE produced was then sorted using the Moflo Astrios cell sorter. Gating was performed to identify and isolate each observed population:

- A population for fluorescent DE droplets (i.e. DE droplets containing fluorescent E. coli)

- A population for non-fluorescent DE droplets, referred to as empty droplets in Figure 7.C.

First, double emulsions were identified in an FSC/SSC plot (Figure 7.C, left graph). Then single DE droplets were selected to avoid sorting doublets (Figure 7.C, middle graph) and finally the DE droplets with high green fluorescence were separated from those with no or low green fluorescence (Figure 7.C, right graph). Among the individual DE droplets, the fluorescent DE accounts for 66% of the events and the empty DE accounts for 7% of the total events. This difference in population shows that the flow cytometric analysis is in agreement with microscopic observations.

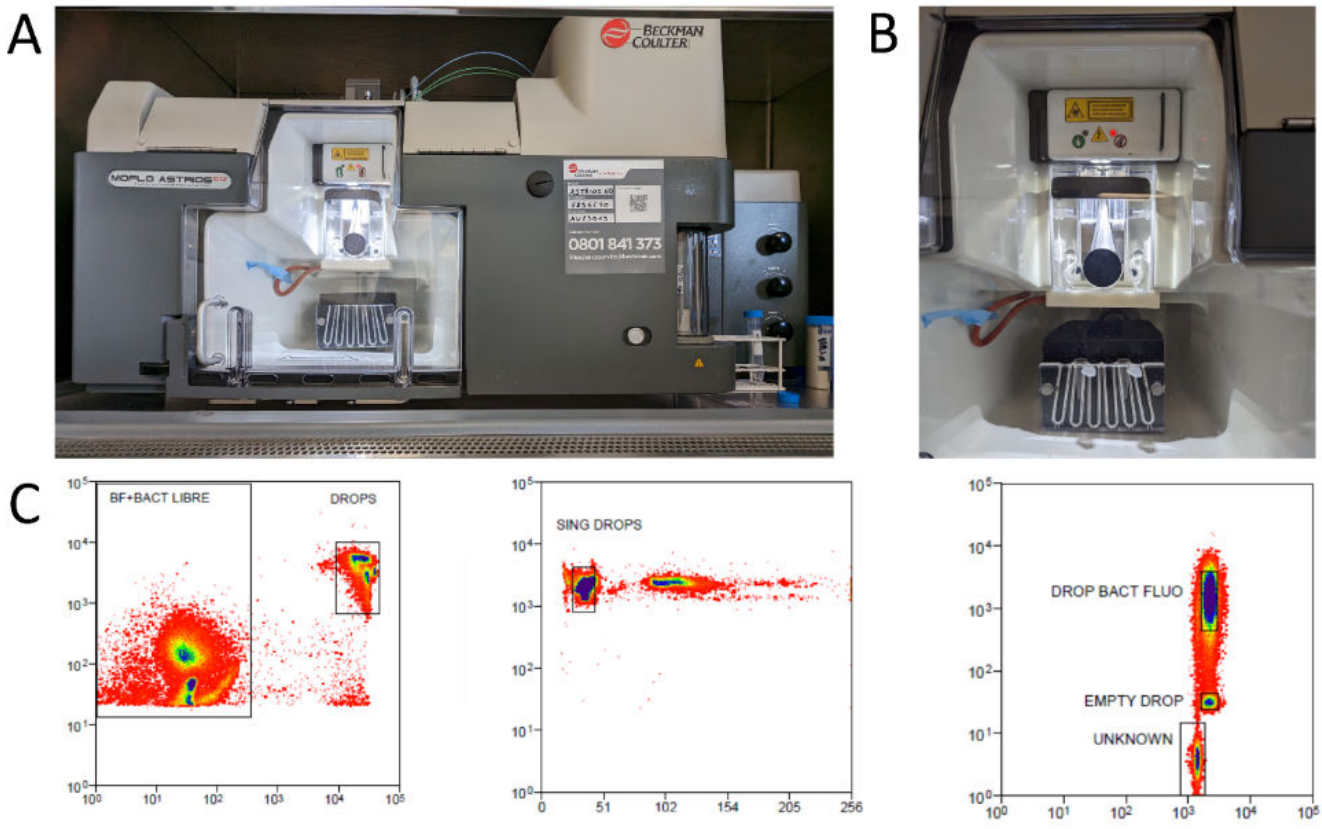

Figure 7: FACS machine used for sorting (A) with a zoom to the sorting area (B), and cytometry analysis and gating (C).

The droplets were then sorted and collected in two 1.5ml tubes according to their association with the DROP BACT FLUO or EMPTY DROP gate. The flow cytometer showed a sorting efficiency between 75% and 95%. After collection, the sorted populations were again imaged by microscopy. This was done to verify the purity of the sorting.

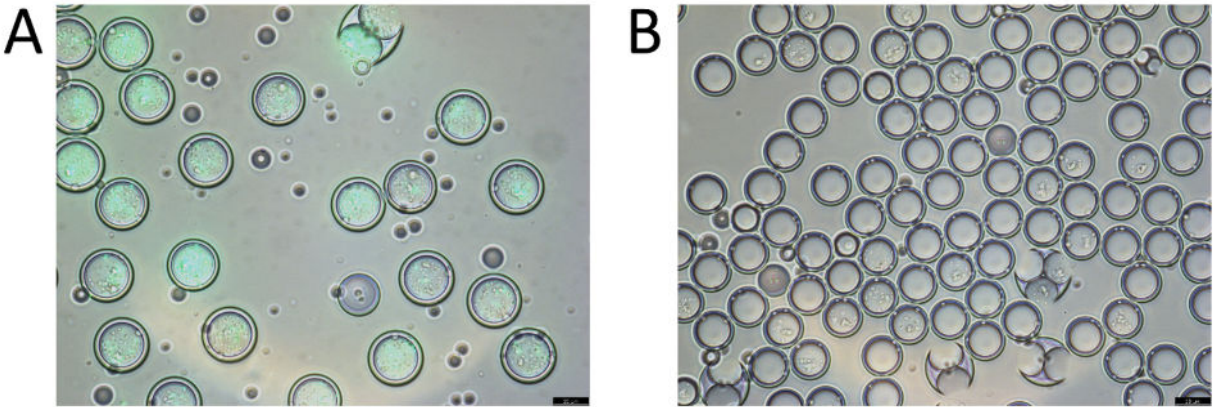

Figure 8: BF and GFP stack image of fluorescent droplets (A) and empty droplets (B) after sorting; scalebar = 20µm.

We can notice that all the DE sorted from the "fluorescent" gate contain fluorescent bacteria, with different levels of fluorescence, as expected. In addition, none of the droplets considered "empty" in the gating strategy are fluorescent, some of them empty and some encapsulating non-fluorescent E. coli.
Surprisingly, the empty droplets appear to be smaller than the fluorescent ones.

CONCLUSION

We demonstrated the potential of the platform to efficiently and rapidly encapsulate (<30min for 200mL of W/O/W droplets) biological compounds into double emulsions. The double emulsions are compatible with Flow Cytometry droplet sorting (commonly known as FACS). The produced droplets have excellent monodispersity and encapsulation efficiency, and the sorting using flow cytometry coupled to electrical charge proved to be efficient, as all sorted "fluorescent" droplets were fluorescent.

These double emulsions were used here to encapsulate bacteria, but their use could be extended to encapsulating single eukaryotic cells or yeast and sorting a specific cellular type among a complex population or selecting cells responding to a specific stimulus.

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