IMPROVING DYE RETENTION IN DROPLET-BASED MICROFLUIDIC EXPERIMENTS BY CHOOSING THE ADEQUATE OIL AND THE APPROPRIATE RATIO OF SURFACTANT

Discover Emulseo's novel fluorinated oil Fluo-Oil 200 to improve dye retention in your droplet-based microfluidic experiments

INTRODUCTION

Droplet-based microfluidics has emerged as an efficient solution for rapid, precise, quantitative and low-cost screening tool for biological and chemical applications. The monodispersed water-in-oil droplets generated in droplet-based microfluidic devices are loaded with biological or chemical agents. The droplets are used as microcompartments. To analyze and/or sort the content of the droplets, dyes are usually added. The droplets are then monitored and sorted according to their fluorescence signal.

A common issue in droplet-based microfluidic experiments involving the use of dyes is dye leakage. The release of the dye into the oil phase and neighboring droplets results in a decrease of the accuracy of fluorescence monitoring. Some of the parameters influencing the retention of dyes in droplets are the nature of the buffer or cell culture medium, the choice of fluorophore and its hydrophilicity, the oil phase and the concentration, nature and molecular weight of the surfactant.

In this study, we present a comprehensive investigation of the impact of oil choice and surfactant concentration on dye leakage during droplet-based microfluidic experiments. The performance of two fluorinated oils and surfactant concentrations on dye retention will be discussed.

I) Impact of oil choice on dye retention

1. Material and methods

- **Oils properties**

In this study, two oils were selected to investigate dye leakage in droplet-based microfluidic experiments: Fluo-Oil 40 and Fluo-Oil 200. Both fluorinated oils have been formulated to ensure stability, biocompatibility, and reproducibility of microfluidic experiments as an alternative to Fluorinert™ FC-40 oil.

	Fluo-Oil 200	Fluo-Oil 40	Fluorinert™ FC-40
Appearance	Transparent	Transparent	Transparent
Biocompatibility	✓	✓	✓
Boiling point (°C)	196	165	165
Viscosity (mPa.s)	4.3	4.1	4.1
Density	1.79	1.85	1.85

Table 1: Fluo-Oil 200, Fluo-Oil 40 and Fluorinert™ FC-40 physical properties.

Table 1 presents the physical properties of the two Fluo-Oils. The properties of both Fluo-Oils are compared to the properties of Fluorinert™ FC-40. The properties of Fluorinert™ FC-40 are the same as Fluo-Oil 40. The density of Fluo-Oil 200 is slightly lower than the density of Fluo-Oil 40 and Fluorinert™ FC-40, whereas its boiling point and its viscosity are slightly higher. During droplet generation, slight adjustments might be needed to obtain similar droplets but the physical properties of the two oils remain very close.

- **Microfluidic experiments**

To evaluate the impact of oil choice on dye leakage, water-in-oil droplets were generated using both oils. Droplet size distribution, stability after incubation (37°C) as well as molecular retention were evaluated.

The PDMS/glass microfluidic chip used to generate the droplets consists of a flow focusing geometry. The channels are coated with Fluo-ST2 (hydrophobic surface treatment). In the study comparing the two oils, the oil continuous phase was prepared by dissolving 4w/w% of neat FluoSurf™-C surfactant in either Fluo-Oil 200 or Fluo-Oil 40. The dispersed aqueous phase used for these experiments was PBS, 20 µM fluorescein in PBS, 2 µM resorufin in PBS or 44 µM resazurin + 40 mM cysteine in PBS. The comparison of the two oils was performed by analyzing a mixture of empty droplets and droplets containing the fluorescent dye. Syringe pumps were used to control the flow rates of the different phases in the microfluidic device (oil phase: 300 µL/h and aqueous phase: 100 µL/h for droplet stability experiments; oil phase: 600 µL/h and aqueous phase: 100 µL/h for dye retention experiments).

Droplet generation was monitored using an inverted microscope. ImageJ software was used for image analysis.

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II) Droplet stability through incubation

Droplet stability through incubation is studied in both oils (Fluo-Oil 40 and Fluo-Oil 200). The size distribution of the droplets is analyzed before and after 3 days of incubation at 37°C.
The results are reported in Figure 1.

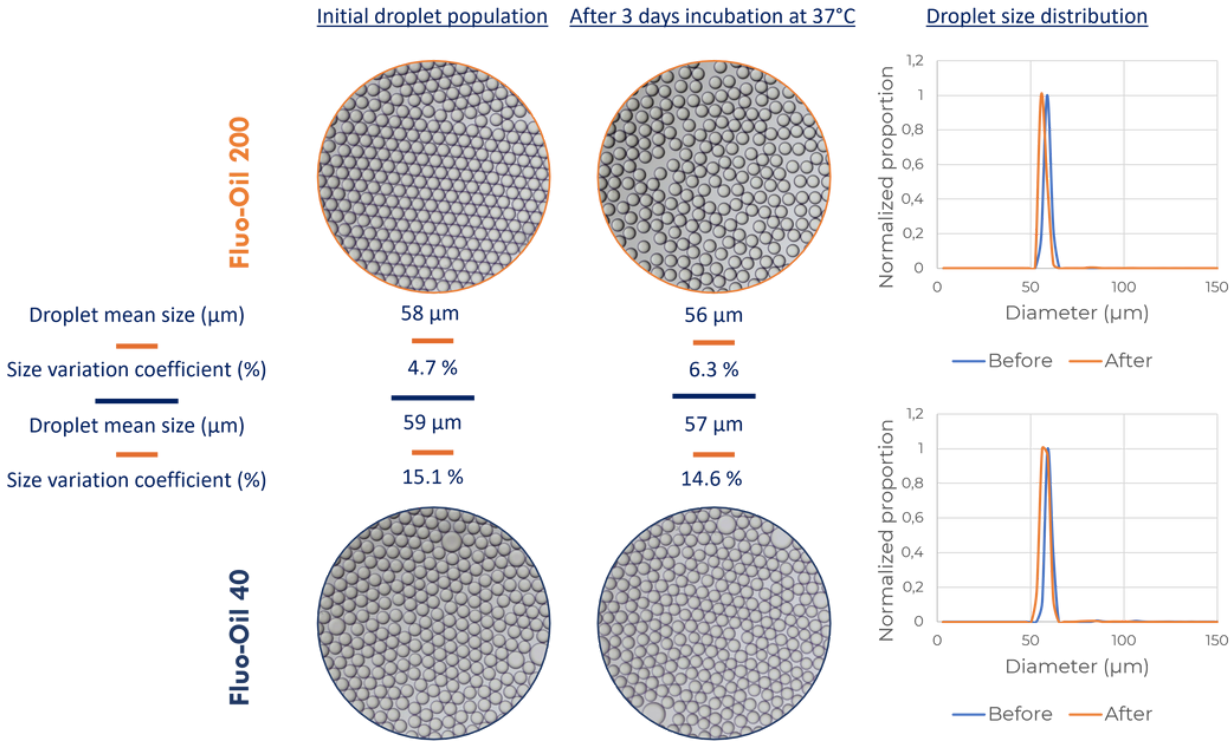

Figure 1: Droplet size distribution and associated pictures of water-in-oil droplets generated in Fluo-Oil 200 and Fluo-Oil 40 before and after incubation at 37°C for 3 days.

The average droplet diameter is about 60 µm for both oils. Statistical analysis of the images showed size variation coefficients of 15.1% and 4.7% for FluoSurf™-C stabilized droplets generated in Fluo-Oil 40 and Fluo-Oil 200, respectively. After incubation, the droplet sizes are similar and the size variation coefficients are 14.6% and 6.3% for Fluo-Oil 40 and Fluo-Oil 200, respectively. The size variation coefficient before and after incubation is lower for droplets generated in Fluo-Oil 200 meaning that the droplets generated in Fluo-Oil 200 are less dispersed than the droplets generated in Fluo-Oil 40. The difference between the two coefficients before and after incubation for each oil is very low, the stability of the droplets generated is constant throughout the incubation.
To summarize, Fluo-Oil 200 shows better results than Fluo-Oil 40 regarding the dispersity of the droplets generated. However, the droplets generated with both oils show excellent stability throughout the incubation.

III) Dye retention

The retention of dye in the droplets during incubation is studied in this part for both oils (Fluo-Oil 40 and Fluo-Oil 200). Two dyes were selected, fluorescein and resorufin.

1. Fluorescein as a model compound

Fluorescein is widely used as a fluorescent dye in microfluidic experiments and is used in this study as a model compound for retention studies.
Two water-in-oil emulsions ["empty" (PBS-loaded) and "full" (20 µM fluorescein in PBS-loaded) droplets] are generated alternately in Fluo-Oil 40 and in Fluo-Oil 200. The mixes containing two populations of droplets ("empty" and "full") are then incubated at 37°C and pictures are taken at different timepoints. The pictures and the results of the statistical quantitative analysis of the evolution of fluorescence intensity in full and empty droplets are reported in Figure 2.

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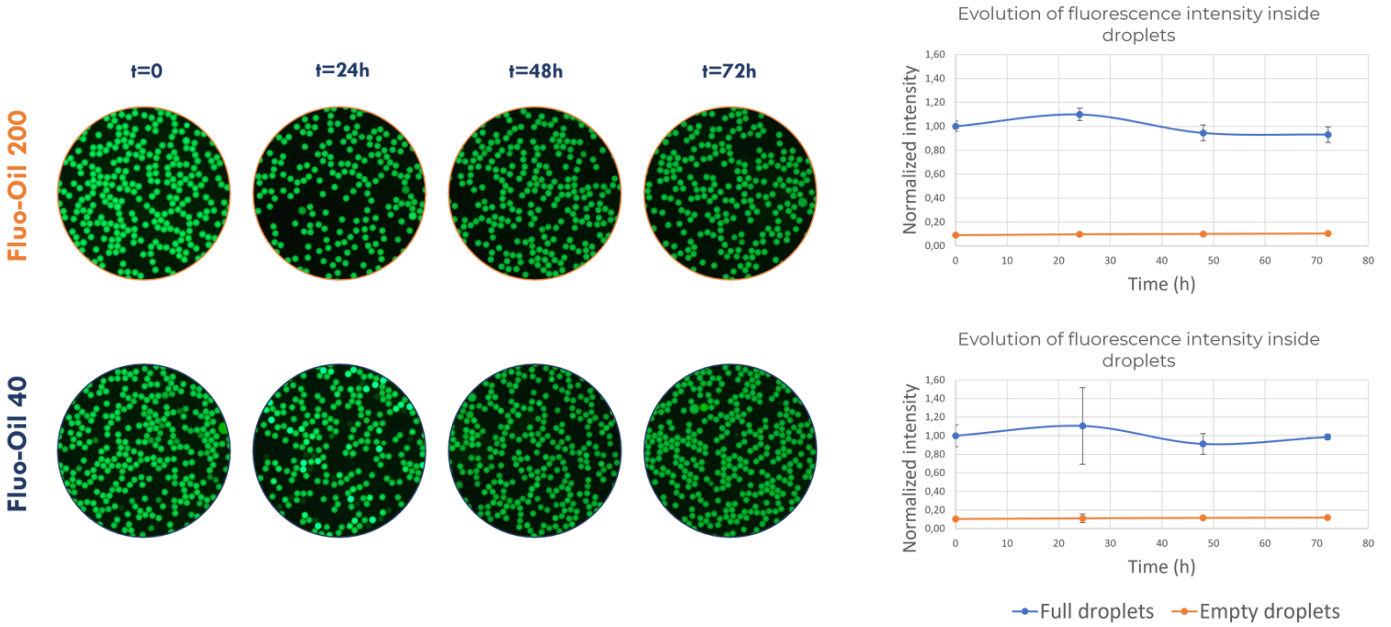

Figure 2: Pictures of the mix of full and empty droplets and evolution of the mean fluorescence intensity throughout incubation at 37°C of water-in-oil droplets generated in Fluo-Oil 200 and in Fluo-Oil 40.

In the pictures shown in Figure 2, the black droplets correspond to the empty droplets while the green droplets correspond to the droplets containing the fluorescent dye: fluorescein. In both oils, at t=0, right after the generation and at t=72h, at the end of the incubation, no crosstalk between the full and empty droplets is observed. Fluorescein dye retention is good in both oils. The evaluation of the mean fluorescence intensity in full and empty droplets confirms these results by showing almost constant values for both oils.
To summarize, fluorescein dye retention is very effective in droplets generated in Fluo-Oil 200 and Fluo-Oil 40.

2. Resorufin: a common cell viability marker in microbiology

Resorufin is commonly used in microbiology as a probe to detect cell viability. In resorufin based assays, molecules with low fluorescence intensity are converted to highly fluorescent resorufin by cellular metabolism. Resazurin is one such molecule. Resazurin is blue and weakly fluorescent but in contact with a viable cell, it is reduced by an oxido-reduction reaction to resorufin, a red-colored highly fluorescent compound as shown in Figure 3.

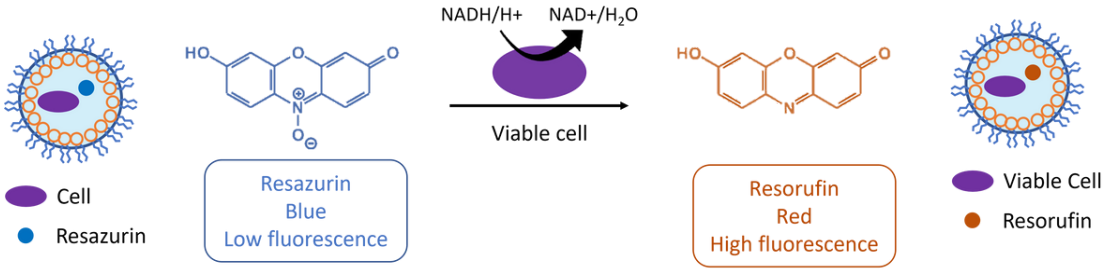

Figure 3: Scheme explaining how resorufin can be used as a fluorescent probe to test cell viability.

This microbiology assay has been applied to droplet-based microfluidics: one cell is encapsulated in one droplet with resazurin; if the droplet becomes fluorescent, the cell contained in the fluorescent droplet is viable. This test is for example suitable for the rapid detection of pathogen agents in food.
In the first part, the retention of resorufin in the droplet was characterized in two different oils. After evaluation of the more suitable oil, the surfactant concentration was optimized. Once the optimal conditions were determined, the retention and conversion of resazurin to resorufin (with cysteine) inside the droplets during incubation was characterized.

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- **Resorufin retention**

First, the retention of resorufin, the fluorescent compound, in the droplets is characterized. As previously, two water-in-oil emulsions ["empty" (PBS-loaded) and "full" (2 µM resorufin in PBS-loaded) droplets] are generated alternately in Fluo-Oil 40 and in Fluo-Oil 200 with 4w/w% FluoSurf™-C. The mixes containing two populations of droplets ("empty" and "full") are then incubated at room temperature and pictures were taken at different timepoints.

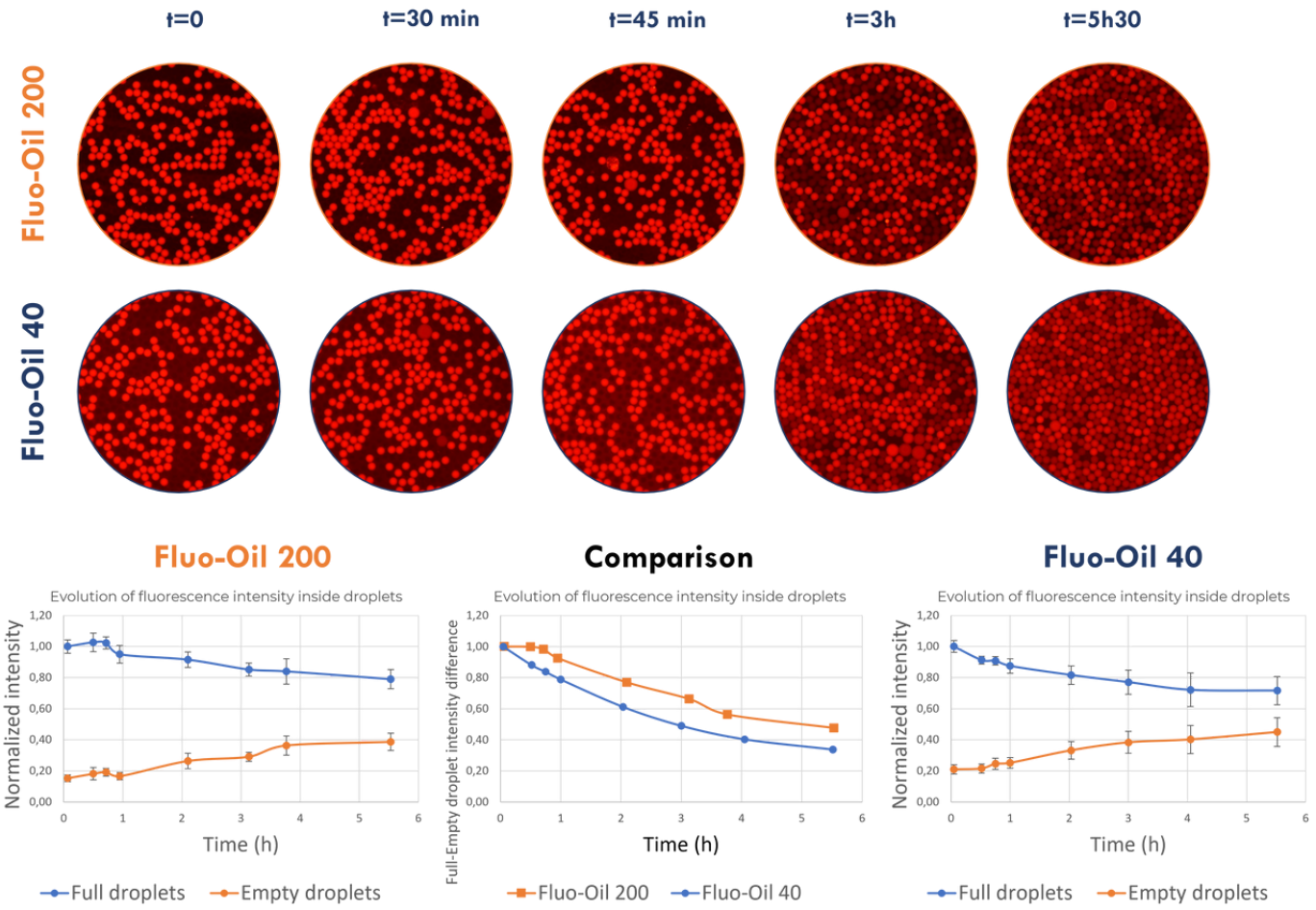

Figure 4: Pictures of the mix of full and empty droplets and evolution of the mean fluorescence intensity throughout incubation at room temperature of water-in-oil droplets generated in Fluo-Oil 200 and in Fluo-Oil 40.

In Figure 4, the qualitative and quantitative evaluation of the fluorescence evolution in the empty and full droplets generated in Fluo-Oil 200 show the beginning of resorufin leakage after 45 minutes of incubation. However, for droplets generated in Fluo-Oil 40, resorufin leakage starts at t=0. A better resorufin retention is therefore observed in Fluo-Oil 200.
Another way to limit dye leakage in droplet-based microfluidic experiments is to tune the surfactant concentration.

- **Impact of surfactant concentration on resorufin retention**

Two water-in-oil emulsions ["empty" (PBS-loaded) and "full" (2 µM resorufin in PBS-loaded) droplets] are generated alternately in Fluo-Oil 200 with three different surfactant concentrations. The mixes containing two populations of droplets ("empty" and "full") were then incubated at room temperature and pictures were taken at different timepoints.

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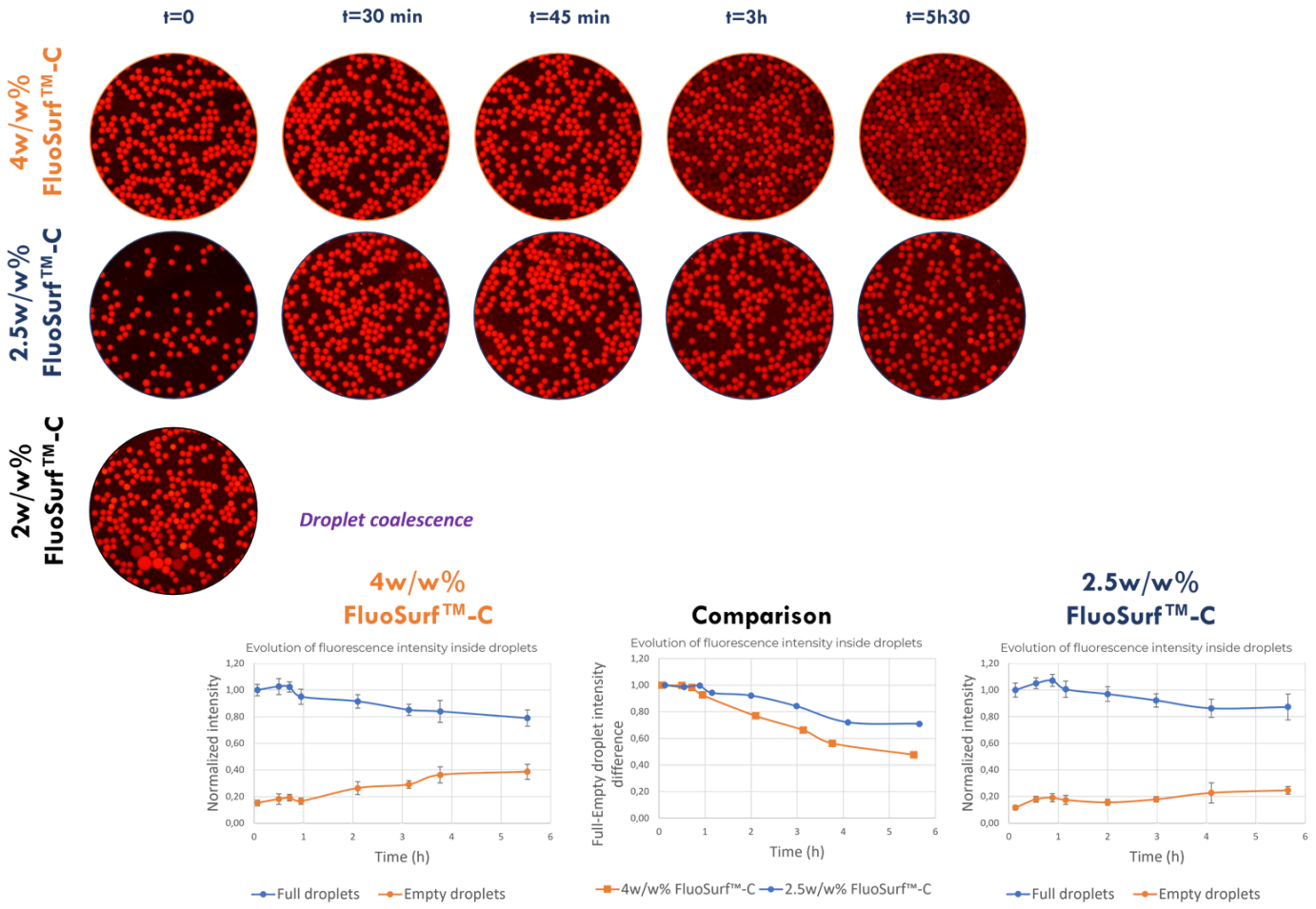

Figure 5: Pictures of the mix of full and empty droplets and evolution of the mean fluorescence intensity throughout incubation at room temperature of water-in-oil droplets generated with 4w/w%, 2.5w/w% and 2w/w% FluoSurf™-C in Fluo-Oil 200.

As previously observed, after 45 minutes, at 4w/w% FluoSurf™-C in Fluo-Oil 200, the resorufin starts to leak from the full droplets into the empty droplets. With 2w/w% FluoSurf™-C in Fluo-Oil 200, the droplets coalesce at t=0. A concentration of 2w/w% is not enough to ensure the stability of the droplets generated. With 2.5w/w% FluoSurf™-C in Fluo-Oil 200, resorufin is retained in the droplets for a longer time. Leaks are limited. Herein, the optimal concentration to stabilize the droplets and to limit dye leakage as much as possible in the conditions of our study is 2.5w/w% FluoSurf™-C in Fluo-Oil 200.

- **Resazurin-based cell viability model assay in droplets generated in Fluo-Oil 200**

In this part, a model viability assay using resazurin is performed in droplets. The optimal experimental conditions (2.5w/w% FluoSurf™-C in Fluo-Oil 200) found in the previous sections are used. Cysteine, a chemical compound able to mimic the activity of a viable cell in the assay is also used. Droplets containing resazurin (44 µM) in PBS and droplets containing resazurin (44 µM) and cysteine (40 mM) in PBS are generated alternately. The droplets are incubated for 4 hours at room temperature. A scheme of the experiment is presented in Figure 6.

At the beginning of the experiment, the droplets containing resazurin only are non-fluorescent; during the incubation, without crosstalk, the droplets should stay non-fluorescent. On the other hand, droplets containing resazurin and cysteine are non-fluorescent at the beginning of the experiment; during incubation, the cysteine converts the resazurin to resorufin and the droplets become fluorescent. The results of the experiments are presented in Figure 6.

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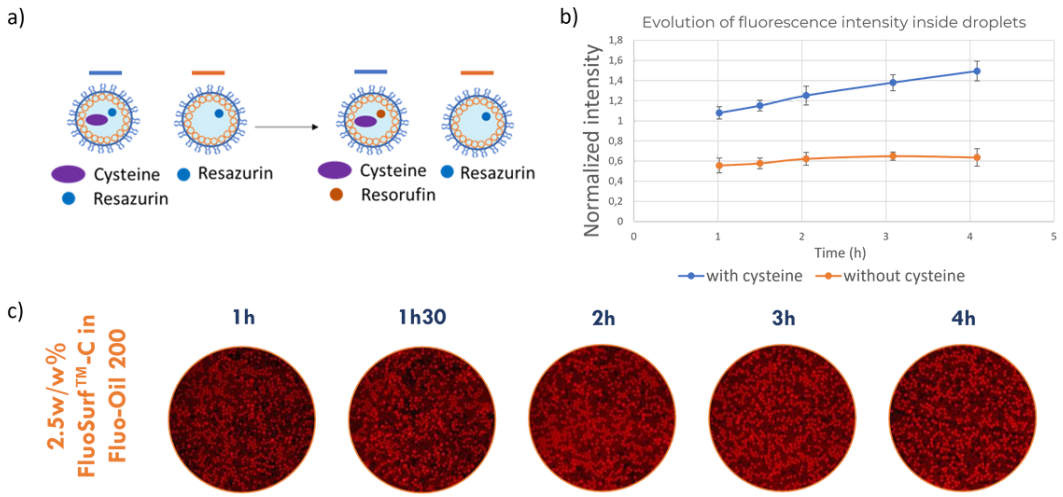

Figure 6.
a) Scheme of the experiment: Resazurin is converted to resorufin only in droplets containing cysteine.
b) Evolution of the mean fluorescence intensity throughout incubation of water-in-oil droplets generated with and without cysteine in Fluo-Oil 200.
c) Pictures of the mix of full and empty droplets throughout incubation.

In Figure 6, after 1 hour of incubation, the fluorescence difference between the two droplet populations (droplets containing cysteine and droplets without cysteine) is very slight. During incubation, the fluorescence level of droplets containing cysteine increases as the latter converts the resazurin into resorufin. This result is corroborated by the characterization of the fluorescence intensity. On the graph, the droplets containing cysteine show a constant increase in fluorescence intensity. Moreover, the droplets without cysteine show a constant fluorescence intensity for 4 hours. This result indicates that there is no crosstalk between the droplets for 4 hours. The recommended incubation time for cell viability experiments is 1-4 hours. Herein, we have demonstrated that by using Fluo-Oil 200 to generate droplets under optimal conditions, resazurin-based cell viability assays can be performed using droplet-based microfluidics.

CONCLUSION

In order to improve dye retention in droplet-based microfluidic experiments, the experimental conditions need to be optimized. This includes optimizing the surfactant concentration, but also the choice of oil. In this study, we showed that using Fluo-Oil 200 to generate the droplets significantly reduces dye leakage in microfluidic experiments.
Cell viability assay using resorufin can therefore be achieved in droplet-based microfluidics by using Fluo-Oil 200 under optimal experimental conditions.

To learn more about surfactants and other formulation products for droplet microfluidics, please visit
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Emulseo, 14 avenue Pey Berland 33600 Pessac, FRANCE
Email: contact@emulseo.com / Tel: +33 (0)5 35 54 10 06 / Website: www.emulseo.com