Application Note

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Rapid Evaluation of Spheroid Formation and Self-Renewal of Patient-Derived Glioblastoma Stem-Like Cells using OneFlow™

Virginie Neirinckx[1], Mariana Tsimacidis[2], Christina Nguyen[2], Quentin Graillet[2], Bernard Rogister[1], Stéphanie van Loo[2] and Natacha Coppieters[2]

1: Laboratory of Nervous System Diseases and Therapy, GIGA Neuroscience, GIGA Institute, University of Liège, Belgium.

2: LiveDrop, Avenue de l'Hôpital 11, 4000 Liège, Belgium.

Abstract

This work investigates glioblastoma stem-like cell (GSC) spheroid generation using the OneFlow™ droplet microfluidics platform. Controlled cell encapsulation enabled quantitative assessment of spheroid morphogenesis, viability, and proliferative potential. In multicellular droplets, GSCs rapidly aggregated into compact spheroids, with diameter reduction and increased roundness reaching a plateau within 24h. A good cell viability was maintained for up to 24h with a marked decline in viability observed beyond 48h in droplets, indicating an optimal extraction window for these cells at 24h. Spheroids recovered at 24h continued to grow and structurally matured in culture plates. Single-cell encapsulation demonstrated that isolated GSCs retain proliferative and spheroid-forming capacity after 72h. Collectively, these results validate OneFlow™ as a precise and efficient system for generating homogeneous 3D GBM models and for probing cellular dynamics, viability constraints, and stem-like behaviour in microfluidic environments.

Introduction

Glioblastoma (GBM) is the most aggressive and common malignant primary brain tumour in adults, characterized by rapid proliferation, diffuse infiltration, and resistance to conventional therapies [1]. Robust and reliable preclinical GBM models are required to address and interfere with these malignant phenotypes, to ultimately improve patient outcomes. Alongside the introduction of the cancer stem cell concept, GBM stem-like cell (GSC) cultures have emerged as powerful *in vitro* tools to study GBM biology, allowing long-term maintenance of initial tumour molecular features and functional properties, including stem-like traits [2], [3]. These GSC cultures grow as three-dimensional (3D) spheroids that can be used for many different cell-based assays, especially for high-throughput assays, including drug screening [4]. However, the generation of reproducible, size-controlled and homogeneous GSC spheroids for robust analysis remains challenging, and the study of GSC phenotype (especially at the single-cell level) is labour intensive and requires large amounts of consumables.

Aims and objectives

In the present study, we used two patient-derived GBM cell cultures (T018 and T033) to evaluate the OneFlow™ instrument as a novel tool for the rapid evaluation of two important criteria: the ability of GSCs to form 3D tumorspheres and to self-renew. In addition, this platform can be used for the generation of large numbers of homogeneous spheroids, ideal for drug screening and the development of personalised medicine while minimizing the amount of starting material required.

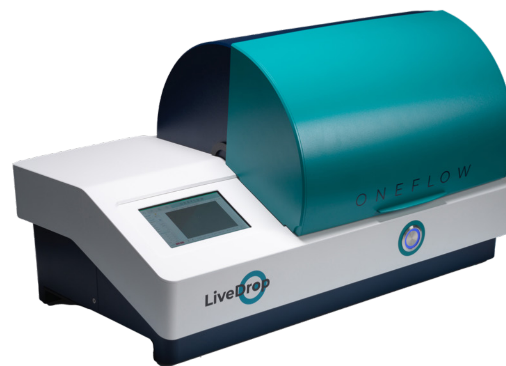

Figure 1. The OneFlow™ Instrument.

Materials and Methods

Cell culture

Human GBM samples were obtained in collaboration with the Neurosurgery department of the Liège University Hospital (CHU of Liège, Belgium) and the Liège University Biobank, in accordance with legal regulations on residual human body material and with approval from the ethics committee of the CHU of Liège. Patient-derived GBM cell cultures (T018 and T033) were established and maintained as previously described [5]. T018 and T033 GSCs were cultured as floating 3D tumourspheres, dissociated with accutase to obtain a single-cell suspension as described previously [6]. Cells were resuspended at a predefined cell concentration to obtain the desired number of cells per droplet and therefore per spheroid. Cell viability was monitored over time by adding SYTOX® Green Nucleic Acid Stain (20 nM) in the encapsulation medium (Molecular Probes, Thermo Fisher, S7020).

OneFlow™

OneFlow™ is a fully integrated droplet microfluidics instrument designed to is to encapsulate biological samples into uniform droplets at high-throughput, enabling workflows such as 3D cell culture and single-cell omics (Figure 1). It produces up to tens of thousands of droplets per second with exceptional uniformity and optimal use of the sample, offering tight control over droplet size and volume. The platform is highly versatile, supporting a wide range of droplet-based operations such as single-cell and small multi-cell encapsulation (up to around 300 cells per droplet), co-encapsulation of multiple reagents or cell types, droplet merging, and reagent addition. OneFlow™ combines flexibility with user-friendly features, including an integrated microscope and camera for real-time visualization, intuitive software, and SMART™ connectors.

Spheroid formation and recovery

For the formation of spheroids, cells are typically encapsulated in large droplets (8-10 nL) using a Drop-250 chip. The microfluidic chip is inserted in a SMART™ cartridge and placed in the OneFlow™ instrument. The chip is connected to the oil reservoir containing perfluorinated oil (Oil-10; LiveDrop, L161-201), PBS is loaded in the SMART reservoir using a pipette and pressure parameters are selected to form water-in-oil droplets. Droplet volume can be measured and adjusted live to obtain the targeted size. GSC cell suspensions were prepared as described above at $2x10^7$ cells/mL or $7.5x10^6$ cells/mL to obtain large or small spheroids (+/- 170 or 65 cells in 8.5 nL droplets), respectively. After encapsulation, droplets were collected in tubes, transferred with oil to a 96-well plate, and incubated for up to 48h in a conventional incubator.

The plate was covered with a Breath-Easy® sealing membrane to reduce oil evaporation while maintaining gas exchange. Spheroid formation was monitored by visualising the droplets under a conventional microscope. Once formed, spheroids were released from droplets using a PolyTetraFluoroEthylene (PTFE) membrane as previously described [7] and resuspended in medium for imaging and analysis (Figure 2).

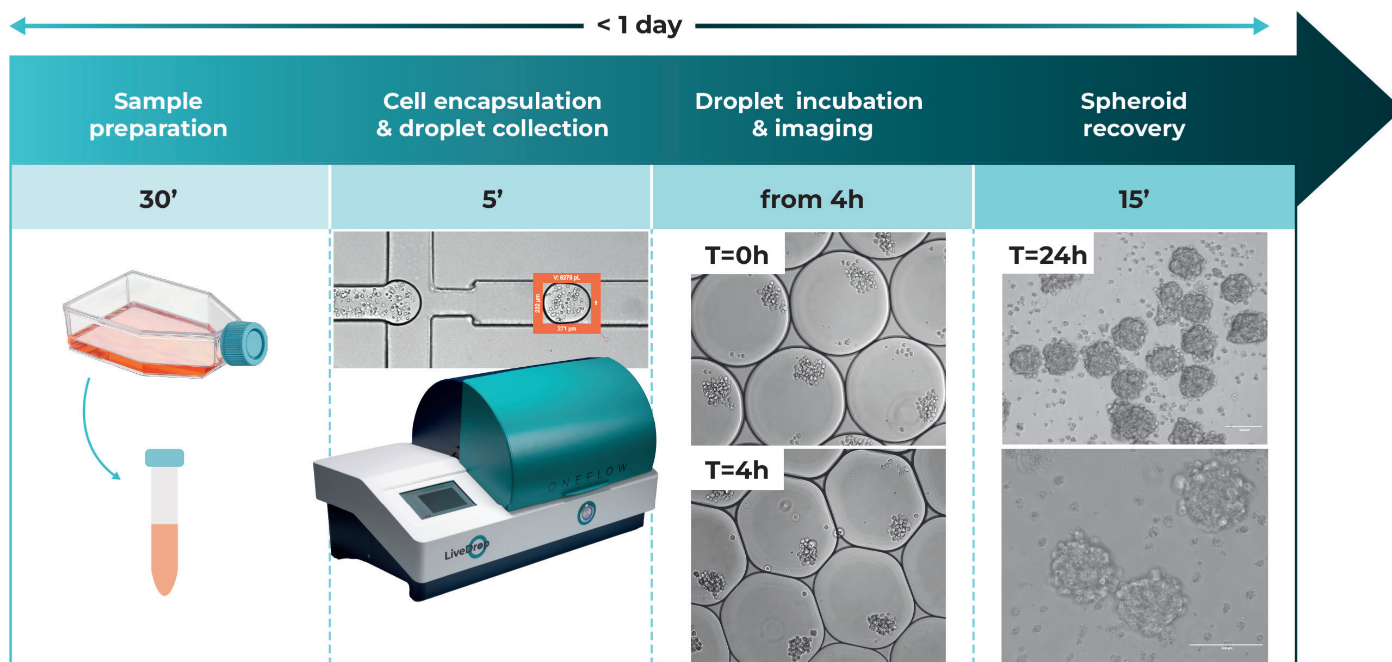

Figure 2. Summary of the workflow for the high-throughput manufacturing of spheroids. *A typical workflow for the formation of spheroids using the OneFlow™ can take less than one day. It involves the preparation of cells at the intended concentration, the encapsulation of cells in droplets using a Drop-250 chip, incubation of droplets from 4h up to several days depending on the cell type, and the release of spheroids from the droplets. Scale bars represent 100 µm. The orange frame is a software tool indicating the size (µm) and the volume (pL) of a selected droplet.*

Self-renewal study

To study the ability of GSCs to self-renew, T018 and T033 were encapsulated at a single cell per droplet, and self-renewal was evaluated after 3 days of incubation in droplets. GBM cells were prepared at $4.4x10^4$ cells/mL and encapsulated as described above in 4.5 nL droplets using a Drop-250. According to the Poisson law, these parameters generate approximately 82% of empty droplets, 16% of droplets with no more than 1 cell and <2% with more than 1 cell per droplet. After cell encapsulation, the emulsions were stored in a 96-well plate and incubated for up to 72h in a conventional incubator. Droplets were imaged, and the number of cells per droplet was evaluated at t=0 and t=72h.

Statistical analysis

GraphPad Prism (version 5.03) was used for statistical analysis and for making graphs. Multiple comparisons were performed with one-way ANOVA and a Bonferroni's Multiple Comparison Test was used for parametric analysis. A p value of ≤ 0.05 was considered as statistically significant. Data were obtained from three independent experiments and more than 100 spheroids per group were analysed.

Results and discussion

Patient-derived GSCs (including T018 and T033) have been previously characterised for their expression of stem-like markers, their ability to form neurospheres using conventional methods and to generate tumours in a xenograft model. The profiles of these cells differed from one another despite identical culture conditions, highlighting biologic variability across individuals, probably associated with different tumour profiles [5], [8], [9], [10]. In the present study, we have evaluated the ability of T018 and T033 to form spheroids in droplet microfluidics. When confined in small spherical droplets, cells are in close proximity and quickly adhere to form aggregates which compact and develop into spheroids. As the number of cells encapsulated into each droplet is tightly controlled, spheroids are homogeneous in size and in shape. The production of homogeneous spheroids at high-throughput (up to 50,000 spheroids in less than 1 minute) offers a great tool for drug screening. It is now well recognized that performing drug testing on 3D culture is critical, since spheroids more closely mimic *in vivo* tumour biology by preserving cell–cell and cell–extracellular matrix interactions, gradients of oxygen and nutrients. In addition, these 3D structures provide a physiologically relevant platform for studying tumour growth, invasion, stem-like features, and therapeutic resistance, thereby serving as valuable tools in preclinical glioblastoma research [11].

First, T018 and T033 were encapsulated at high number of cells per droplet (170 cells) to generate large spheroids. After 24h of incubation in droplets, T018 and T033 cells formed spheroids. However, T018 spheroids appeared more compact than spheroids formed with T033 cells (Figure 3A). To assess the compactness of spheroids over time, cells were encapsulated and images were taken just after encapsulation and after 4h, 24h and 48h. In parallel, cell viability was assessed by adding Sytox Green which penetrates and labels cells with permeable membranes. At t = 0, cells from both patients were observed in closed proximity within droplets. During incubation, spheroids appeared increasingly compact while green cells could be detected after 48h which indicates a decrease in cell viability after prolonged incubation within droplets (Figure 3B). The spheroid diameters and roundness were measured using Fiji software. As illustrated in Figure 3C, spheroid diameters decreased over time, while roundness increased. These results indicate that spheroids are compact with good cell viability and are ready to be released from the droplets for downstream analysis in just 24h.

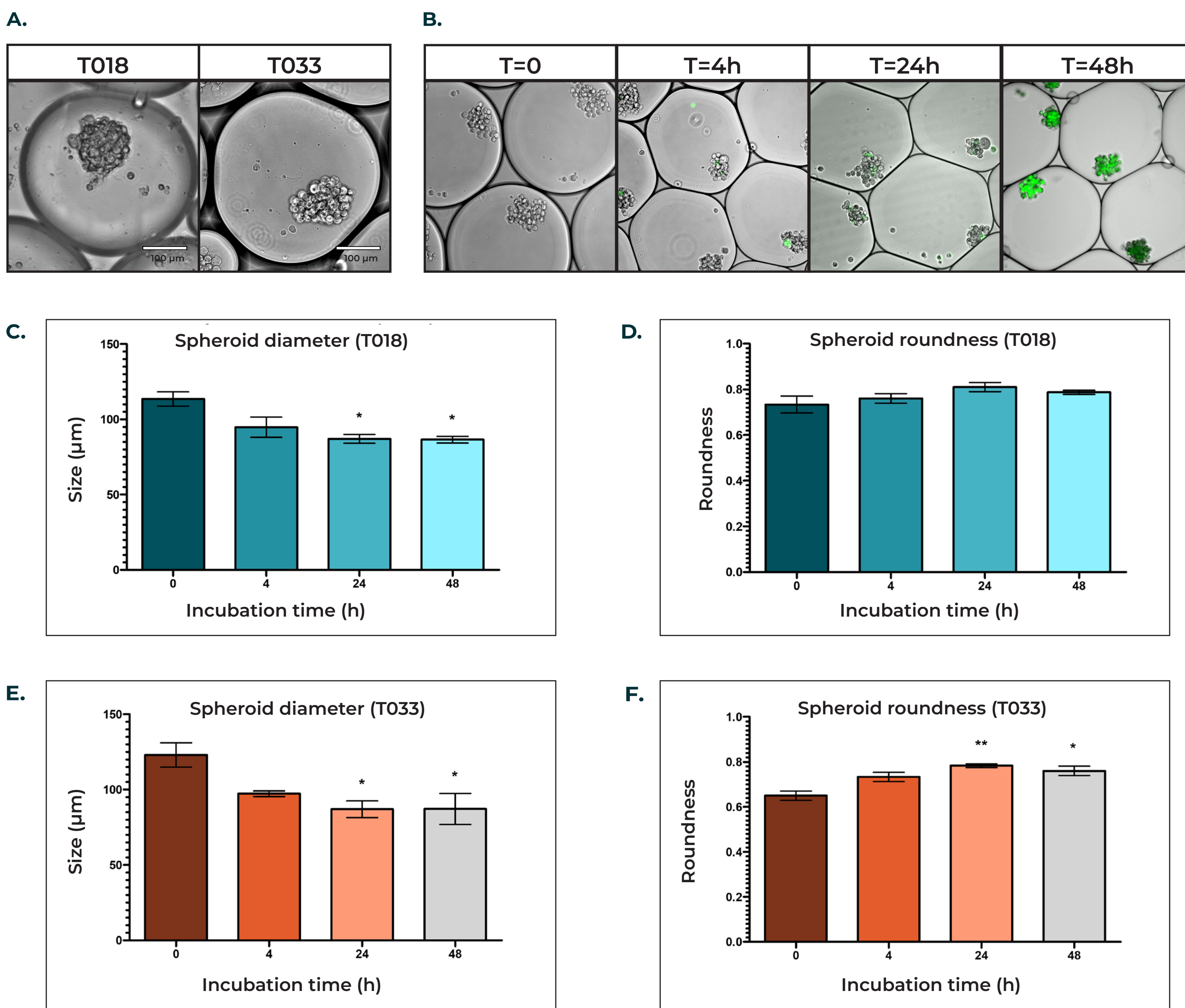

Figure 3. Formation of spheroids with patient-derived GBM cells using droplet microfluidics. *A) Representative images of spheroids of patient-derived GBM stem cells (T018 and T033) developed in droplets after 24h. Scale bars represent 100 µm. B) Representative images of T018 cells directly after encapsulation in droplets and after 4h, 24h and 48h. Sytox Green (green) was added to the droplets to monitor cell viability. Very few cells were labelled with Sytox Green up to 24h, with clear cell death observed only after 48h of incubation in droplets. Scale bars represent 170 µm. The spheroid diameter (C & E) and roundness (D & F) were measured with Fiji software for T018 (blue bars) and T033 (orange bars) directly after encapsulation and after 4h, 24h and 48h inside droplets. Data were obtained from three independent experiments, and are presented as mean +/- SEM. * $p < 0.05$, ** $p < 0.01$ relative to t = 0.*

As 24h of incubation provided optimal conditions for obtaining viable and compact spheroids with patient-derived GSCs, cells were released from the droplets after 24h and imaged just after release (t = 0) and after 24h and 48h in culture to monitor spheroid growth (Figure 4). Just after release from the droplets, spheroids were homogeneous in size and shape. As already observed in droplets, spheroids obtained with T033 cells seemed less compact than spheroids obtained with T018 cells. As expected, spheroid size and roundness increased overtime.

However, spheroid sizes became less homogeneous with increasing time in culture for both T018 and T033. The most likely explanation is spheroids merging, leading to larger spheroids. However, it is also possible that some of the cells included in the spheroids have slightly different stemness profile which could alter tumour growth. This hypothesis should be further investigated by comparing the gene expression profiles and the protein expression of larger spheroids compared to their smaller counterparts.

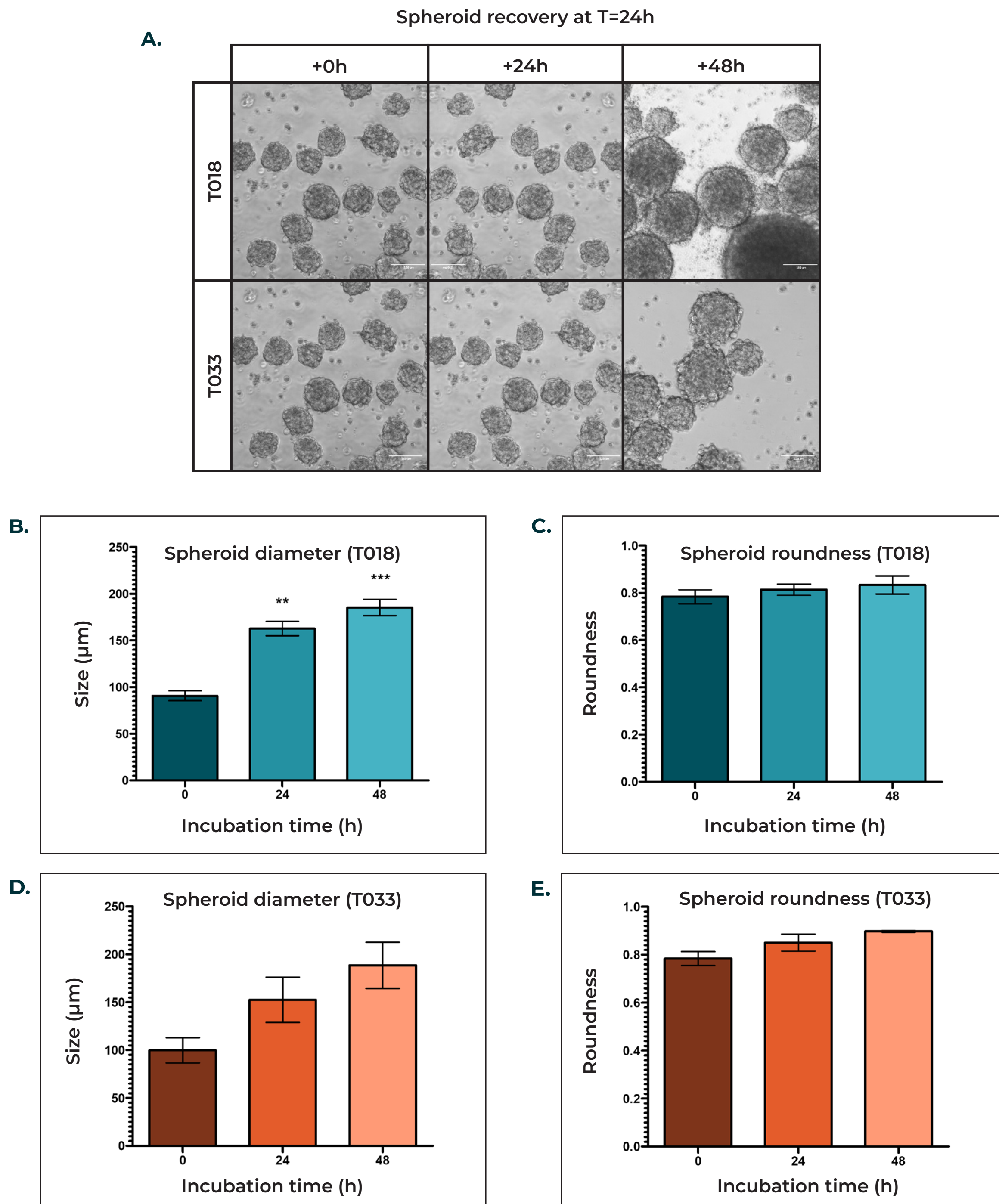

Figure 4. Spheroid growth after release from droplets. *A) Representative images of spheroids developed from T018 and T033 cells encapsulated in droplets for 24h before being released and imaged directly thereafter (+0h) and after an additional 24h and 48h in culture. Scale bars represent 100 µm. Spheroid diameters (B & D) and roundness (C & E) were measured with Fiji software for T018 (blue bars) and T033 (orange bars) directly after release from droplets (0h) and after 24h and 48h in culture. Data were obtained from three independent experiments, and are presented as mean +/- SEM. ** p < 0.01, *** p < 0.001 relative to t = 0.*

After confirming the ability of T018 and T033 to form tumorspheres, a similar workflow was used to evaluate their ability to self-renew. Cells were encapsulated in droplets as single cells, and images were taken at t = 0 and after 72h to count the number of cells per droplet. Based on the Poisson distribution, approximately 82% of droplets were expected to be empty, 16% to contain a single cell, and fewer than 2% to contain more than one cell. At t = 0, this prediction was confirmed by manual cell count both for T018 (81.93%, 16.17% and 1.9%) and T033 (82.83%, 15.27% and 1.89%). As shown in Figure 5, after 72h of incubation, the percentage of droplets containing a single cell decreased and the percentage of droplets with 2 or more cells increased. These results indicate that cells were capable of dividing and confirm the ability of these patient-derived GSCs to self-renew.

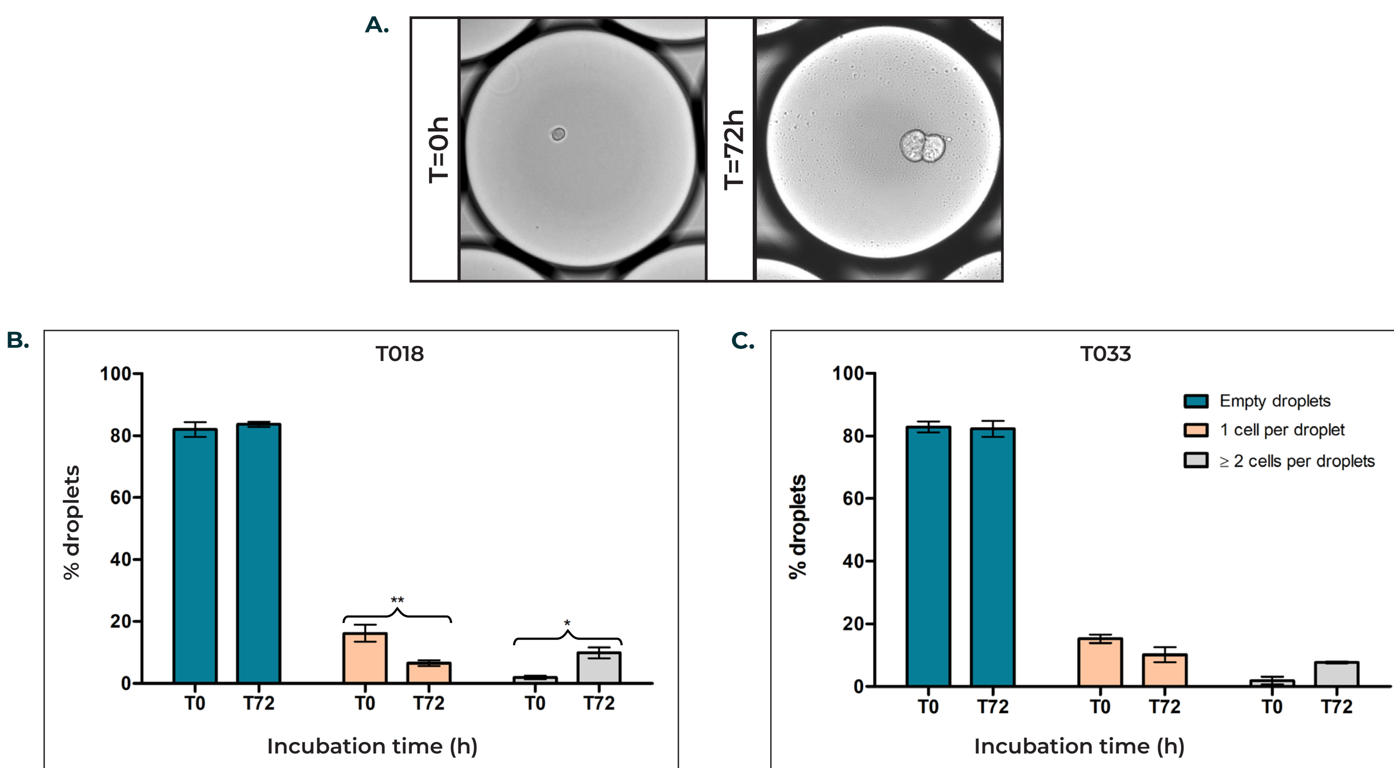

Figure 5. Evaluation of the ability of cells to self-renew. *Patient-derived GBM cells (T018 and T033) were encapsulated as single cells in droplets and incubated for 72h. Droplets were imaged and the number of cells per droplet was evaluated. (A) Representative images of droplets containing GBM cells (T018) just after encapsulation (t = 0h) and after 72h of incubation (t = 72h). (B & C) Percentage of droplets containing either no cells (empty droplets, blue bars), a single cell (orange bars) and two or more cells (grey bars) just after encapsulation (t = 0) and after 72h for T018 (B) and T033 (C). Data were obtained from three independent experiments, and are presented as mean +/- SEM. * $p < 0.05$, ** $p < 0.01$ relative to t = 0 within each group.*

Conclusion

Results from this study demonstrate that microfluidic droplet biology enables rapid evaluation of tumoursphere-forming capacity and assessment of functional properties, such as self-renewal, at the single-cell level. Indeed, we showed that patient-derived GSCs could rapidly form compact spheroids within droplets and were able to self-renew. Spheroids were homogenous in size and shape, produced at high-throughput (> 50,000 spheroids in less than 1 minute) and able to grow in culture once released from droplets. In addition, cells obtained from two different donors showed different behaviour with one sample forming more compact spheroids and showing a tendency for faster self-renewal. The OneFlow™ instrument is a user-friendly platform for the fast and reproducible production of thousands of homogeneous spheroids, which has the potential to become the new standard for drug screening and the development of personalised medicine.

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Acknowledgments

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