Application Note 13

Cyto-Mine® - an Automated Platform for Cell Line Development (CLD)

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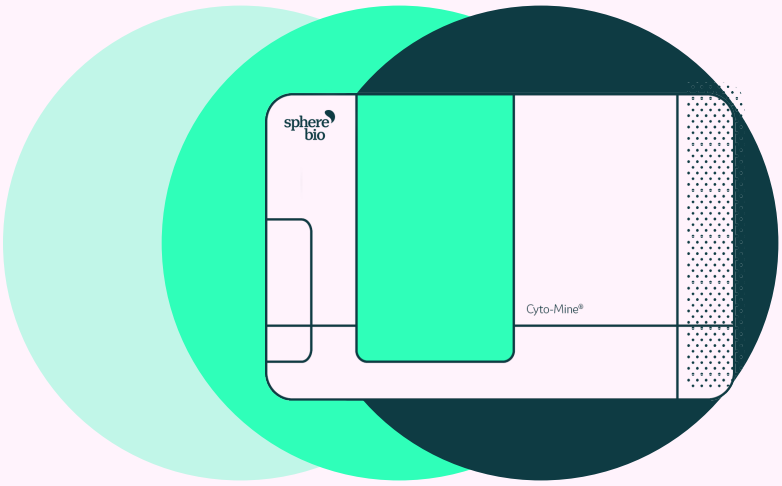
Background

This Application Note demonstrates the suitability of the Cyto-Mine® platform for isolating and cloning cells within a Cell Line Development workflow. We highlight how the automated Cyto-Mine® workflow has comparable cell viability and proliferation to traditional, manual Limiting Dilution Cloning methodology.



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Introduction

Over the last three decades antibody-based therapeutics have become the predominant class of new drugs within the pharmaceutical industry. The success of these therapeutics is due to their high specificity, adaptability and reduced side effects compared to older small-molecule based approaches. Thus, the pharmaceutical industry has invested a great deal of effort into the development of stable cell-lines for antibody expression, optimized for manufacturing of these therapeutics (1-2).

Despite multiple methods and instruments developed to be utilized in the CLD process, this field still faces significant challenges. These factors include complex, extended workflows, labour-intensive processes, and high reagent costs. Thus, high-speed, miniaturized, automated systems, able to screen and isolate the best performing single cells have become essential in making CLD processes more efficient and cost-effective. However, single cell isolation during cloning processes can put strain on a cell's viability and productivity. Therefore, techniques used for screening, titer assessment and isolation of rare cells should be designed to have minimal detrimental effect on cell health.

The Cyto-Mine® Single Cell Analysis and Monoclonality Assurance system is an automated picodroplet-based platform (Figure 1). Here single-cells are encapsulated within picoliter-sized aqueous reagent droplets in an oil carrier fluid (Cyto-Surf® B) and incubated to allow accumulation of secreted antibodies. Productivity of captured cells is evaluated inside picodroplets using various methods including FRET assays. Droplets containing single cells with high productivity for a protein of interest are isolated and dispensed into microtiter plates (MTP). This process results in isolating individual high-producing cells into MTP wells with dispensing accuracy of 98.9% (3). To mitigate any adverse impact on cell viability, the overall Cyto-Mine® workflow has been streamlined to just a few hours.

In this Application Note we examine the effect of each step of the Cyto-Mine® workflow on the viability of cells and compare the data to traditional Limiting Dilution Cloning (LDC).

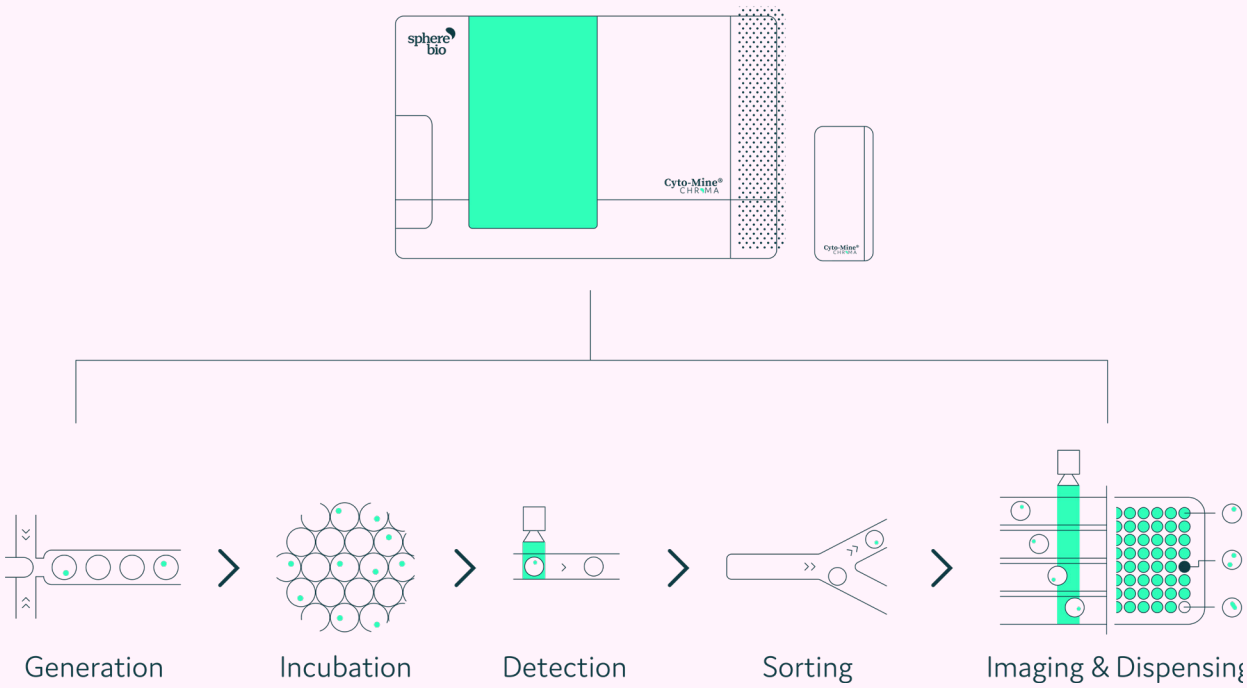


Figure 1. A Cyto-Mine® workflow. Integration of single cell screening, sorting, isolation, and verification using a fully integrated microfluidic process.

Methods

Mouse IgG Förster Resonance Energy Transfer (FRET) assay

FRET assays were set up (see 4 for details). Briefly, FRET probes (AF488 and AF594 labelled anti-mouse IgG) were used to detect target mouse IgG in the supernatant of cells within the droplets. The binding of the FRET probes to the target antibody results in formation of a three-molecule complex, leading to specific and sensitive detection of secreted IgG at single cell level.

Cyto-Mine® Cell Outgrowth

Single cells isolated by Cyto-Mine® were dispensed into 96 or 384-well plates and their outgrowth was evaluated after 2 weeks of culture. All experiments were designed to work within a single cell-per-droplet range ($0.15x10^6$-$0.3x10^6$ cells/mL) based on Poisson distribution calculation. To minimize clone-specific bias, all experiments were conducted on multiple lines or clones of mouse hybridomas, each secreting different levels of mouse IgG.

Limiting Dilution Cloning (LDC) Outgrowth

LDC was performed as described previously (3). LDC (λ=0.5) was set up in parallel to every Cyto-Mine® Cell outgrowth experiment to provide a baseline for proliferation of cells and for data comparison to Cyto-Mine cell outgrowth. Briefly, cells were serially diluted to a final density of 5 cells/mL, and seeded at 100 µL/well, resulting in an expected 40% plate occupancy (at least one cell per well) based on a Poisson distribution.

Results

The Cyto-Mine® workflow begins with sample preparation, where cultured cells are washed to remove secreted antibodies (Figure 1). The cells are then resuspended in encapsulation medium containing FRET reagents and filtered through a cell strainer to exclude any cell aggregates. To determine any adverse effect of sample preparation and the presence of encapsulation/FRET assay reagents on cell viability, cells were prepared in encapsulation medium (containing FRET reagents) and left at room temperature for 5 hours (mimicking total exposure time in Cyto-Mine® for the full process).

Following incubation, cell viability and proliferation was assessed using NucleoCounter NC-3000 and LDC, respectively (Figure 2) and compared to the same measurements made on the original cell culture. Data confirmed that cell preparation and subsequent incubation did not affect cell viability when compared to the original cell cultures, indicating that these steps are not toxic to cells (Figure 2A). Furthermore, the cell outgrowth data highlighted no significant effect of cell preparation and incubation on proliferative ability of cells (Figure 2B and C).

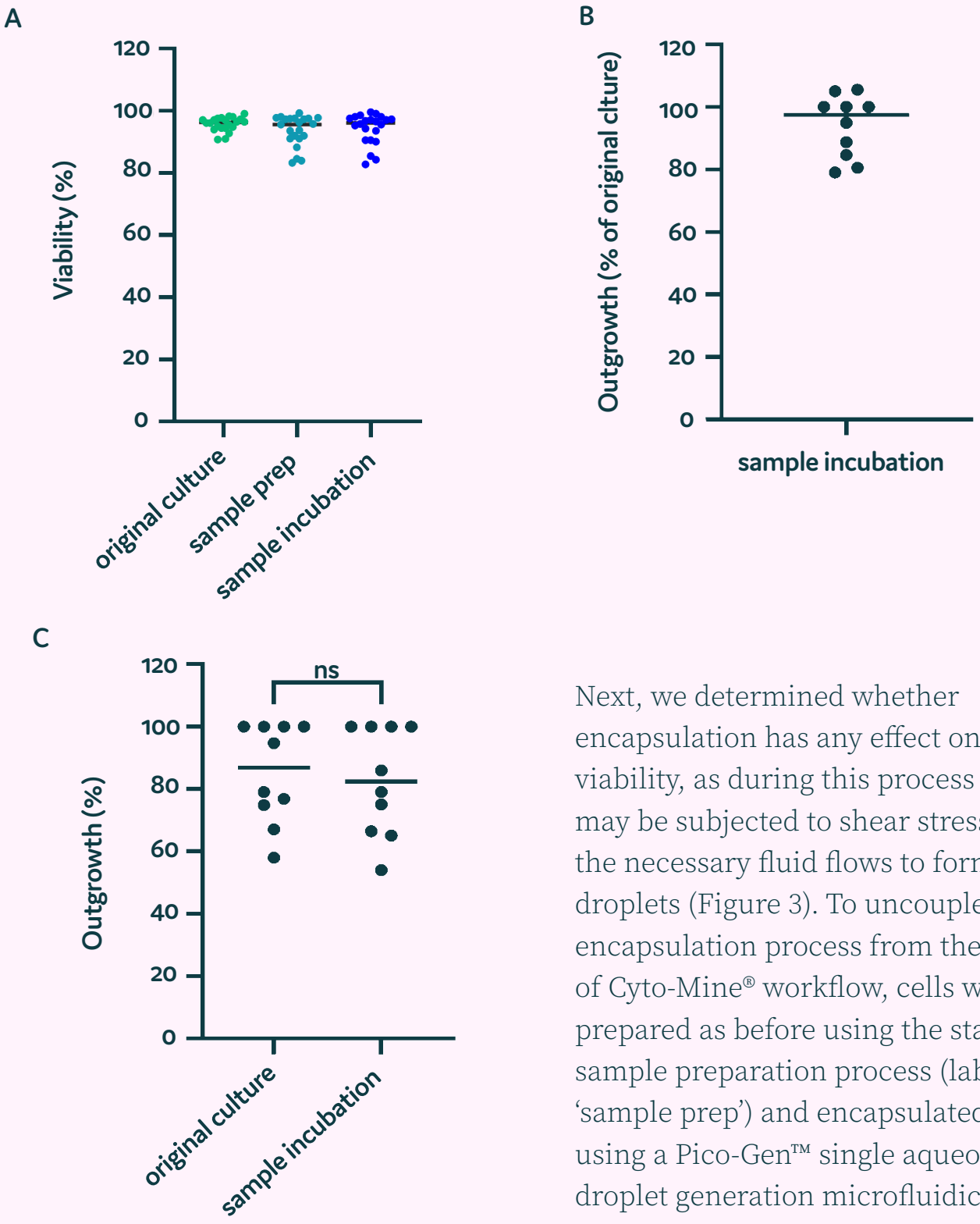


Figure 2. Effect of sample preparation on cell viability. The viability of five different hybridoma clones was determined (A) in original culture cells, after Cyto-Mine® sample preparation and after sample incubation for 5h. Viability was measured by NucleoCounter-3000, using staining with Acridine Orange (AO) and DAPI. n=21 separate experiments. (B) Outgrowth (LDC) of cells from original sample, or from sample after 5h incubation. n=10 (C) Outgrowth as in (B) experiment was normalized to its LDC control. n=10 separate experiments. Data analyzed in GraphPad Prism 10.0.1 using one-way ANOVA revealed no significant difference.

Next, we determined whether encapsulation has any effect on cell viability, as during this process cells may be subjected to shear stress from the necessary fluid flows to form droplets (Figure 3). To uncouple the encapsulation process from the rest of Cyto-Mine® workflow, cells were prepared as before using the standard sample preparation process (labelled 'sample prep') and encapsulated using a Pico-Gen™ single aqueous droplet generation microfluidic biochip and manual syringe pump (see 5 for details), resulting in droplets with similar volume (~450pL) and composition as on Cyto-Mine®.

Droplets were then incubated for 2 hours at 37°C (mimicking incubation time in Cyto-Mine®) before cells were chemically released from the droplets using Pico-Break™ (labelled post-experiment). Cell viability and proliferation were determined with NucleoCounter NC-3000 and by LDC, respectively. As shown in Figure 3, the encapsulation did not negatively affect cell viability (Figure 3A), and cells post release from droplets were able to proliferate to produce colonies (Figure 3B).

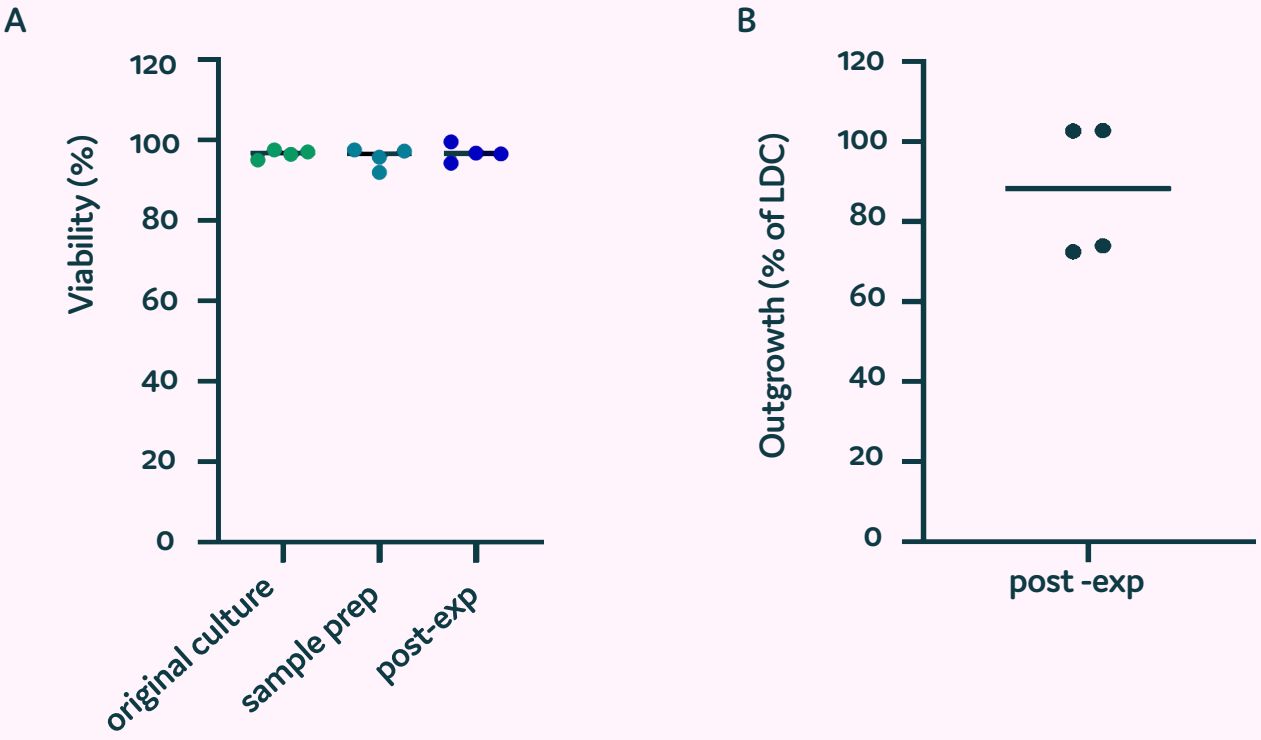


Figure 3. Effect of encapsulation on cell viability. Four different hybridoma clones were prepared in picodroplet encapsulation generation. Cells were encapsulated using manual syringe pump (pre-experiment). Following a 2h incubation, the emulsion was broken to recover cells (post-experiment). (A) Viability of cells was measured using NucleoCounter-3000 and (B) subjected to LDC in 96-well plates.

For typical Cyto-Mine® CLD workflows, it is sufficient to conduct a 2- hour incubation for sensitive detection of protein secretion. However, for other applications or slower secreting cells longer incubation in Cyto-Mine® may be necessary. As such we wished to determine the feasibility of longer incubation of cells within Cyto-Mine®. To this end, cells were sorted and dispensed after 2, 4 or 16 hours of incubation in Cyto-Mine® and clonal outgrowth was determined following standard procedure (Figure 4). The first scoping experiment clearly showed that although shorter incubation delivered improved outgrowth, prolonged incubation in picodroplets *in-situ* (on instrument) was feasible (Figure 4A).

Further analysis of experiments focusing on prolonged incubation (16h) only, demonstrated that prolonged incubation results in ~50% of LDC outgrowth performance (Figure 4B). Interestingly, the FRET signal looked similar in its magnitude and size of positive population for cells regardless of their incubation time (data not shown), further confirming feasibility of this approach for future secretion studies.

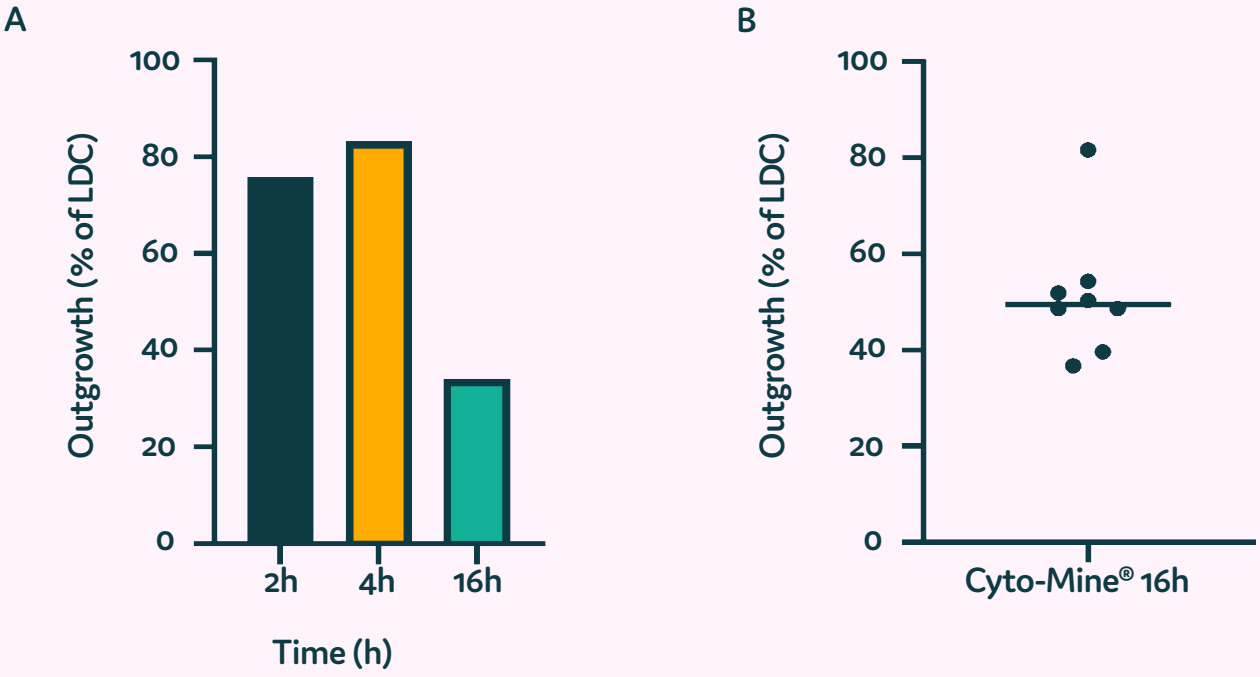
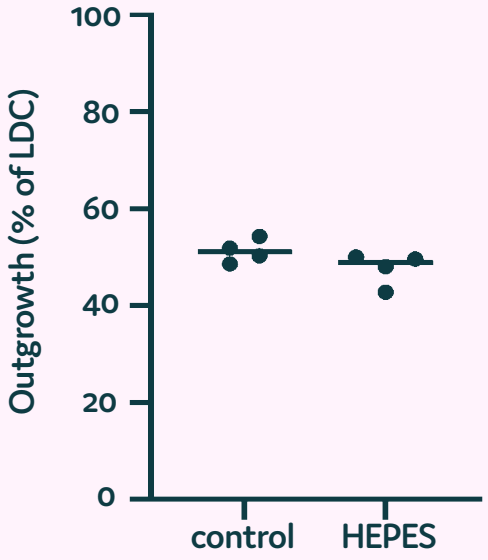


Figure 4. Effect of prolonged incubation of cells in Cyto-Mine® on cell viability. (A)- outgrowth from representative time-course experiment, incubations of 2h, 4h or 16h on Cyto-Mine®. (B) Statistical analysis of Cyto-mine® experiments (n=8), when cells were incubated for 16 hours in droplets. Comparison of outgrowth data when normalized to paired LDC is shown below.

As prolonged cell culture normally requires exogenous CO_2 to maintain the pH in culture media, we tested whether the presence of an organic buffer such as HEPES would improve cell growth following Cyto-Mine® based cell selection. Interestingly, supplementing culture media with this buffer did not significantly affect Cyto-Mine® cell outgrowth compared to control condition, indicating that use of buffers which optimize pH may not improve cellular proliferation after overnight incubation in Cyto-Mine® (Figure 5).



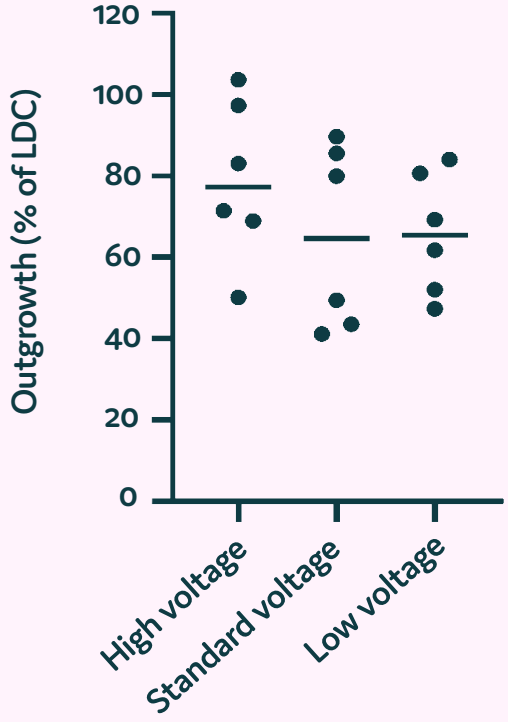


Figure 5. Effect of HEPES buffer on cell viability during prolonged incubation in Cyto-Mine®. Cells were incubated in Cyto-Mine® for 16h in the absence (control) or absence of HEPES buffer (50 mM), before being dispensed into 96 well plates. Outgrowth of cells was assessed after 2 weeks. n =4 experiments, each comprised of two Cyto-Mine® dispensed plates and two LDC plates.

Figure 6. Effect of sorting voltage on cell viability. Cells were exposed to high (1600 mV), standard (1200 mV) or low (950 mV) voltage during sorting on Cyto-Mine®. Outgrowth of cells dispensed from Cyto-Mine® was determined following 2 weeks of culture. This assessment was done on the same batch of cells during the same Cyto-Mine® experiment, resulting in three rounds of sorting/experiment. Multiple experiments (n=6) were conducted to collect statistically significant measurements and the different voltage settings were done in a different order in every experiment to minimise bias towards order of events. Data analysed in GraphPad Prism 10.0.1 using one-way ANOVA revealed no significant difference between the three groups of measurements.

Cyto-Mine® utilizes electric fields to generate forces to displace droplets perpendicular to the flow trajectory during sorting, allowing positive droplets to be eventually dispensed into MTP. Applying high voltage fields on living cells may subject these to stress, negatively impacting cell viability and survival. Although cells within picodroplets are somewhat electrically shielded from stress posed by high voltage, we determined whether the maximum voltage used during the sorting process in Cyto-Mine® has any effect on cell survival (Figure 6).

To this end, different levels of voltage (high- 1,600 mV; medium (standard)- 1,200 mV; low- 950 mV) were applied during sorting, and cell outgrowth was determined in comparison to the LDC control (Figure 6). As evident from Figure 6, all voltage settings resulted in similar level of outgrowth, indicating that changing the Cyto-Mine® voltage used for sorting does not have any significant effect on cell survival. One of the main requirements of a CLD workflow is the ability to isolate a single cell, thus Cyto-Mine® is designed to dispense a single droplet in each well of the output MTP in a reliable and

accurate manner. Every droplet is dispensed within a nanolitre volume drop of carrier oil (Cyto-Surf® B), that will remain with the dispensed cell in the MTP well.
To determine whether the presence of this oil has any adverse effect on cell outgrowth, we seeded cells (using LDC protocol) into plates containing single empty droplet per well (no cells), that were dispensed using Cyto-Mine®. As control, cells were seeded according to LDC procedure (plates containing media only). As shown in Figure 7A, no significant effect on cell outgrowth was detected in plates containing oil compared to control cultures, and cells were often seen growing in close proximity to the occasionally formed oil drops (Figure 7B), further confirming its biocompatibility.

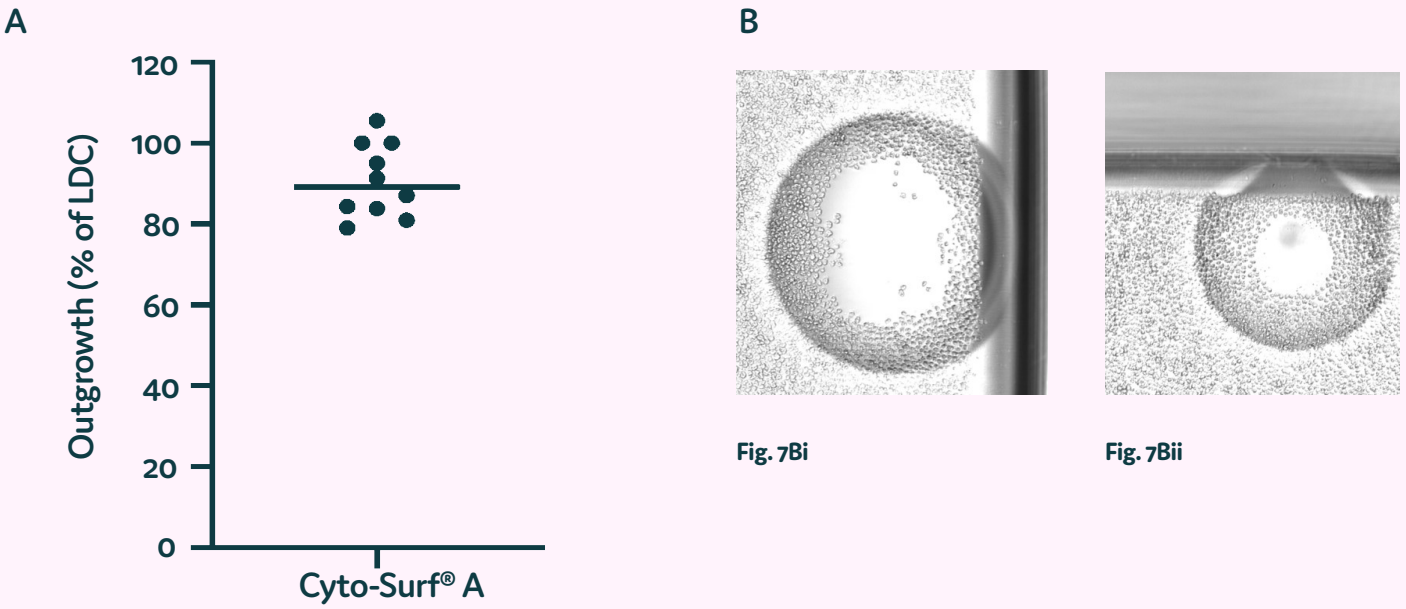


Figure 7. Effect of presence of Cyto-Surf® A on cell viability. Empty unoccupied droplets were sorted and dispensed (by Cyto-Mine®) into 96 well plates, pre-filled with small volume of culture media. Control plates were set up, where the wells were filled with media only. Cells were then seeded either into control plates or into plates containing droplets. Following a 2-week culture, the outgrowth of cells was analyzed. (A) The outgrowth data were normalized to LDC data. n= 8 experiments, each comprised of two Cyto-Mine® dispensed plates and two LDC plates. (B) Shows images of oil drops detected during analysis from wells containing grown colony. Images were taken using NyOne cell imager.

Finally, when outgrowth data, collected over three years from cells isolated by Cyto-Mine®, were compared to data from paired LDC assays, only a small difference (4.9%) in the average cell proliferation was observed between the two methods (Figure 8A). This was further highlighted when the data from each Cyto-Mine® experiment was normalized to its LDC control assay, demonstrating that Cyto-Mine® mean value for outgrowth is close to its LDC control (95.13%) (Figure 8B).

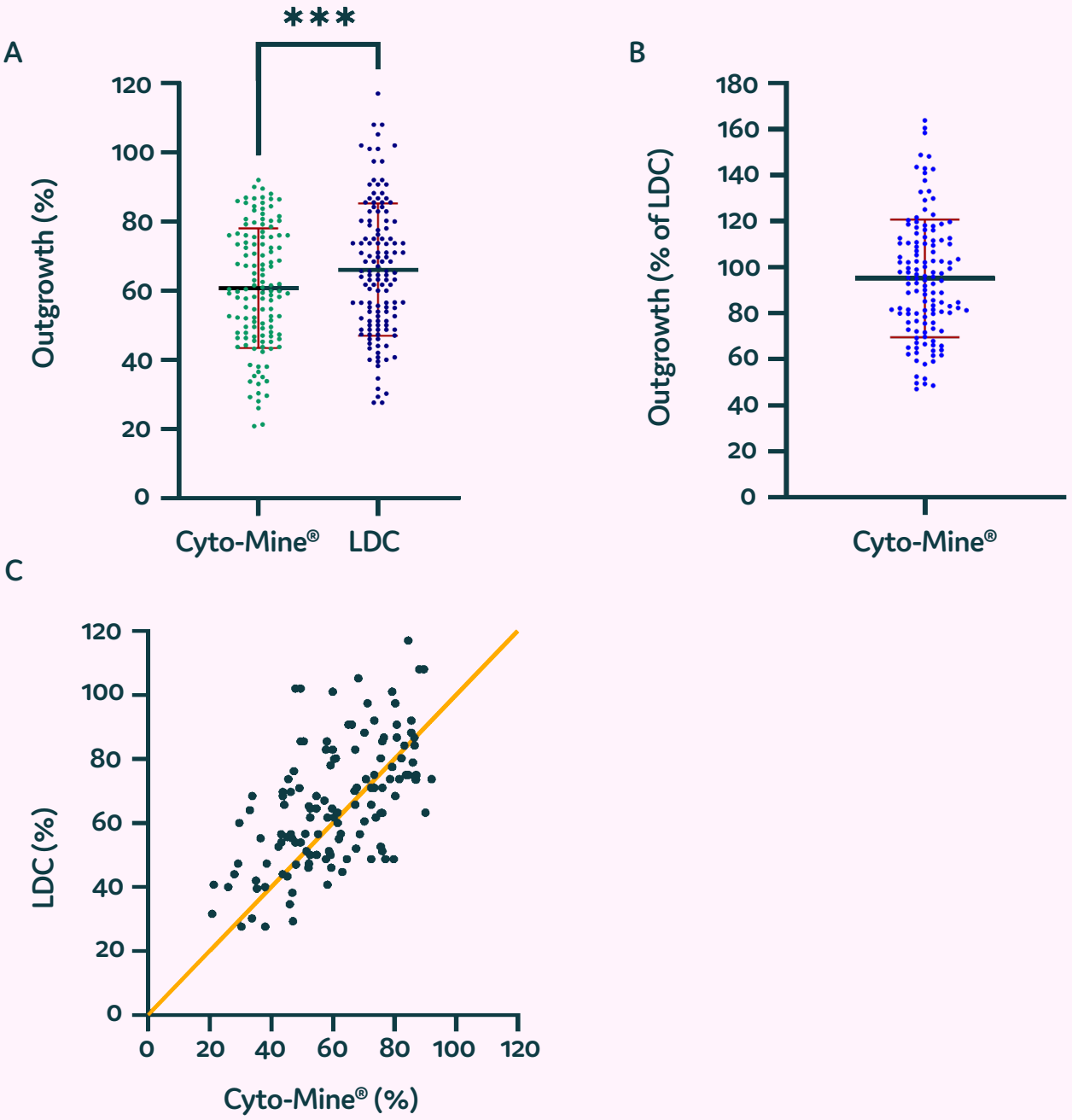


Figure 8. Statistical analysis from 130 different experiments presented as Cyto-Mine® versus LDC outgrowth. (A) Outgrowth data for cells dispensed by Cyto-Mine® was compared to LDC. Mean Cyto-Mine® = 60.7%, mean LDC= 66%. Statistical analysis performed in GraphPad Prism v. 10.0.1; ***P-value= 0.0004. (B) Cyto-Mine® outgrowth data was normalized to the relevant LDC data. Mean Cyto-Mine®= 95.13%. (C) Cyto-Mine® outgrowth data was compared to the relevant LDC outgrowth data. A correlation between the two datasets was determined using GraphPad Prism v. 10.0.1. n=130 experiments, each comprised of two Cyto-Mine® dispensed plates and two LDC plates.

Comparing the two datasets also highlights correlation between the two sets of data (corelation coefficient Pearson r=0.5846), suggesting that single cell isolation using Cyto-Mine® has minimal effect on cell growth compared to the manual cloning processes (Figure 8C).

Conclusion

In this Application Note we studied the viability of cells when exposed to different stages within a Cyto-Mine® workflow for cell line development applications and determined any detrimental effect on cellular proliferation. The data presented in this Application Note underscores the suitability of Cyto-Mine® in isolating and cloning cells within a CLD process workflow. Furthermore, we demonstrate that this process has minimal impact on cell viability and proliferation when compared to the manual LDC method.

References

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