Application Note 09

Cell Engineering in Picodroplets

Introduction

Picodroplet microfluidics is rapidly becoming the technology of choice for single-cell analysis, providing an easy-to-use tool to encapsulate single cells in picolitre-volume aqueous droplets (picodroplets) at high throughput rates of thousands of droplets per second. This powerful and sensitive technique offers significant cost and efficiency advantages, including:

High-throughput workflow: they provide the ability to isolate and analyze hundreds of thousands to millions of cells in one experimental run.

Precisely defined microenvironment: they compartmentalize single cells in a controlled environment for individual engineering, screening, and characterization.

Highly concentrated volumes: they increase detection sensitivity with higher concentrations of key ingredients trapped in picodroplets, reducing diffusion.

Low operating costs: they miniaturize critical steps to a picolitre volume, resulting in reduced reagent costs.

Integration and automation: they offer a low-risk, low-effort option to achieve streamlined workflows and reduce labor-intensive processes.

An additional advantage of this approach is that picodroplets provide a cell friendly culturing environment. Depending on the assay and application of interest, cells can be cultured in these picodroplets for a couple hours, to up to several days. The ability to culture the cells for various lengths of time has opened opportunities for a wide range of applications including antibody discovery, cell line cloning and cell engineering.

This application note provides an overview of how picodroplets can be used in cell engineering. We begin by evaluating the compatibility of reagents for chemical-based

transfection with picodroplets. Then introduce editing steps of increasing complexity to address the various viral and non-viral delivery systems utilized in the research community. These steps progress from conventional plasmid-based transfection to viral-based transfection and conclude with CRISPR-Cas9 delivery into mammalian cells using automated instrumentation.

Compatibility of cells and reagents with picodroplets

Before undertaking the genome editing experiments, a selection of widely used transfection reagents were chosen for compatibility testing with picodroplets technology, including liposome-cationic lipids, non-liposomal lipids, activated dendrimers, and lipopolyplexes (Figure 1A). During this test, the transfection reagents were mixed in a ratio of 1:100 with encapsulation media to mimic standard transfection protocols, and 400 pL picodroplets were generated using syringe pumps. Then picodroplet stability was assessed under microscopy after 2 and 24 hours incubation at 37°C (Figure 1B). The results confirm that picodroplet generation and stability are not affected by different transfection reagents across all experiments.

A

Group of reagents	Specific reagent	Supplier
Liposomes-cationic lipids transfection	Lipofectamine & Lipofectamine 3000	Thermo Fisher Scientific
Non-liposomal lipids	Fugene	Promega
Activated den-drimers	PolyFect	Qiagen
Lipopolyplexes	TransIT	Mirus

B

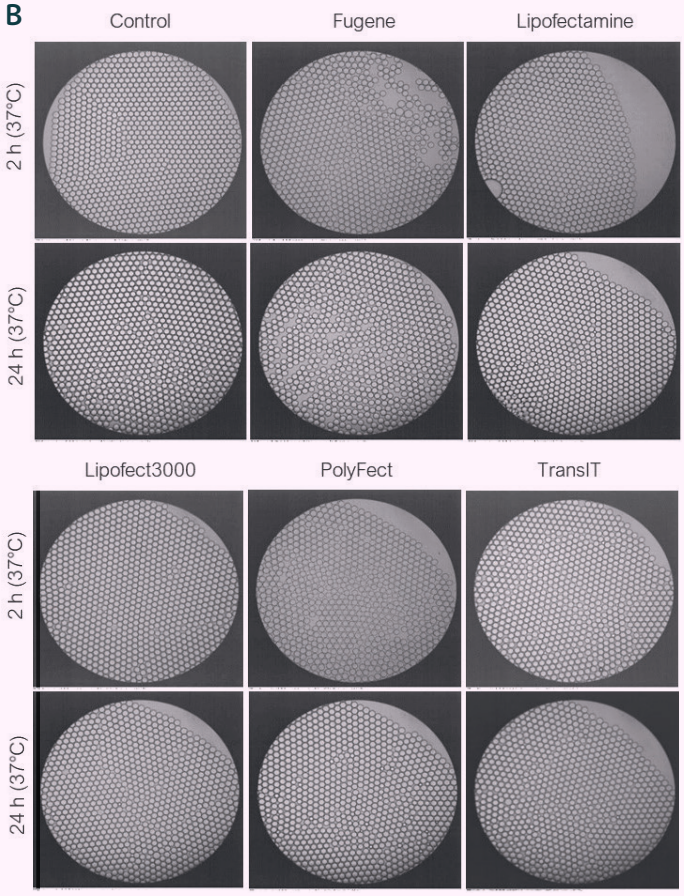


Figure 1. Compatibility of picodroplets with different transfection reagents.

Cell engineering in picodroplets experiments

Cell engineering in picodroplets was then successfully performed using different viral and non-viral delivery systems. These include plasmid-based transfection, viral-based transfection and CRISPR-CaS9 delivery into mammalian cells using automated instrumentation.

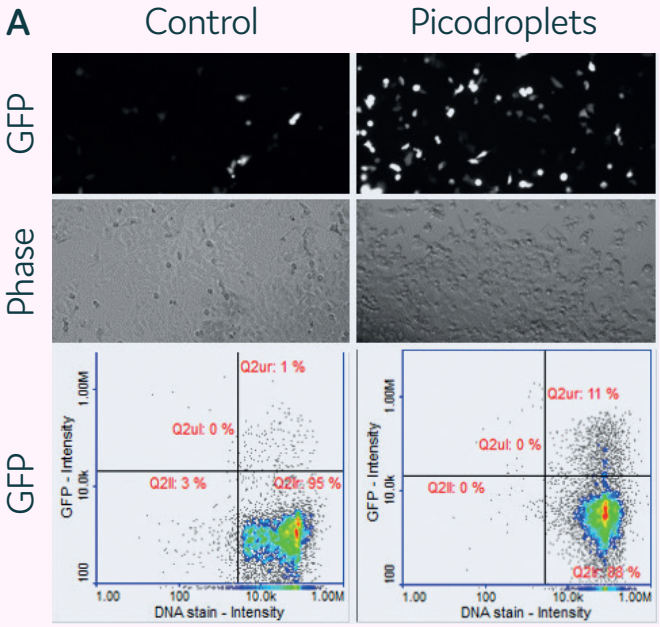


Plasmid-based transfection in picodroplets

In a plasmid-based transfection study, HCT116 cells ($2.5x10^5$ cells) and a transfection cocktail of 0.05 µg DNA (GFP)/0.1 µL Lipofectamine 3000 /150 µL media were co-encapsulated in 300 pL picodroplets (Figure 2A). As a control, pre-seeded cells were transfected with an identical transfection mix according to the manufacturer's protocol.

Picodroplets containing cells and transfection reagents were incubated for 2 hours at 37°C. The cells were recovered from picodroplets by chemically inducing the coalescence of the picodroplets, using Pico-Break™. The recovered cells were washed by centrifugation, then further cultured in fresh culture medium. Fluorescent images were taken after culturing for 24 hours to assess success rate of cell transfection. After 48 hours of culturing, quantitative measurements of GFP protein expression levels were carried out for both control and the picodroplet samples (Figure 2B).

Micrographs and quantitative measurements of GFP expression of transfected cells in NucleoCounter® NC-3000™ Advanced Image Cytometer. The quantitative summary of six different experiments indicates that transfection worked efficiently in picodroplets and may potentially be higher in picodroplets than conventional bulk transfection.



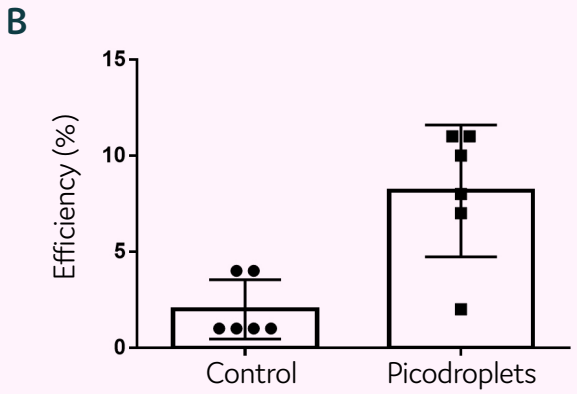


Figure 2. Plasmid-based transfection of HCT116 mammalian cells in picodroplets.

Viral transduction in picodroplets

Jurkat cells (1x10^6 cells) and EGFP-Lenti Genecopoeia virions (MOI=2) were co-encapsulated in 400 pL picodroplets. The picodroplets were then incubated for 48 hours at 37°C and imaged using a fluorescent microscope. Transduction efficiency was assessed by measuring GFP expression in Sphere Bio's Pico-Mine® system.

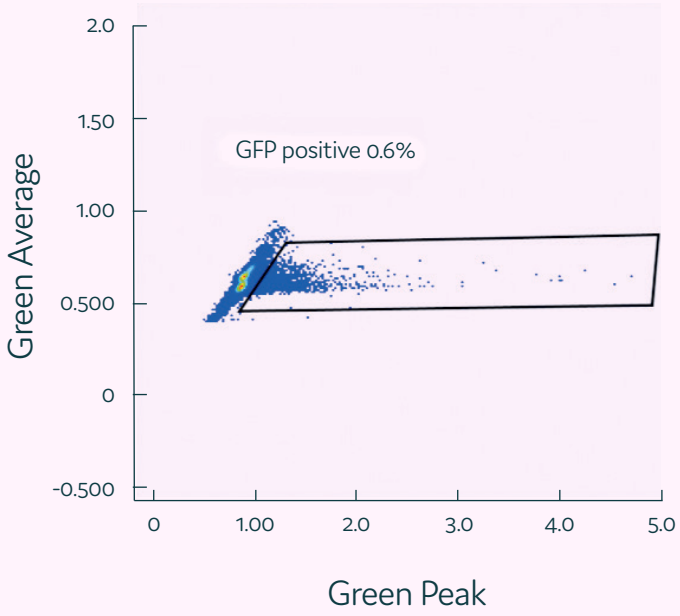


Figure 3. Viral transduction of mammalian cells.

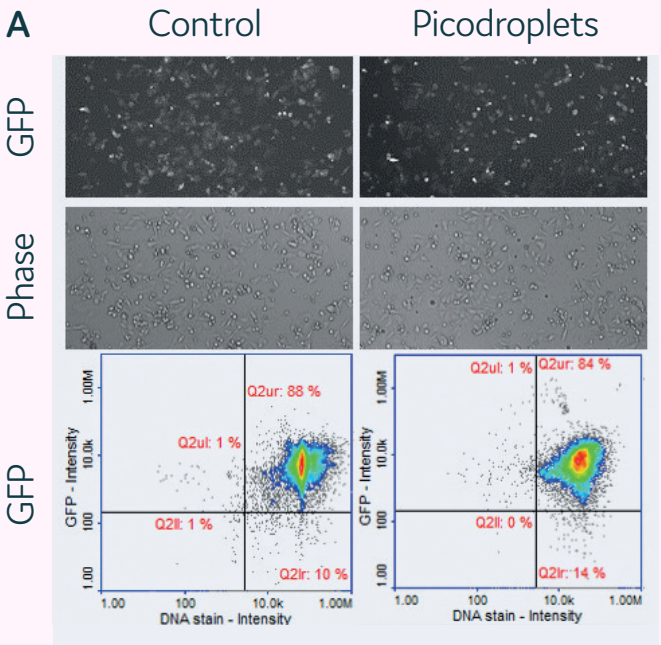
mRNA transfection in picodroplets

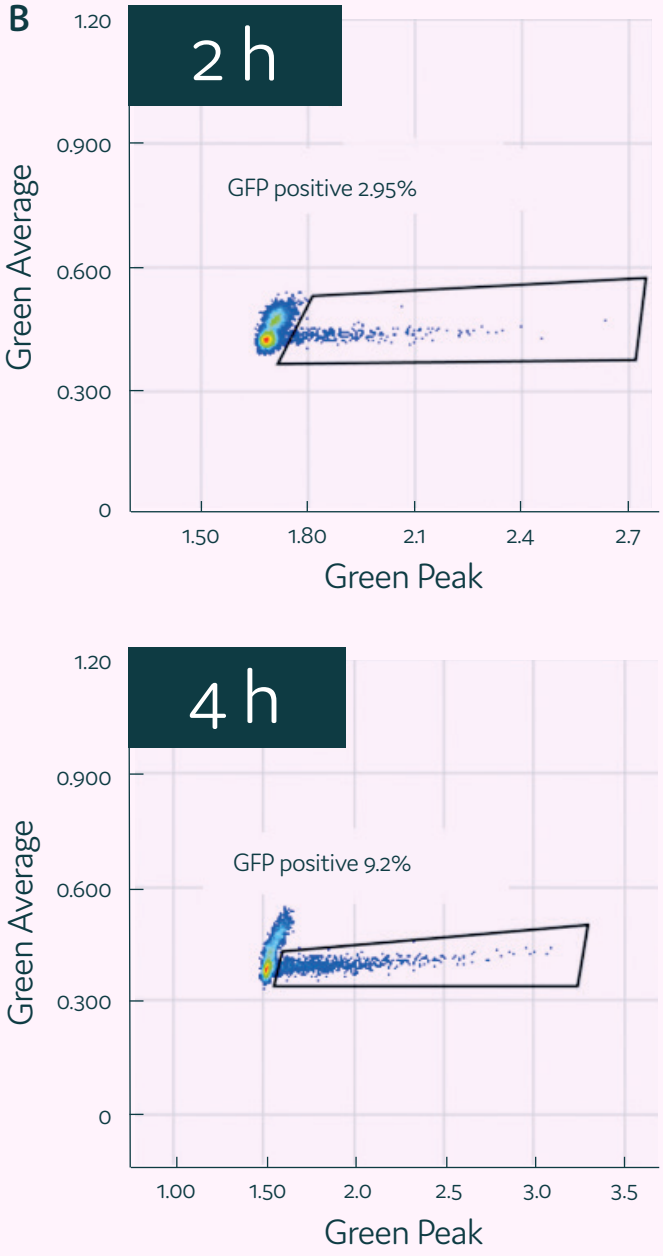
Next mRNA was chosen to assess feasibility of its delivery into mammalian cells in picodroplets. To this end HCT116 cells (2.5x10^5 cells) and a transfection cocktail (200 ng of GFP mRNA + DharmaFect Duo 0.4 µL/150 µl sample) were encapsulated into 450 pL picodroplets (Figure 4A). In parallel, cells were reverse-transfected with an identical transfection cocktail according to the manufacturer's protocol (control).

Cells in picodroplets were then incubated for 4 hours at 37°C. Cells were then recovered from picodroplets using Pico-Break™, washed by

centrifugation, and further cultured in fresh culture medium. After culturing for 24 hours, expression of GFP protein was assessed using the NucleoCounter® 3000 quantification protocol or by fluorescent microscopy. It was demonstrated to show similar expression levels between standard and picodroplet-based methods (Figure 4A).

Figure 4. mRNA transfection of mammalian cells in picodroplets.





C

Plate #	Recovery (%)
1	84.78
2	95.40
3	71.59
4	82.28
5	79.75
average	**82.76**
stdev	**8.6**

To evaluate the capability of Cyto-Mine® in detection and isolation of GFP-transfected cells, HCT116 cells and transfection mix were encapsulated into picodroplets using Cyto-Mine®, then GFP expression of the encapsulated cells was then assessed after 2 or 4 hours incubation (Figure 4B). After four hours, when GFP signal reached its maximum, positive picodroplets were sorted and dispensed into 96-well microtiter plates. The proportion of wells that produced colonies of cells was evaluated after two weeks of outgrowth (Figure 4C), showing successful completion of the workflow that starts from picodroplet-based mRNA transfection and finishes with the efficient outgrowth of sorted single cells.

Genome-editing of mammalian cells in picodroplets

CRISPR-Cas9 technology has triggered a step-change in the range, precision, and efficiency with which genomes can be edited. However, the production and screening of gene-edited cell lines remain inefficient. Our final experiment presents a complete CRISPR RNP workflow (Figure 5A), starting from picodroplets-based gene delivery, then incorporating a high throughput screening and isolation step using Sphere Bio's Cyto-Mine® Single Cell Analysis System, thus eliminating the need for multiple handling and selection steps, as in classic genome-editing workflows.

Here, HCT116 Fire-line cells (from Horizon Discovery/PerkinElmer) were used as a model system for gene insertion mediated by the CRISPR-Cas9 system. In this system, HCT116 cells were initially engineered to have genomic insertion of mutated GFP gene, resulting in lack of its expression (due to cellular degradation of its mRNA). The phenomenon can be reversed by repairing this point mutation (Figure 5B). To demonstrate genome editing in picodroplets, HCT116 cells ($3.5x10^5$ cells) and transfection mix composed of Cas9 RNP as well as template for repair of mutated genome-incorporated GFP sequence (crRNA1/tracrRNA complex 100 pM, ssODN 200 pM, DharmaFect Duo 0.4µl/150 µL sample) were co-encapsulated in 450 pL picodroplets. In parallel, cells

were reverse-transfected with an identical transfection mix according to manufacturer protocol (control).

Picodroplets containing cells and transfection reagents were incubated for 2 hours at 37°C. Cells were then recovered from picodroplets using Pico-Break™, washed by centrifugation, and further cultured in fresh culture medium. To compare the efficiency of both protocols, expression of GFP protein was assessed using the NucleoCounter® 3000 quantification protocol or by fluorescent microscopy after culturing for 48 hours (Figure 5C). As shown in Figure 5C, both protocols successfully resulted in a similar yield of cells expressing fluorescent protein.

In a separate experiment, picodroplets pre-generated and incubated at 37°C for 48 hours, to allow edited cells to express GFP, were then loaded into Cyto-Mine® to analyze and sort GFP-positive picodroplets at high throughputs (Figure 5D). Subsequently, sorted picodroplets containing GFP-positive cells were dispensed into 96-well microtiter plates, and the proportion of wells that produced colonies of cells were evaluated after two weeks of recovery (Figure 5E). The results of the fluorescent microscopy analysis of these colonies determined that most of them were expressing GFP (Figure 5F), demonstrating successful application of a picodroplet-based integrated platform to perform a complete genome-editing workflow.

Figure 5. Genome-editing of mammalian cells in picodroplets.

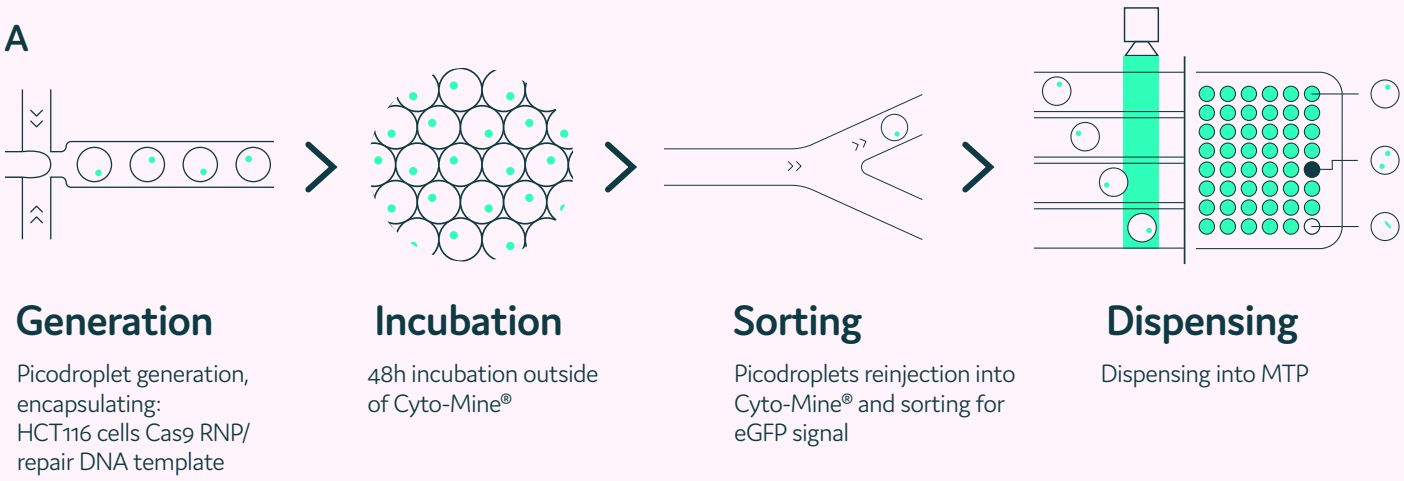
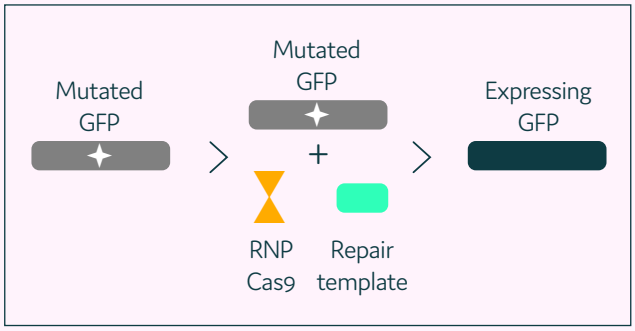


Figure 5 continued. Genome-editing of mammalian cells in picodroplets.

B



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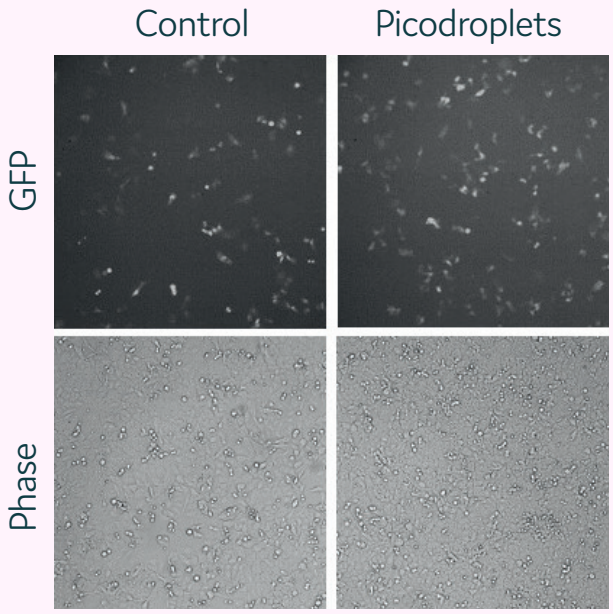

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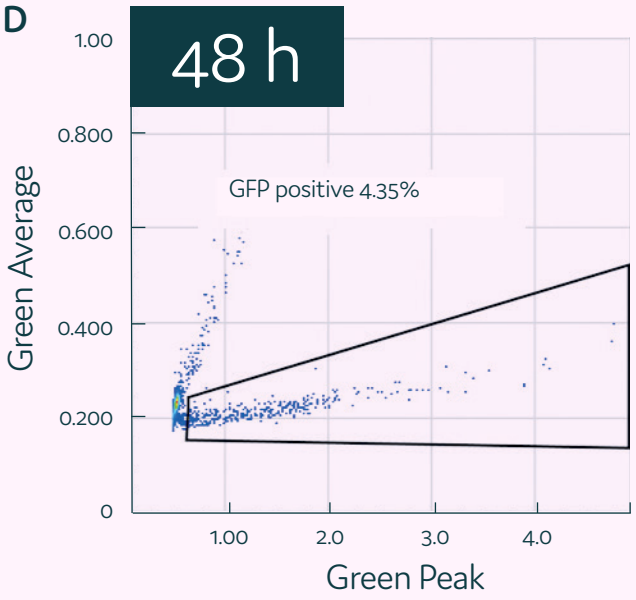
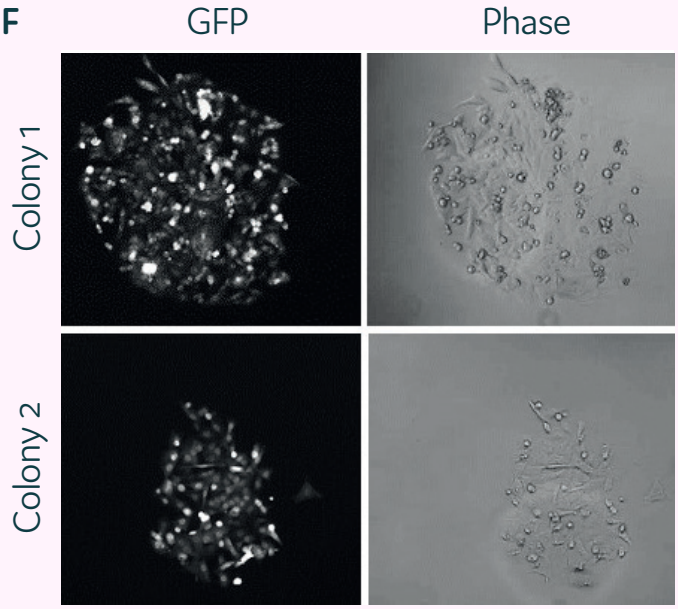


E

Plate #	Recovery (%)
1	20.5
2	13.5
3	15.2
4	11.5
5	18.8
6	14.6
average	**15.7**
stdev	**3.4**

F



Conclusions

This application note demonstrates the viability of picodroplet microfluidics as a technique for both cell engineering and screening in genome editing. Across the series of experiments, editing was detected via a fluorescent readout, and the positive single cells were sorted, dispensed into microtiter plates, and grown into single cell-derived clones. Progressively, manual editing steps were replaced with integrated picodroplet-based instrumentation, including the configurable Pico-Mine® system and the fully automated Cyto-Mine® system, presenting a streamlined solution for cell engineering applications.

References

1. Han, Xin, et al. "CRISPR-Cas9 Delivery to Hard-to-Transfect Cells via Membrane Deformation." Science Advances, vol. 1, no. 7, 2015, doi:10.1126/sciadv.1500454.
2. Josephides, Dimitris, et al. "Cyto-Mine: An Integrated, Picodroplet System for High-Throughput Single-Cell Analysis, Sorting, Dispensing, and Monoclonality Assurance." SLAS TECHNOLOGY: Translating Life Sciences Innovation, vol. 25, no. 2, 2020, pp. 177–189., doi:10.1177/2472630319892571.
3. Matula, K. et al. (2020): Single cell analysis using droplet microfluidics. Adv. Biosys. 4: 1900188.

Notes

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