Application Note 10

High Throughput CAR-T Cell Function Verification in Microfluidic Picodroplets

Introduction

Over the last few years, there has been an increasing interest in cell therapy as a strategy to harness the immune system to fight tumors. Technologies include reprogramming of T cells collected from patients to produce personalized medicines. T-cell therapy capitalizes on the body's immune systems ability to recognize and kill tumor cells. This ability can be lost or overwhelmed as a cancer develops. By extracting and manipulating a patient's T-cells, the ability to attack cancer cells can potentially be restored. Rapid, accurate assays to qualify the potency of the engineered T-cells against the target tumor cell are required to support the effective and efficient development of therapeutics.

This study presents a new picodroplet-based approach for the functional validation of CAR-T cells. The easy-to-use and robust method combines the benefits of Sphere Bio's high-throughput picodroplet technology with a granzyme B assay for studying cell-mediated cytotoxicity (Figure 1). Engineered T cells and target cells are co-encapsulated in picolitre-volume aqueous droplets (picodroplets) in an oil emulsion, along with granzyme B substrate. Granzyme B release by the T cell, a readout of the cells ability to recognize and kill the cancer cell, can be captured to determine the level of potential anti-cancer activity. In doing so, this method enables rapid cell product efficacy readouts to streamline the QC release of cell product.

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About Picodroplet Technology

Picodroplet-based technology has emerged as an attractive microfluidic-based technique for single cell functional analysis, offering several advantages over conventional tools.

Picodroplets provide a unique microenvironment in which to perform high throughput analysis of cell secretion and cell-cell interaction studies at a single cell level. Miniaturized picolitre volumes facilitates rapid mixing and minimized sample dilution, increasing detection sensitivity while reducing sample requirement and reaction time.



Figure 1. Workflow depicting high throughput CAR-T cell function verification in microfluidic picodroplets.

About Granzyme B detection in Picodroplets

The release of Granzyme B in picodroplets is detected with a commercially available Granzyme B assay kit (SensoLyte® Granzyme B Activity Assay Kit, Anaspec, Fremont, CA) adapted for use in picodroplets. The assay is based on the cleavage of a Granzyme B substrate peptide, labelled with a 5-FAM fluorophore and a QXL®-520 fluorophore quencher. In the intact, uncleaved, state, no fluorescence is emitted upon excitation of the fluorophore - due to the quenching effect of the nearby QXL®-520 molecule (Figure 2A). In the presence of Granzyme B the peptide substrate is cleaved, releasing the quenching molecule, and resulting in a fluorescent signal (Figure 2A and 2B).

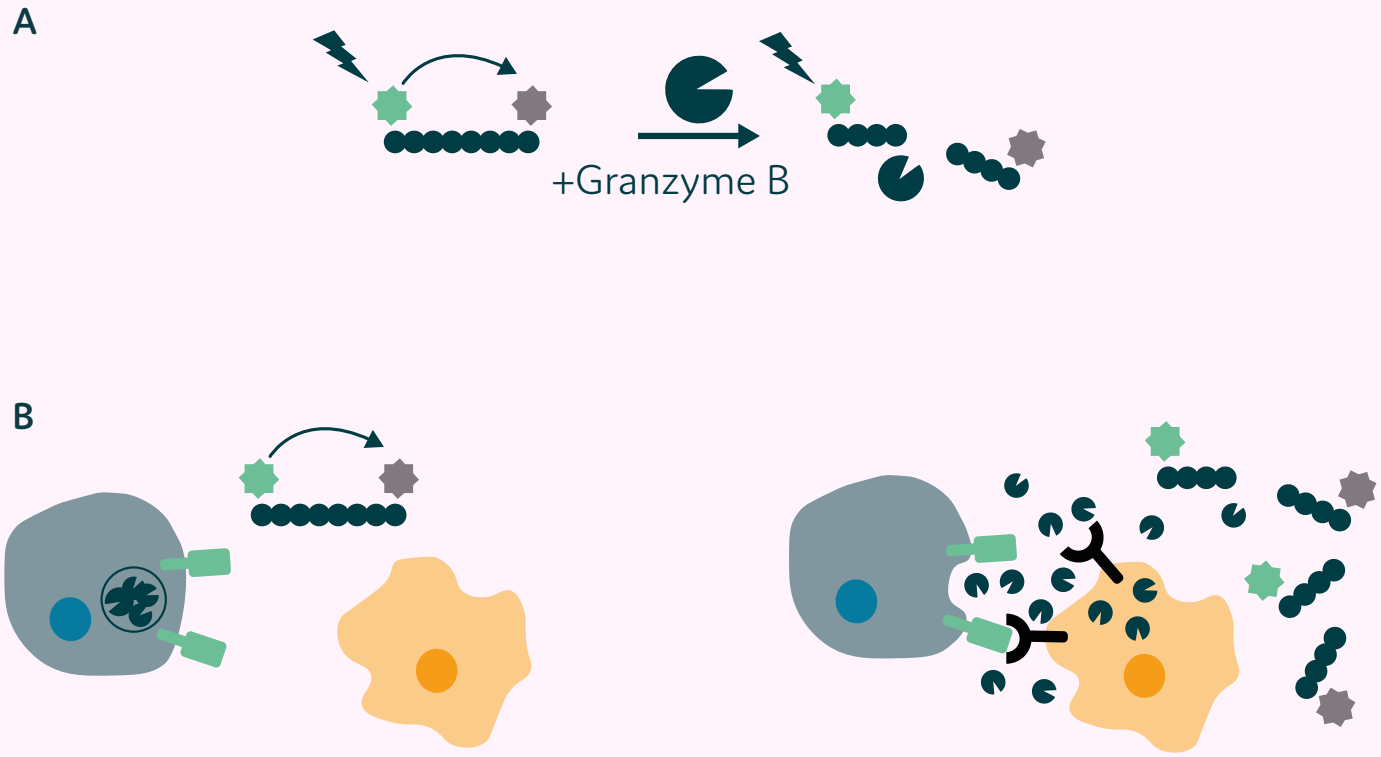

Figure 2. Principle of Granzyme B detection assay. A) Granzyme B substrate peptide labelled with 5-FAM (green star) and a QXL®-520 fluorophore quencher (grey star). No fluorescence is detected on excitation due to Fluorescence Resonance Energy Transfer (FRET) from 5-FAM to QXL®-520 (left side). On cleavage with Granzyme B the quenching molecule is removed, and fluorescence is emitted from 5-FAM (right side). B) Application of Granzyme B activity assay to CAR-T cell/target cell interaction. Left: CAR-T cell and non-target cell, no CAR mediated signal, no Granzyme B release, 5-FAM fluorescence quenched. Right: CAR-T cell interaction with target cell results in CAR mediated signal, Granzyme B release, substrate peptide cleavage, release of the quencher and fluorescence is then detected.

Granzyme B detection in Picodroplets

To demonstrate the general feasibility of the assay, we initially generated two populations of picodroplets of ~450 pL volume with our Pico-Capture® instrument. One population contained only the fluorogenic Granzyme B substrate peptide (negative control), while the other co-encapsulated recombinant Granzyme B together with the substrate peptide.

Figure 3 shows micrographs of the two populations in brightfield (left) and green fluorescence (right) after 2h incubation at 37°C. Only picodroplets containing both Granzyme B and the substrate peptide (bottom) show clearly detectable fluorescence after a 2h incubation, while picodroplets with substrate peptide only (top) exhibit only very low to no background fluorescence.

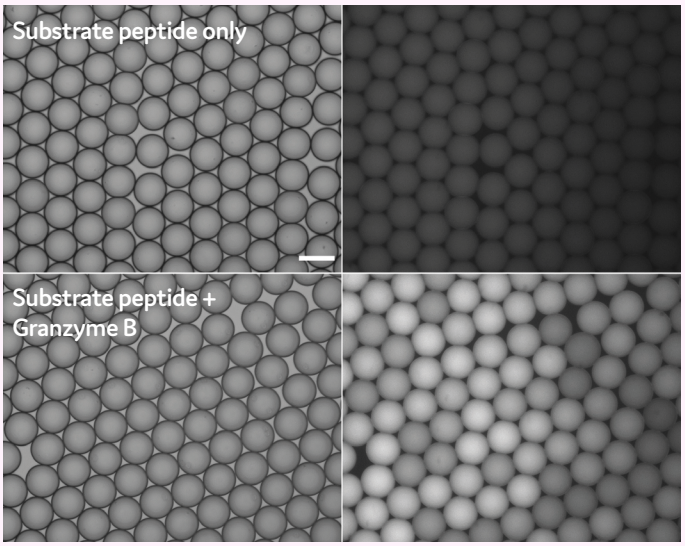

Figure 3. Detection of Granzyme B activity in picodroplets using a fluorogenic 5-FAM/ QXL®-520 substrate peptide. Brightfield (left side) and Fluorescence micrographs (right side) 2h post generation. Scale bar: 100 µm.

Granzyme B detection assay with T cells and target cells

In the next step we co-encapsulated polyclonal human donor T cells genetically modified to express a CAR directed against Prostate-Specific Membrane Antigen (PSMA), with target cells expressing PSMA (PC3-LN3-PSMA) and the fluorogenic Granzyme B substrate peptide.

We used an in-house custom-made biochip with two separate aqueous inlets to keep CAR T and target cells apart until immediately prior to encapsulation. Cell concentrations were adjusted so that only ~20% of picodroplets contained a CAR T cell, while >75% received at least one target cell (according to a Poisson distribution). Note, 150,000 out of 1 million generated picodroplets will contain one CAR-T cell and at least one target cell. Consequently, ~15% (0.75 x 0.2) of picodroplets contained at least one CAR-T and one target cell. As a control we co-encapsulated CAR T cells with PC3-LN3 cells lacking PSMA expression, together with the fluorogenic peptide substrate under otherwise identical conditions.

Picodroplets were collected in an Eppendorf tube and imaged after 2, 4, and 24 hours of incubation under a fluorescence microscope to detect Granzyme B activity. Figure 4 shows fluorescent micrographs of picodroplets containing non-expressing control cells (top) and PC3-LN3-PSMA target cells (bottom), imaged after 2, 4, and 24 hours (left to right).

Picodroplets containing PC3-LN3 control cells showed very little Granzyme B activity after 2-4h. In picodroplets with PC3-LN3-PSMA cells, multiple fluorescent picodroplets are indicative of Granzyme B released by CAR-T cells in response to encountering one or more target cell(s). Only after prolonged incubation (24h) is increased fluorescence also observed in the negative control, possibly due to unspecific stimulation or Granzyme B leaking from dead or apoptotic CAR-T cells.

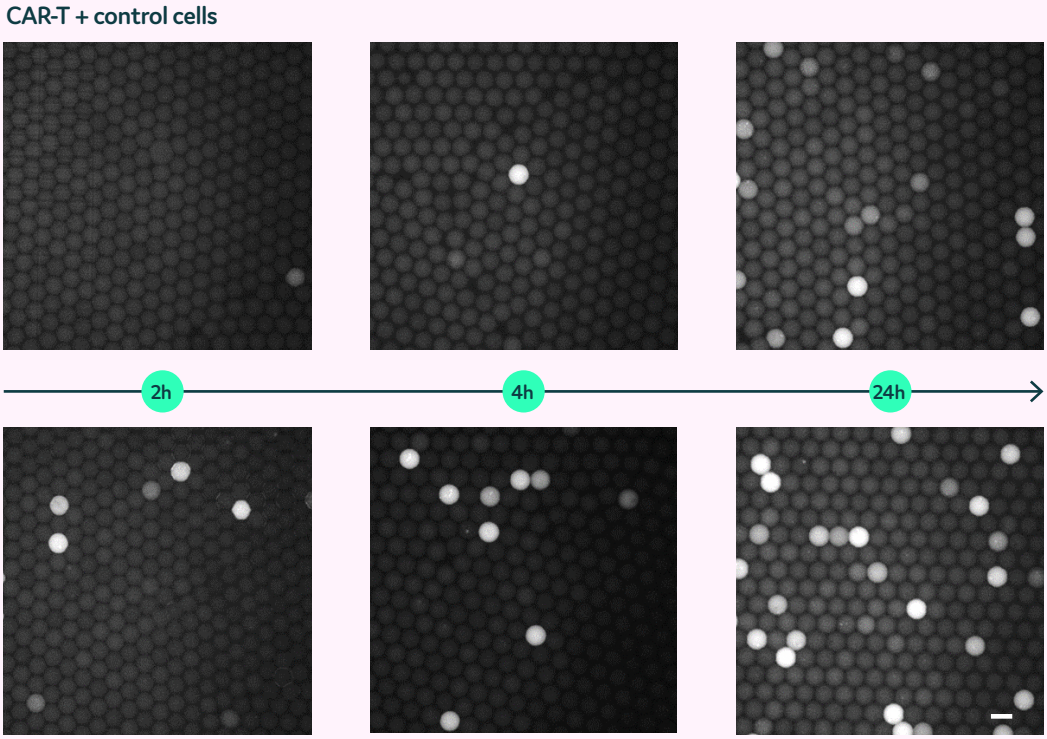

Figure 4. Granzyme B assay in picodroplets with co-encapsulated CAR-T and target cells (bottom) or control cells (top). Fluorescence micrographs of picodroplets after 2, 4, and 24 h (left to right). Scale bar: 100 µm.

Granzyme B detection using Cyto-Mine®

After establishing that Granzyme B release by CAR-T cells can be detected with the 5-FAM/QXL®-520 fluorogenic peptide substrate we proceeded to establish detection and sorting of Granzyme B positive picodroplets using Sphere Bio's Cyto-Mine® Single Cell Analysis System. We co-encapsulated CAR-T cells with PC3-LN3-PSMA target cells and the 5-FAM/QXL®-520 peptide substrate in 450 pL sized picodroplets as described above with Sphere Bio's Pico-Capture® instrument. The emulsion was collected and loaded into a Cyto-Cartridge® for injection, analysis and sorting on the Cyto-Mine® platform.

The fluorescence signal was detected immediately after all picodroplets had been injected into Cyto-Mine® (t=0), as well as after an additional incubation of 1 and 2h at 37°C inside the instrument. Figure 5 shows scatter plots of picodroplet size (y-axis) versus green fluorescence (x-axis) obtained at the indicated time points. At the earliest time point, a clear fluorescent signal was detectable in ~1.25% of picodroplets. This positive population increased over time up to 2.95% of picodroplets after 2h. As only 15% of picodroplets contain both a CAR-T cell and at least one target cell, this corresponds to an actual positive rate of ~20%.

We then proceeded to sort 5,000 positive picodroplets to visually confirm the presence of a fluorescent signal in these picodroplets. After sorting, we carefully removed the Cyto-Cartridge® from the instrument and placed it under a fluorescence microscope to image the picodroplets in the dispensing chamber.

Figure 6 shows a brightfield (left) and fluorescent micrograph (right) of picodroplets in the dispensing chamber of the Cyto-Cartridge®. A clear enrichment of fluorescent picodroplets is evident. Analysis with ImageJ software using a Hough Circle Transform algorithm resulted in a total count of 1,112 picodroplets. 170 of these picodroplets were deemed to contain little to no fluorescence (i.e., false positive, based on near background signal and a dark picodroplet border); consequently, 942 picodroplets (~85%) were true positives. This corresponds to a 28-fold enrichment compared to the non-sorted population with 2.95% positives after 2h incubation.

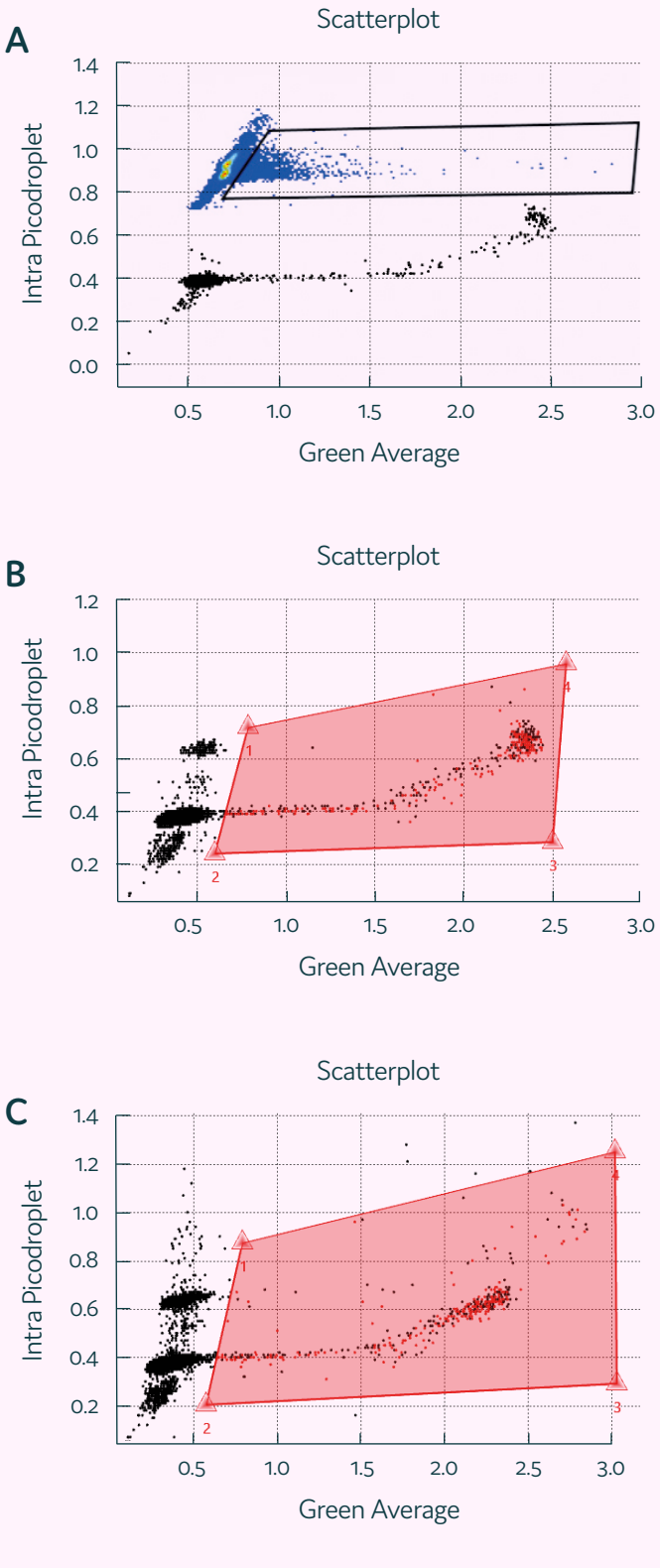

Figure 5. Detection of Granzyme B activity in picodroplets with Cyto-Mine®. Screenshots of Cyto-Mine® software during detection/sorting, showing scatterplot of picodroplet size (Intra picodroplet, ms) against Fluorescence signal (Green Average, V) at t=0 (A), 1h (B) and 2h (C). The middle and bottom panel also show polygon gates used for determining percentage of positive picodroplets and for sorting.

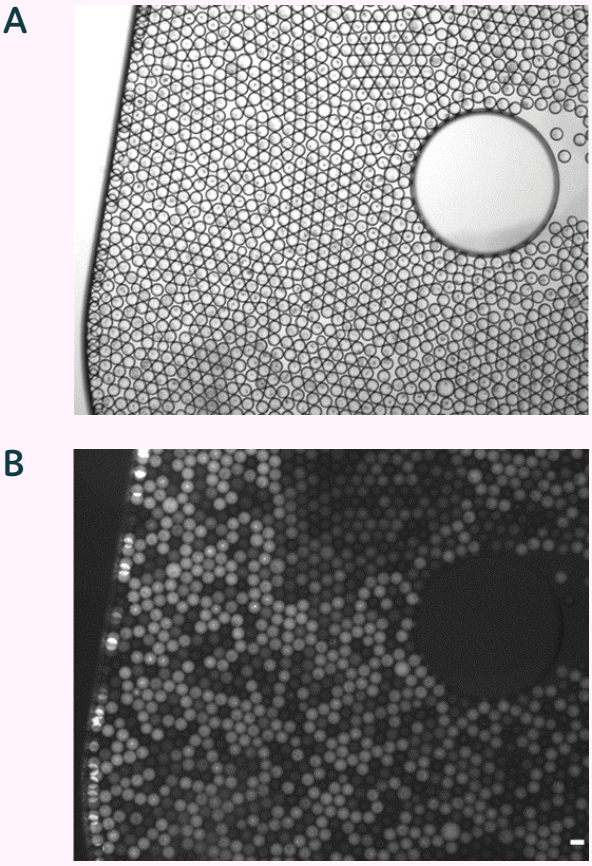

Figure 6. Brightfield (A) and fluorescent micrograph (B) of sorted picodroplets in dispensing chamber. Scale bar 100 µm.



Figure 7. Cyto-Mine® - Sphere Bio's automated and fully integrated single cell analysis system.

Conclusions

This study demonstrated that researchers can use picodroplet technologies to monitor CAR-T activation at relevant early timepoints after co-culture. This has been accomplished by adapting established assays to the picodroplet format.

Current strategies rely on screening in bulk cultures to establish CAR-T cell product profiles. Readouts of cytotoxicity take time and are not currently included in release criteria for cell products. This information is gathered after the fact or not at all. The ability to run a short assay, to give a readout of potential efficacy fitness of a cell product could be of great value to cell therapy development. Picodroplet technology enables screening at the single cell level in a high throughput and rapid process. Coupling this utility to granzyme B release by co-encapsulated CAR-T and target cells offers the potential to release highly functional products with the best chance of impact for patients.

In addition to rapid release analysis for a cell product, this co-encapsulation approach could be utilized by cell therapy developers to interrogate other readouts of cellular fitness and cytotoxicity. This could include other proteomic markers of cytotoxicity or direct cytotoxicity of the target cells. Bulk T cells cultures display a variety of phenotypic and metabolic subsets. Investigating the cytotoxic efficacy at a single T cell level has the potential to inform on each of these subsets individually and guide T cell engineering towards more potent subsets. An additional role for co-encapsulation in picodroplets could be rapid screening of candidate CAR/TCR gene modified cells for drug development.

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Notes

Notes

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