Rapid understanding of functional heterogeneity and serial-killing function of cellular therapies, with single-cell resolution, at scale

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Introduction

Cell-based therapies represent a promising approach in cancer treatment and cure, yet their development, donor and patient screening, and immuno-monitoring remain challenging. These applications require analytical tools capable of assessing cell function at a resolution high enough to highlight heterogeneity within subpopulation of cells that are commonly included in each product batch.

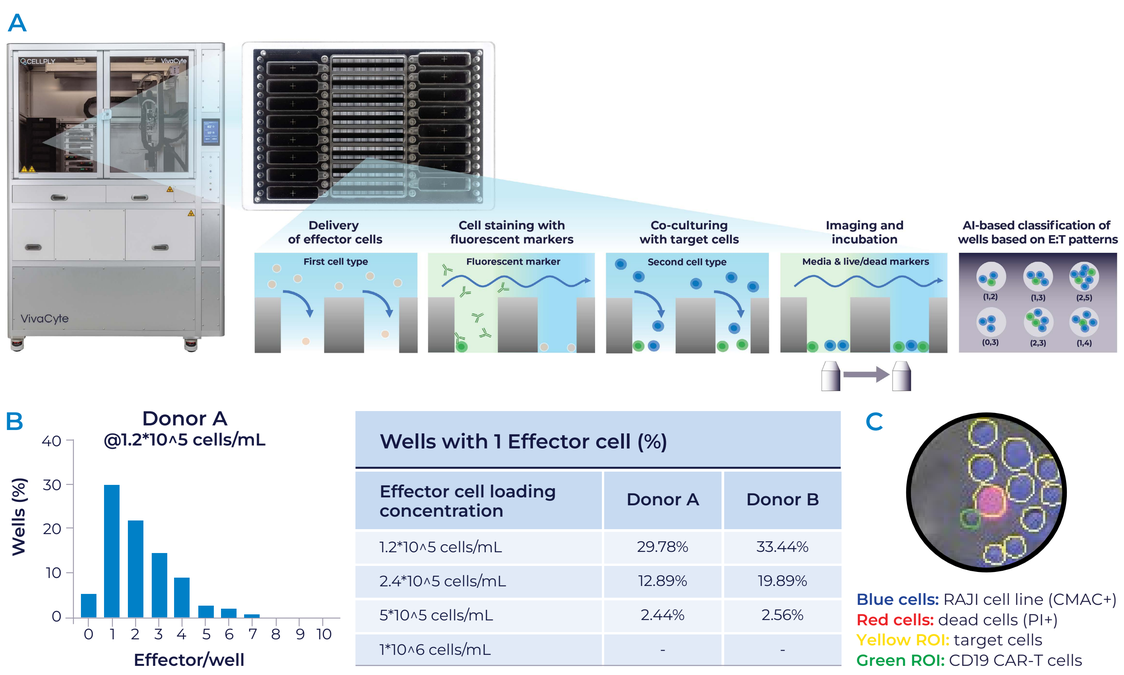
Hence, single-cell methods that capture this heterogeneity are essential to understand the role of the various subpopulations of immune cells and to optimize potency and persistence. Here, the automated VivaCyte platform was used to deeply characterize an anti-CD19 CAR-T product and to simultaneously measure immune cell function (killing and serial killing activity), and phenotype (surface marker expression) at a scale of hundreds of single CAR-T cells in parallel, linking function to phenotype without prior sorting.

VivaCyte technology

VivaCyte is a walk-away analytical system that automates immune-cell function characterization from sample preparation to image processing (**Figure 1A**). The core technology is the CC-Array® microfluidic plate which allows generating up to 19,200 miniaturized co-cultures per device within an array of microwells, each of 0.5 nL volume. Integrated microfluidic channels support a rapid liquid exchange (RLE) method, which automatically delivers cells into the microwells. Cells are randomly seeded in microwells, generating a Poisson distribution in terms of number of cells per well, based on the cell loading concentration (**Figure 1B**). RLE allows medium in each microwell to be replaced without displacing the cells previously loaded. This enables automatic staining with fluorescent markers (e.g. antibodies) and dead markers (e.g. Propidium Iodide, PI) to detect the cytotoxic activity of immune cells on tumor cells. Time-lapse fluorescence imaging is performed at predefined time points, providing the primary readout for monitoring single cell killing. Then, microwells are tagged and selected automatically as region of interest (ROI) by an AI-based image analysis which detects and counts the cells contained in each microwell. A blue marker, applied to the target cells prior to loading (CellTracker™ Blue CMAC Dye) was used to distinguish effector from target cells in each microwell[(**Figure 1C**).

Single cell killing and phenotype

Immune and tumor cells were loaded in microwells in sequence, then anti-CD4/CD8 antibodies were added at 0 h, followed by PI. The antibody staining was repeated at the end of the assay. Cells were monitored over 24 h, and at each time point VivaCyte analytical software identified microwells containing one effector cell (anti-CD19 CAR-T) and multiple target cells (Raji cell line). Effector cells were classified as inactive, killers (≥1 target cell killed), or serial killers (≥3 target cells killed) based on the number of dead target cells (PI+) counted in each microwell and normalized to the average number of dead cells in effector-free microwells. As shown in **Figure 2**, Donor B exhibited a higher frequency of serial killer cells than Donor A, with statistically significant differences observed at selected time points. Serial killing provides a direct single-cell measure of functional potency, distinguishing rare highly active effector cells that are not identified in bulk assays. The Unified Killing Score (UKS) represents the weighted mean number of target cells killed by a single effector cell in a standardized assay, enabling direct comparison of donors (**Figure 3**). Fluorescent anti-CD4 and anti-CD8 antibodies extend the readout to surface phenotype, providing simultaneous functional and phenotypic profiling at the single-cell level without pre-sorting. This links each effector cell's killing activity to its CD4/CD8 identity and shows which cell subset drives the cytotoxic response over time. Two CAR-T batches from donors A and B, indistinguishable by a conventional bulk serial killing analysis (data not shown) carried out through a rechallenge assay, were compared. Donor B sustained a consistently higher frequency of serial killers throughout the study ($p<0.05$), with its CD8+ serial killers peaking at 36.3% at 24h versus 28.7% for donor A. CD4+ and CD8+ kinetic profiles differed substantially between donors: CD8+ killers and serial killers consistently exceeded CD4+ levels in donor A, whereas in donor B CD4+ serial killers were initially more frequent than CD8+ cells that prevailed at later timepoints (**Figure 4**).



Wells with 1 Effector cell (%)		
Effector cell loading concentration	Donor A	Donor B
1.2*10^5 cells/mL	29.78%	33.44%
2.4*10^5 cells/mL	12.89%	19.89%
5*10^5 cells/mL	2.44%	2.56%
1*10^6 cells/mL	-	-

Figure 1. **(A)** The fully integrated, walk-away VivaCyte system. The CC-Array® microfluidic chip features 16 microfluidic channels with 1,200 microwells per channel, for a total of 19,200 microwells per chip. Automation allows consistent co-culture formation of E:T ratios, while rapid media exchange and reagent infusion enable multiparametric analysis on the same cells. **(B)** AI powered image analysis automatically classifies microwells by number of effector and target cells. **(C)** Random cell loading into microwells follows Poisson statistics. The table represents the percentages of microwells containing one single effector cell in relation to cell loading concentrations for the two Donors analyzed.

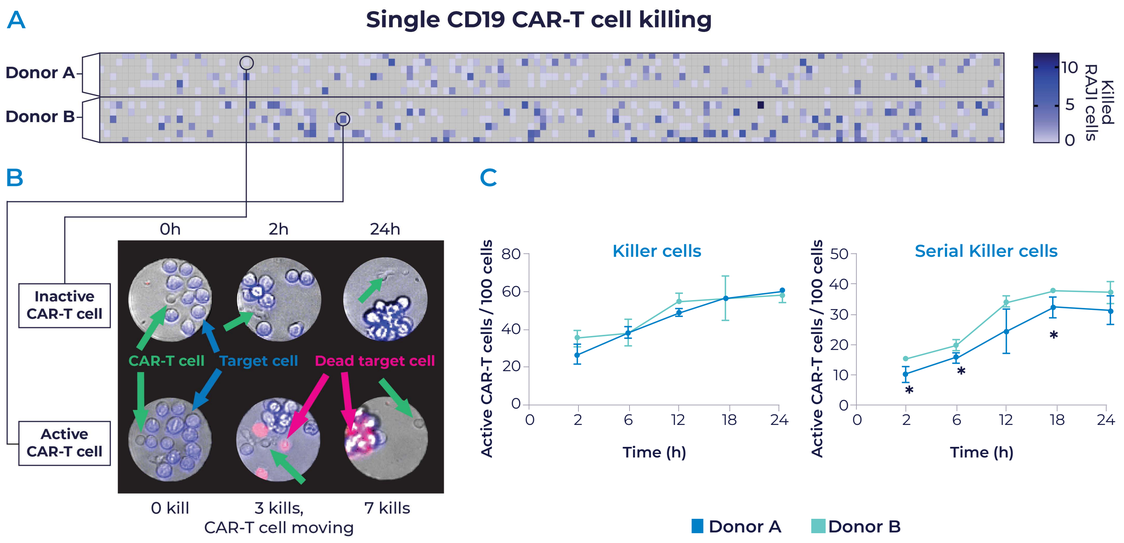
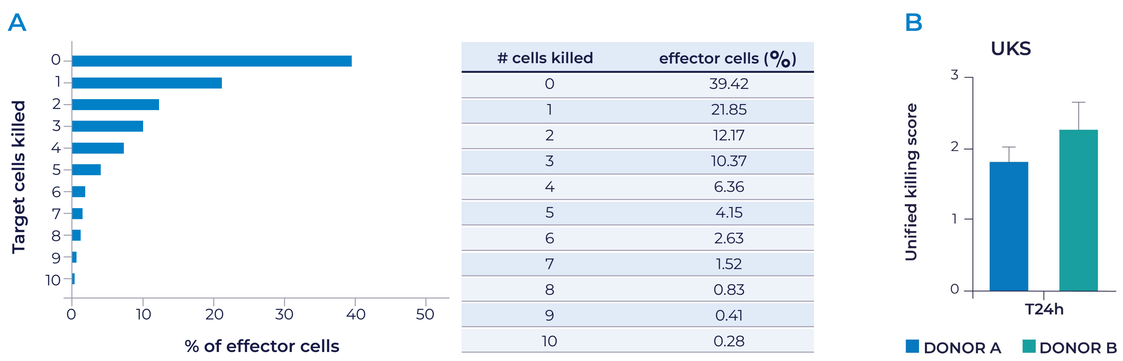


Figure 2. **(A)** The heatmap displays the number of dead RAJI cells (PI+) of microwells containing a single anti-CD19 CAR-T cell, providing a quantitative measure of single cell cytotoxic activity after 24 h and indicating enhanced cytotoxic function in Donor B. **(B)** Time-lapse visualization of the single cell killing activity of single CAR-T cells. **(C)** Killer (left) and serial killer (right) CAR-T cells quantified during the 24h hours of co-culture for two donors. The data show a higher frequency of effector killer cells in Donor B compared with Donor A (* $p\leq0.05$).



# cells killed	effector cells (%)
0	39.42
1	21.85
2	12.17
3	10.37
4	6.36
5	4.15
6	2.63
7	1.52
8	0.83
9	0.41
10	0.28

$$\text{Unified Killing Score} = \frac{1}{100}\sum \text{Cells killed}_i * \%\ \text{of effector}_i$$

Figure 3. **(A)** Single cell killing data used to calculate the Unified Killing Score after 24 h, using CAR-T donor B against RAJI cell line as target. **(B)** UKS comparison across donor A and donor B.

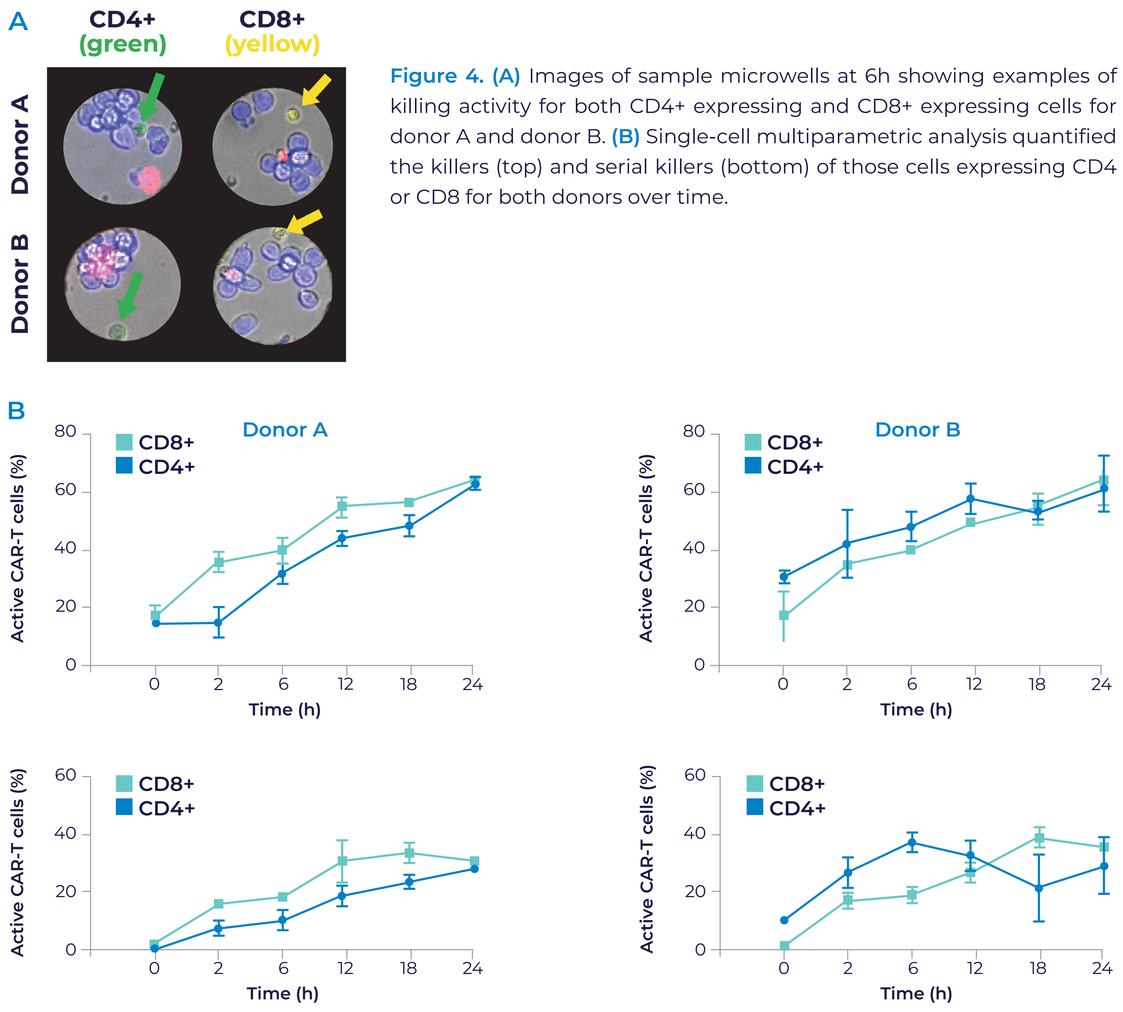


Figure 4. **(A)** Images of sample microwells at 6h showing examples of killing activity for both CD4+ expressing and CD8+ expressing cells for donor A and donor B. **(B)** Single-cell multiparametric analysis quantified the killers (top) and serial killers (bottom) of those cells expressing CD4 or CD8 for both donors over time.

Conclusions

This study presents a novel single-cell immune profiling technology applied to CAR-T cells from two donors using an immune cell killing assay. Killing dynamics revealed donor-to-donor differences, with higher overall activity in donor B, and enabled linking function to CD4+ and CD8+ subsets without prior separation. The VivaCyte platform enables single-cell analysis of CAR-T cytotoxicity against tumor target cells, providing i) information about killing activity of individual cells, highlighting the functional heterogeneity of cell therapy products, ii) rapid analysis of the serial killing activity which can be used for donor/patient selection or to optimize the development and manufacturing of potent CAR-T products, and iii) multiparametric analysis enabling the measurement of the potency of cell subsets.