Universal Pharmacokinetic Assessment of Anti-CD19 CAR-T Using Biocytometry

Martin Cienciala[1], Hynek Kasl[1], Pavel Fikar[1], Laura Berne[1], Mona Luo[1], Monika Holubova[2,3], Daniel Lysak[2], Daniel Georgiev[1,4]

[1] Sampling Human Inc., Berkeley, California, USA, [2] Department of Hematology and Oncology, University Hospital Pilsen, Pilsen, Czech Republic, [3] Laboratory of Tumor Biology and Immunotherapy, Biomedical Center of the Faculty of Medicine, Charles University, Pilsen, Czech Republic, [4] Faculty of Applied Sciences, University of West Bohemia, Pilsen, 30100, Czech Republic

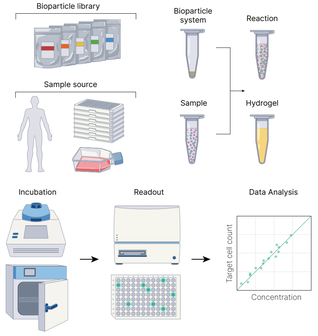
Introduction

Quantification of functional CAR-T cells within a clinical sample is seemingly straightforward but proves to be quite challenging in practice. Conventional flow cytometry often relies on the detection of secondary antigens to quantify CAR-T cells. This approach lacks a definitive ground truth, leading to potential inaccuracies in the interpretation of CAR-T cell presence[1]. Moreover, the dynamics of CAR-T cell therapies necessitate prolonged monitoring to evaluate therapeutic efficacy and patient response over time. To accommodate this requirement, anti-idiotypic antibodies are commonly employed. However, these antibodies, while providing sensitive recognition of CAR constructs (anti-FMC63, LOD ~ 10^{-3}), do not directly correlate with the functional activity of CAR-T cells[2].

This study utilized biocytometry, a novel approach for identification of cellular immunophenotypes. We incorporated the CD19 antigen to enable universal detection of anti-CD19 CAR-T cells. Comparative analyses with conventional flow cytometry were conducted to assess specificity using healthy donor samples. Furthermore, we examined the clinical relevance of this approach in two patients with acute lymphoblastic leukemia (ALL) undergoing treatment with Kymriah and Tecartus. The evaluation extended to both peripheral blood mononuclear cells (PBMCs) and peripheral blood samples.

Biocytometry Workflow

The biocytometry workflow implements basic mixing and incubation steps combined with high-throughput spectrophotometry to easily accommodate the simultaneous measurement of a wide range of immunophenotypes. Provided as a standardized kit, the assay ensures excellent reproducibility and ease of operation, enabling laboratory technicians at any experience level to conduct the analysis[3].



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[2] Schanda, N., et al. (2021). Sensitivity and Specificity of CD19 CAR-T Cell Detection by Flow Cytometry and PCR. Cells, 10(11), 3208.

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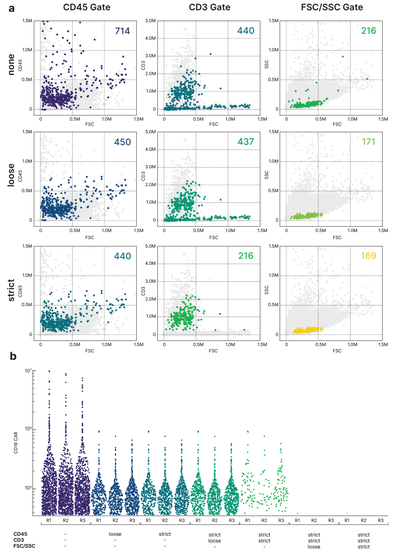
Status Quo

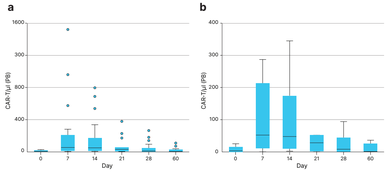
Challenges in establishing ground truth for CAR-T cell enumeration

Enumeration of CAR-T cells using flow cytometry exhibits considerable variability based on the number and rigor of gating steps applied. (**a**) Quantitative analysis reveals a broad range in event counts per 10,000 total cells, from 714 events when gating is based solely on high expression in the CD19 CAR channel, to as low as 169 events when additional gates for CD45, CD3, and FSC/SSC are implemented. This variation underscores the significant challenge in establishing a consistent ground truth for CAR-T cell enumeration. (**b**) Analysis of samples from healthy donors indicates that incorporation of multiple gating steps is crucial for enhancing the specificity and thus the limit of blank (LOB) characteristics.



Multicenter variability in CAR-T cell enumeration

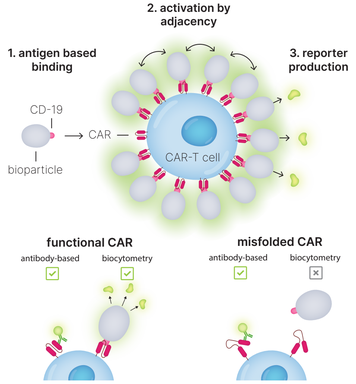
Samples from 24 patients undergoing CAR-T therapy were analyzed across multiple centers with varying expertise in flow cytometry as part of a harmonization process. (**a**) This analysis revealed significant variability in CAR-T cell enumeration. (**b**) After removing outlier data, the findings suggest that the effective monitoring of persistence is constrained to a duration of three to four weeks.



Results

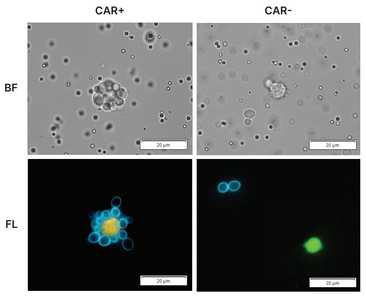
Biocytometry enables robust detection of functional CAR-T cells

Biocytometry offers a novel approach for multiparametric immunophenotyping in suspension, employing a bioparticle system engineered to adhere specifically to designated surface markers and activate when adjacent bioparticles are present. Upon activation, these bioparticles begin the production of a reporter molecule. The intensity of the reporter signal is then quantified using a microplate reader, with the strength of the signal directly proportional to the number of cells detected. Our study utilized bioparticles displaying the extracellular domain of CD19, enabling precise identification of cells expressing the functional anti-CD19 CAR construct.

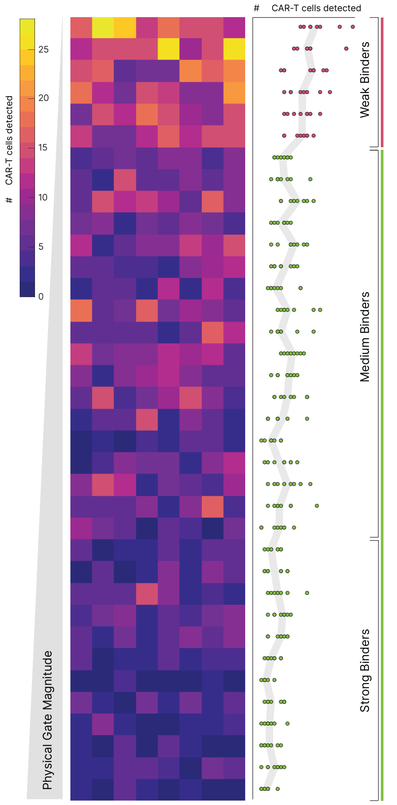


Binary classification of analyzed cells in a sample

Biocytometry leverages multivalent antigen detection, necessitating the formation of multiple bonds between bioparticles and CAR+ cells for effective detection. This approach markedly enhances specificity beyond that of traditional monovalent antibody interactions. It eliminates the need for secondary markers and consistently achieves a zero limit of blank. Additionally, physical gating is utilized to standardize the binary classification of cells based on CAR expression, thereby removing subjective elements from cell analysis. Microscopy images illustrate the physical interaction between CD19 CAR+/- cells and bioparticles.



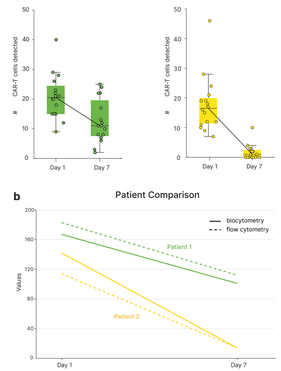
Physical gating can distinguish between strong and weak binders



The biocytometry workflow employs adjustable physical gating to discriminate cells by antigen surface density, requiring stronger cell-bioparticle interactions for detection as gate settings are tightened. In this experiment, samples from a patient undergoing CAR-T therapy were analyzed under various physical gating settings to investigate the impact of stringency on cell detection. Reactions were divided across a standard 96-well plate and analyzed. The number of CAR-T cells detected is illustrated on a heatmap, accompanied by a corresponding scatter plot. Results showed a decrease in detected cells with tighter gating criteria, effectively isolating cells with higher CAR surface density.

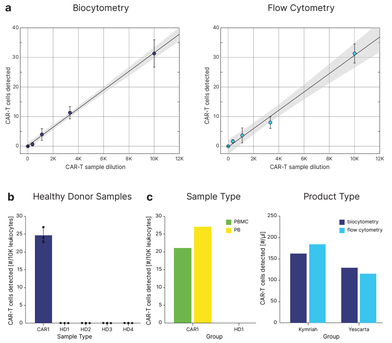
Pharmacokinetic assessment in patients with ALL

(**a**) Peripheral blood monocytes from two patients treated with Kymriah and Yescarta were collected on days 1 and 7 post-administration and cryopreserved for pharmacokinetic study assessment. Each sample was digitized (n = 16) and analysed using biocytometry. (**b**) Differential CAR-T cell expansion dynamics were observed and correlated with values obtained through flow cytometry, revealing variations in cellular response between the patients.



Biocytometric enumeration demonstrates high correlation with flow cytometry

(**a**) In a direct comparison, biocytometry and flow cytometry exhibited a strong correlation ($r = 0.96$, $p < 0.01$). (**b**) Biocytometry consistently yielded no signal in samples from healthy donors. (**c**) Moreover, the workflow demonstrated compatibility across various sample matrices (PBMCs, peripheral blood, frozen samples) and product types (e.g., Kymriah, Tecartus).



Conclusion

Biocytometry offers a novel quantification approach for anti-CD19 CAR-T cells, utilizing CD19 antigen for universal identification of functional CAR-T cells within a sample. This method achieves a zero limit of blank without relying on secondary markers, thereby increasing the accuracy of measurements. The inclusion of physical gating standardizes results and eliminates subjective elements from analysis, ensuring reproducibility across various clinical environments. It can also be used to differentiate between CAR-T cells with low or high surface densities of the CAR construct. Furthermore, the homogeneous assay workflow of biocytometry offers significant advantages to clinicians, featuring a streamlined, high-throughput protocol that can be easily executed by laboratory technicians with minimal training.

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