Uncovering CAR T Cell Spatial Dynamics and Activation States with Integrated Spectral and Imaging Flow Cytometry

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Abstract

Chimeric antigen receptor (CAR) T cell therapies have transformed treatment for hematologic malignancies, yet accurate detection and characterization of CAR T cells by flow cytometry remains challenging. In this study, we demonstrate high-sensitivity CAR detection with minimal nonspecific binding using the BD 1-step CAR Detection Reagent. High-parameter spectral flow cytometry enabled clear distinction between CAR T cells spiked into donor PBMCs and endogenous T cells, allowing assessment of activation, exhaustion, and memory phenotypes across both populations. This approach reliably identified rare primary CAR T cells (<0.1%) within complex samples. Additionally, imaging flow cytometry using the BD FACSDiscover™ S8 with BD CellView™ Image Technology revealed two distinct CAR spatial configurations: circular and polarized. In vitro studies using Jurkat T cells expressing CAR-BCMA showed that the polarized phenotype increased upon stimulation with the U266 target cell line, indicating a connection to activation. Polarized CAR T cells also exhibited CD3 complex polarization and elevated TIM-3 expression, suggesting a distinct activation profile compared to circular CAR T cells. These findings highlight the role of immunological synapse formation in CAR spatial organization and activation. Multiomic analyses of these populations may further elucidate their functional differences and inform the development of more effective CAR T cell therapies. Overall, our results underscore the power of integrating advanced flow cytometry and imaging technologies to characterize CAR T cell phenotypes and spatial dynamics with unprecedented resolution.

Methods

Using BD CellView™ Image Technology to assess CAR spatial organization in *in vitro*-expanded CAR T cells from a healthy donor

Peripheral blood T cells from a healthy individual were activated with anti-CD3/CD28 Dynabeads in the presence of human recombinant IL-2. Following stimulation, the cells were transduced with a CAR construct targeting human B-cell maturation antigen (BCMA). After further cell expansion in IL-2, approximately 25% of the cells expressed BCMA-CAR on the cell surface and were cryopreserved for subsequent analysis. CAR spatial organization was examined by imaging flow cytometry using the BD FACDiscover™ S8 Cell Sorter with BD CellView™ Image Technology [Figure s 1, 2].

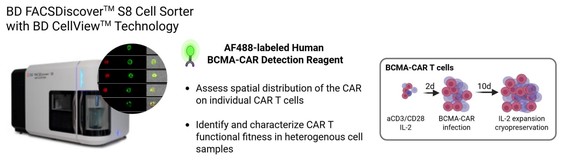


Figure 1: Detection of BCMA-CAR using the BD 1-step BCMA CAR Detection Reagent (Alexa Fluor 488) and imaging flow cytometry. Activated T cells were transduced with a BCMA-CAR construct, expanded *in vitro* and analyzed on the cytometer.

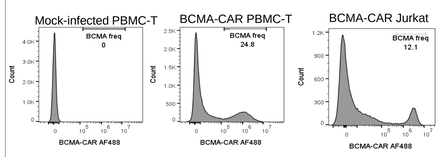


Figure 2: BCMA-CAR expression levels on T cells. Lentiviral **m**ock-infected T cells served as staining negative control (left) while positive staining was observed in BCMA-CAR-transduced primary T cells (middle) and Jurkat T cell line (right).

Besides BD 1-step CAR-BCMA AF488 Detection Reagent, the CAR T cells were stained with six additional antibodies. This allowed us to assess inhibitory receptors expression on CD4 and CD8 T cell subsets. The fluorochrome panel was designed to achieve clear population resolution while minimizing spectral spillover into the imaging channels Img1 and Img2.

Panel compatible with imaging and used to evaluate CAR T functional fitness

Fluorochrome	Target	Purpose	Imaging
AF488	BCMA-CAR	CAR detection	Img1
RB705	LAG-3	T cell inhibitory receptor	Img 3
BUV305	CD3	T cell lineage	
BV421	Tigit	T cell inhibitory receptor	
BV605	PD-1	T cell inhibitory receptor	Minimal spills
BV786	CD8	Cytotoxic CD8 T cell	into imaging
APC	CD4	T helper CD4 T cell	channels
R718	TIM-3	T cell inhibitory receptor	
FVS780	NA	Live/dead discrimination	

To examine CAR distribution on the cell surface, BCMA-CAR$^+$ total intensity signal was used to stratify cells into groups. One group displayed a strong, punctate BCMA-CAR AF488 signal characterized by high delta center of mass between SSC imaging and BCMA-CAR AF488 fluorescence. The other group exhibited a more diffuse and circular CAR distribut**ion**. Together, the results indicated differences in CAR spatial organization on the CAR T cells **[Figures 3, 4, 5]**.

FACSDiscover imaging parameters

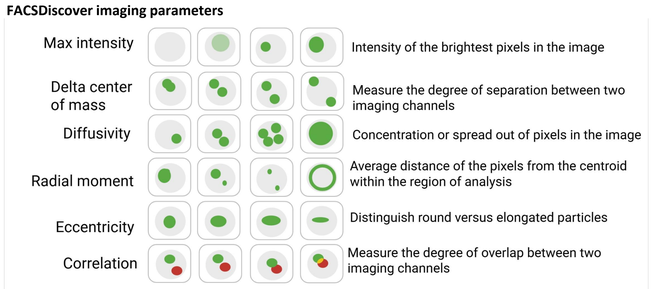


Figure 3: Illustration of spatial patters obtained from the analysis of imaging fluorescence and morphological parameters. Total intensity (not represented) measures total fluorescence signal in a particular imaging channel.

Using imaging parameters to reveal relationships between CAR spatial organization and CAR T functional states

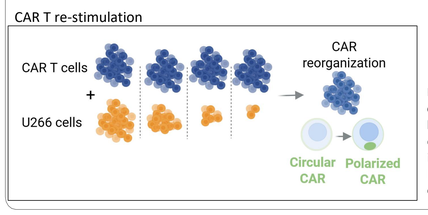
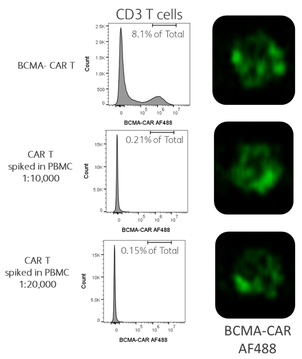


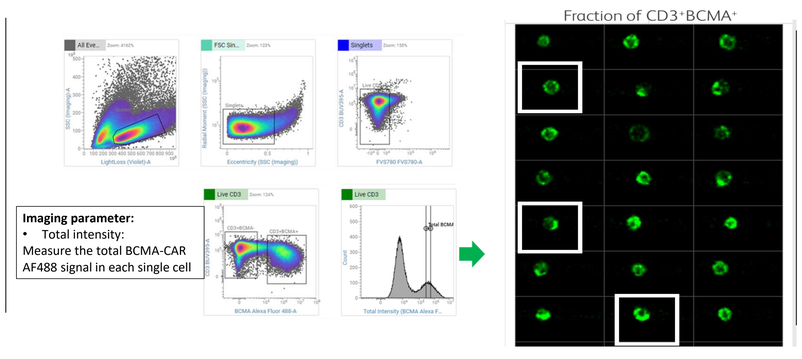
Figure 4: Schematic showing co-cultures of CAR T cells and U266 B-lymphoblastoid cell line. U266 cells express BCMA antigen and were used in different concentrations to trigger BCMA-CAR activation in primary CAR T cells.

Results – BCMA-CAR detection in heterogenous samples and visualization of CAR spatial organization

5A CAR T detection sensitivity



5B CAR T spatial organization varies from cell-to-cell



5C CAR spatial organization is uncoupled from expression level

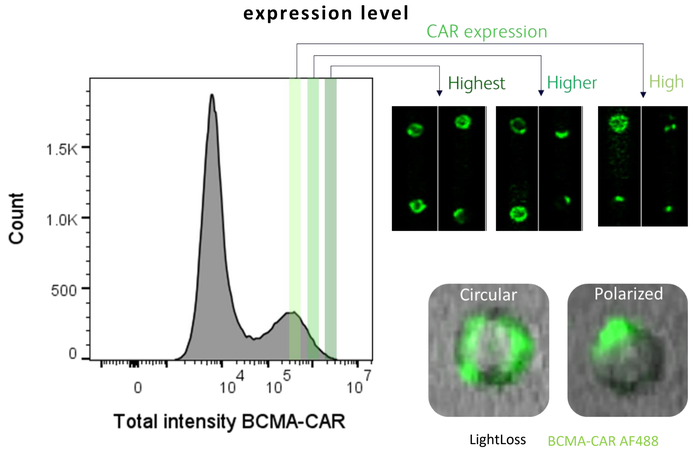
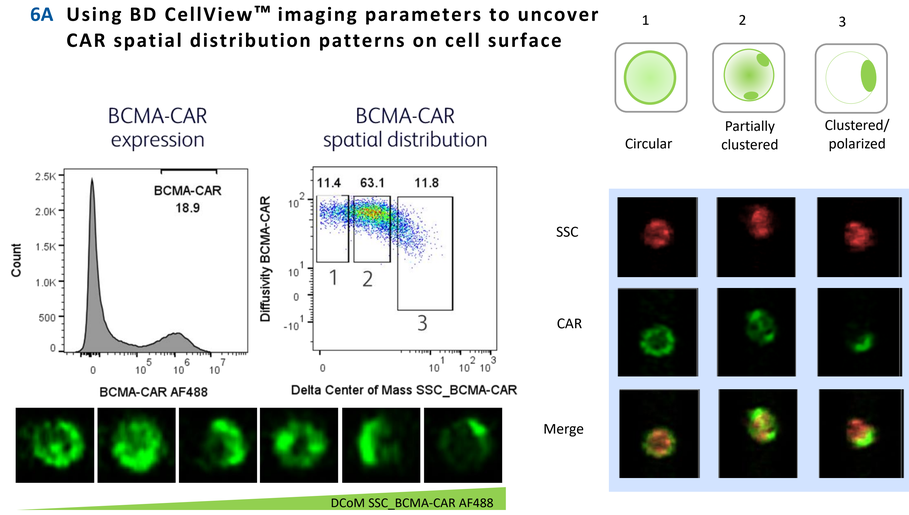


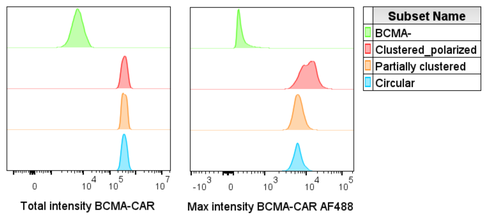
Figure 5: BCMA-CAR detection and visualization with BD CellView™ imaging parameters. A) BCMA-CAR T cells were analyzed after being spiked into PBMC preparations.. The BD 1-step BCMA-CAR Detection Reagent reliably label CAR T cells at frequencies as low as 0.15% of the total. Images from cell alone (top) or heterogenous samples (middle and bottom) showed comparable quality. **B)** Assessment of CAR spatial organization**.** After gating on live T cells, we selected cells with similar overall BCMA-CAR AF488 total intensity, ensuring that images displayed represent cells with relatively comparable expression levels. Under these conditions, we observed CAR distribution on cell surface varied bletween cells. **C)** CAR distribution patterns were uncoupled from expression levels.While most BCMA-CARs displayed a uniform surface distribution, a subset of the cells exhibited a punctate pattern. Both conformations were consistently observed a range of BCMA-CAR expression levels.

Results – Relationship between CAR spatial organization and CAR T cell functional states

6A Using BD CellView™ imaging parameters to uncover CAR spatial distribution patterns on cell surface



Imaging parameters:
- DCoM SSC_BCMA-CAR : Measure the separation between SSC and BCMA-CAR
- Diffusivity: Measure how spread out the BCMA-CAR AF488 signal is
- Max intensity : Display the intensity of the brightest BCMA-CAR pixels in the image



6B CAR circular conformation correlates with T cell degranulation

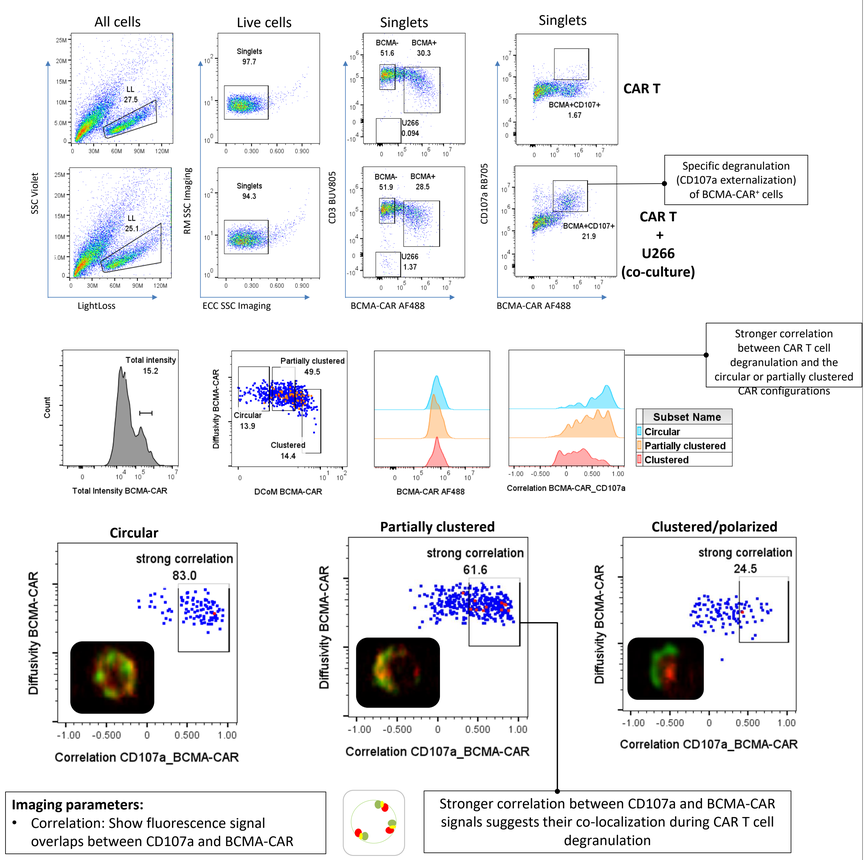


Figure 6: Imaging parameters reveal relationships between CAR spatial patterns and CAR T cell functional states. A) The delta center of mass parameter which measures separation of BCMA-CAR surface signals from side scatter (SSC)-derived intracellular granularity, distinguished a punctate (clustered/polarized) from a uniform (circular) BCMA-CAR distribution. Circular patterns displayed higher BCMA-CAR diffusivity whereas punctate patterns contained cells with the highest BCMA-CAR max intensity signals (histogram overlays). As shown in Figure 5C, these distinct BCMA-CAR patterns were not directly associated with expression levels. **B)** CAR T cell re-stimulation with BCMA positive U266 cells. CAR T cells were cultured alone or co-cultured with U266 cells in the presence of brefeldin, monensin and anti-CD107a -RB705 for 5h. Analysis revealed specific CD107a externalization in BCMA-CAR T cells co-cultured with U266 cells, indicating CAR engagement triggered CAR T cells degranulation. Notably, circular BCMA-CAR patterns correlated strongly with CD107a signals, suggesting co-localization of BCMA-CAR and CD107a at cell surface.

Imaging parameters:
- Correlation: Show fluorescence signal overlaps between CD107a and BCMA-CAR

Stronger correlation between CD107a and BCMA-CAR signals suggests their co-localization during CAR T cell degranulation

7A Association between CAR spatial organization and functional transcriptome

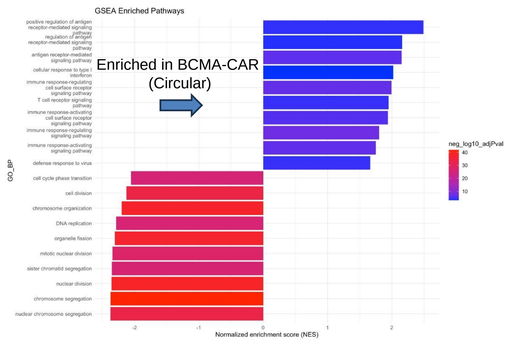


Figure 7: Single-cell whole transcriptome profiling of sorted circular and polarized BCMA-CAR T cells. Using the BD Rhapsody™ HT Xpress System 5,000 cells from each cell group were captured and deep-sequenced. Gene Set Enrichment Analysis (GSEA) comparing circular versus polarized revealed that circular BCMA-CAR cells were enriched for immune response-activating signaling pathways, in particular T cell signaling pathways.

Conclusions

- BCMA-CAR T cells exhibit distinct spatial patterns, appearing either uniform (circular) and punctate (clustered/polarized), independent of overall CAR expression levels.
- Imaging parameters distinguish these spatial patterns, in which circular distributions show higher diffusivity, while punctate distributions exhibit higher max intensity signals.
- Functional assays revealed a link between CAR organization and activity, in which circular BCMA-CAR patterns correlated strongly with CD107a externalization upon target engagement, indication active degranulation.
- Single-cell transcriptomics confirmed functional differences, with circular BCMA-CAR T cells enriched for immune response-activating signaling pathways compared to clustered/polarized cells.
- Together, these results suggest that CAR spatial organization, rather than expression level alone, emerges as a marker of CAR T cell functional states. Insights into how CAR distribution relates to function may inform engineering strategies to promote favorable spatial organization, enhancing efficacy as well as enable enrichment of CAR T cells with higher activity before infusion or provide an early biomarker of functional state, supporting patient stratification and real-time assessment of therapeutic potential.