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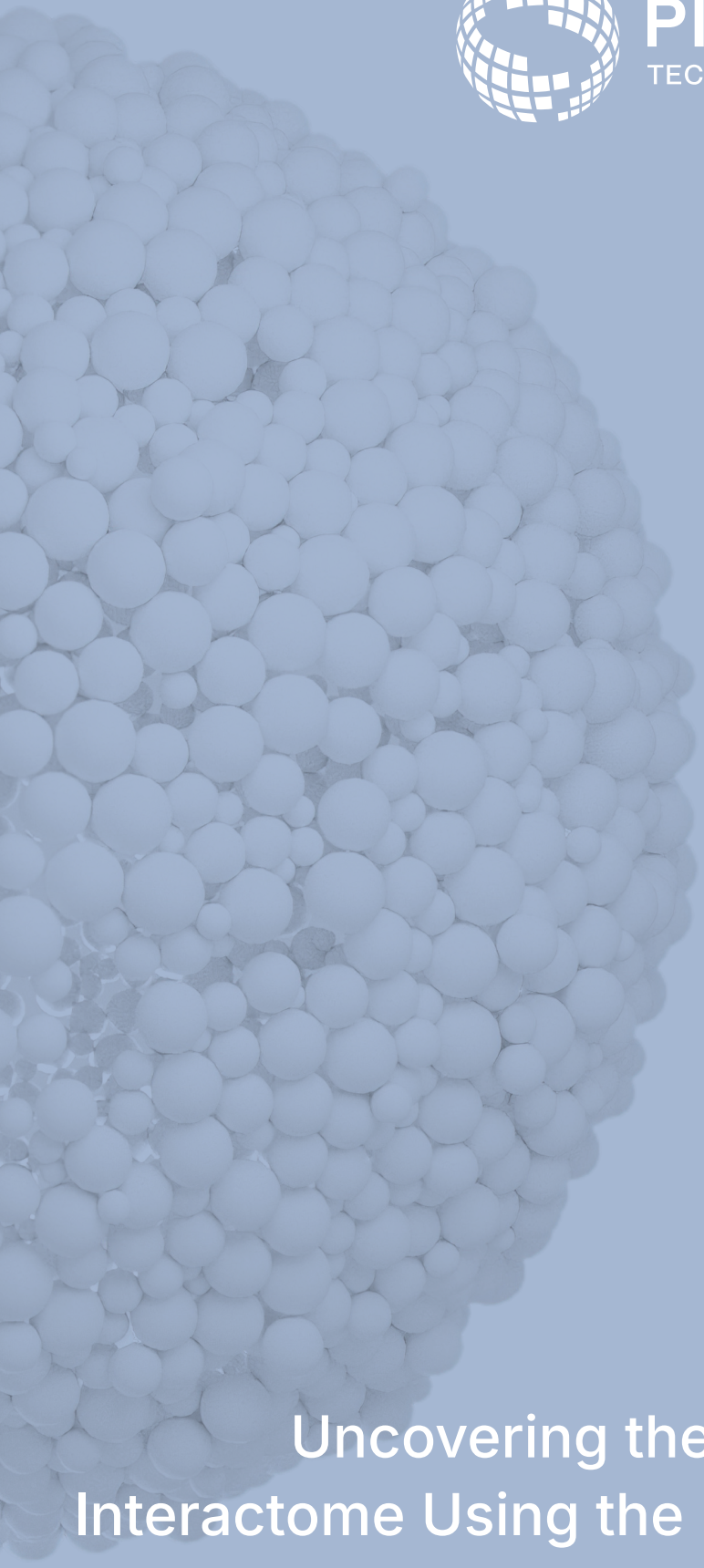

Uncovering the CD19 CAR T Cell Protein Interactome Using the Proximity Network Assay

APPLICATION NOTE

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DISSECTING THE CAR T CELL MEMBRANE

The Proximity Network Assay enables highly multiplexed, single cell protein interactomics studies in CAR T cells

ABSTRACT

The functional behavior of CAR T cells is intimately linked to the nanoscale organization of their membrane proteins, yet traditional omics methods struggle to capture this complexity at single cell resolution. In this study, we applied the high-multiplex, sequencing-based Proximity Network Assay from Pixelgen Technologies to deeply profile CD19 CAR T cells—both in resting conditions and during engagement with tumor targets. This technology enables simultaneous quantification of protein abundance and spatial organization across thousands of cells, revealing how membrane architecture shapes activation, exhaustion, and tumor interaction.

We identified the proxiome of the CD19 CAR receptor at steady state, observed dynamic proteomic remodeling during tumor encounter, and visualized key phenomena such as trogocytosis and cell–cell conjugate formation. Crucially, the spatial context provided by this assay allowed us to distinguish protein upregulation from antigen acquisition via trogocytosis, a distinction critical for functional interpretation. These findings demonstrate how the Proximity Network Assay can be used to decode CAR T cell biology with unprecedented granularity, supporting more informed therapeutic development.

HIGHLIGHTS

- Leverage the Proximity Network Assay to map the abundance and nanoscale organization of 155 surface proteins in thousands of single cells.
- The CD19 Chimeric Antigen Receptor localizes in a complex protein network associating with signaling receptors like CD5, TCRab and SLAMF6, while segregating from lipid raft associated proteins.
- The Proximity Network Assay differentiates between protein upregulation and trogocytosis. Activated and exhausted CAR T cells display distinct patterns of trogocytosis.
- The Proximity Network Assay allows detailed characterization of CAR T - Tumor cell conjugates.

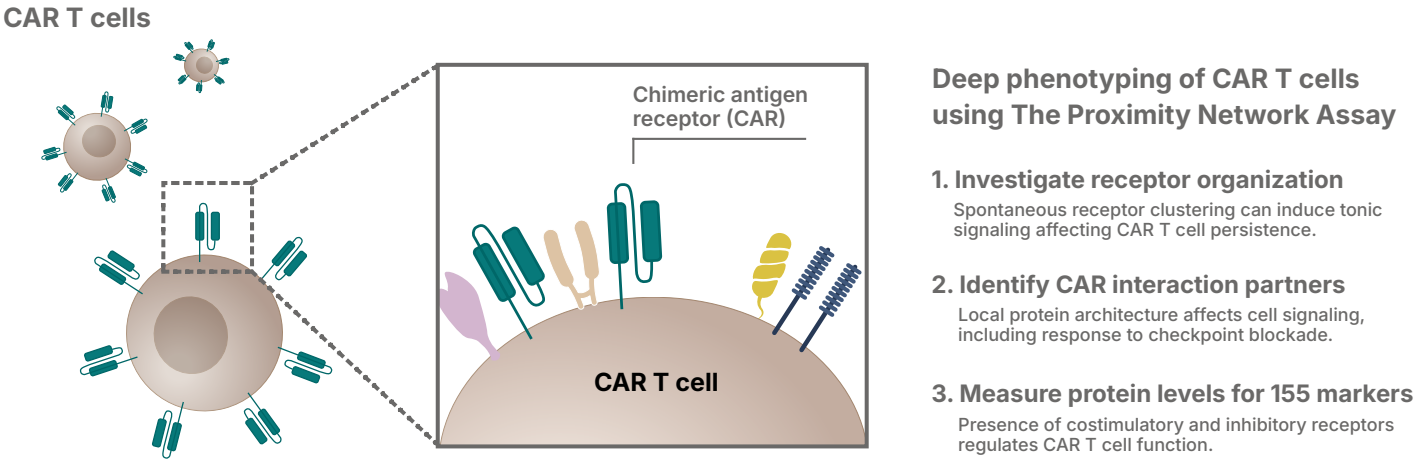

Figure 1. Deep phenotyping of CAR T cells using the Proximity Network Assay. The Proximity Network Assay reveals new details of the biology of CD19 CAR T cells by simultaneously measuring the abundance, clustering and pairwise colocalization of 155 surface proteins. This allows for detailed phenotyping and functional characterization of CAR T cells, providing information about receptor interaction partners, protein transfer between cells, cell:cell interactions, protein up- and downregulation and more.

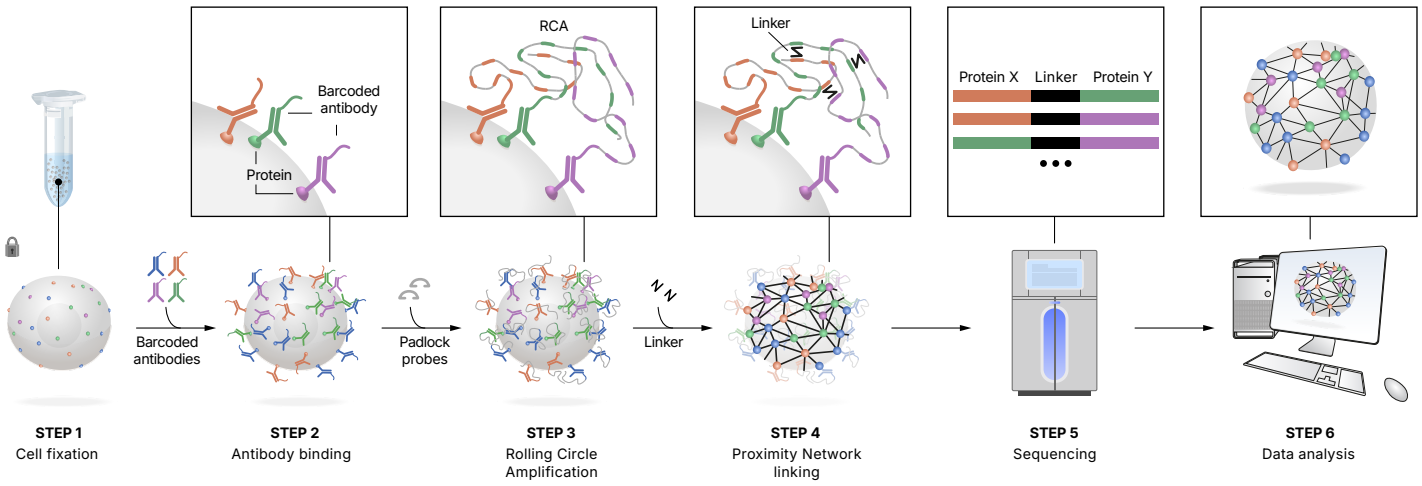

Figure 2. Workflow for the Proximity Network Assay. Cells are put in solution and fixed using 1 % PFA in order to immobilize the surface proteome (Step 1). After this, the cells are stained with a 155-plex antibody panel, where each individual antibody is tagged with a unique oligonucleotide sequence (Step 2). These unique sequences are elongated using rolling circle amplification (Step 3), after which sequences located next to one another are connected physically using a short linker sequence, ultimately forming a complete protein network on the cell surface (Step 4). The linker sequences, which now contain information about the two proteins they connected, are amplified and sequenced using Next Generation Sequencing (Step 5). Cell surface maps are computationally reconstructed for each individual cell using the open-source software Pixelator (Step 6).

BACKGROUND

Chimeric antigen receptor (CAR) T cell therapy has emerged as a transformative approach in cancer immunotherapy, particularly for hematological malignancies[1]. By engineering T cells to express synthetic receptors that target tumor-associated antigens, such as CD19 in B-cell malignancies, CAR T cells can be redirected to recognize and eliminate cancer cells with remarkable specificity and potency[2]. Despite clinical successes, challenges such as antigen escape, T cell exhaustion, and limited efficacy in solid tumors highlight the need for a deeper understanding of CAR T cell biology.

A growing body of evidence suggests that the spatial organization of membrane proteins plays a pivotal role in T cell activation and signaling[3-5]. For CAR T cells, the nanoscale distribution and clustering of CAR molecules at the plasma membrane can influence tonic signaling, receptor triggering and immune synapse formation[6-8]. Membrane microdomains and the dynamic assembly of receptor complexes are thought to modulate signal strength and duration, thereby impacting T cell function, persistence, and cytotoxicity[9-11].

To interrogate the molecular context of CAR T cell receptors, proteomics approaches, particularly interactomics, offer valuable insights. Interactomics enables the systematic mapping of protein-protein interactions, revealing the dynamic networks that underlie receptor function. By identifying interaction partners of the CD19 chimeric antigen receptor, it is possible to uncover key regulatory nodes and potential targets for enhancing CAR T cell efficacy.

METHODS

In this study, we leveraged the Proximity Network Assay to dissect the nanoscale membrane landscape of CD19 CAR T cells, both at rest and during tumor interaction (Fig 2). CD19 CAR T cells (CD19 scFv-FLAG-TM-CD28-CD3ζ) from PROMAB, and Raji cells from DSMZ, were profiled using the Immuno-155 Proxiome Kit from Pixelgen Technologies spiked in with an anti-FMC63 detection antibody. This high-multiplex, sequencing-based assay maps protein organization at single cell resolution by linking DNA-barcoded antibodies into spatial interaction networks. The result is a detailed view of immune receptor organization across thousands of cells simultaneously.

Data was preprocessed using the publicly available pixelator pipeline (part of nf-core as 'nf-core/pixelator'), generating protein abundances and proximity scores. Downstream analysis was performed using the open-source R package pixelatorR (also available as a Python package called 'pixelgen-pixelator') and publicly available R packages.

Cell Prep kit

- Pixelgen Proxiome Kit, Immuno 155 (cat. PROXIMM001)

Cells

- CD19 CAR T cells, ProMab (cat. PM-CAR1007)
- Raji, DSMZ (cat. ACC 319)

Sequencing

- P4 flow cell, NextSeq 2000, Illumina

PROXIOME OF THE CD19 CHIMERIC ANTIGEN RECEPTOR

To understand how membrane context influences CAR T cell function, we mapped the interactome of CD19 CAR T cells using the Proximity Network Assay. This approach enables high-dimensional profiling of membrane protein colocalization at single cell resolution. CAR expression was detected in ~40% of T cells (Fig 3A,B). In these cells, the CAR colocalized with proteins involved in T cell activation and immune synapse formation, including TCRαβ, CD5, CD6, CD44, and ICAM-1/2/3, suggesting its integration into signaling-competent regions (Fig 3C,D). In contrast, the CD19 CAR was segregated from tetraspanins like CD53 and CD81 and lipid raft-associated proteins such as CD52, CD59, and CD55. This exclusion mirrors the resting TCR, which similarly localizes outside lipid rafts until activation, highlighting a possible shared mechanism of spatial regulation in native and synthetic receptors.

Membrane Organization in CAR T Cells

Receptor Design Impacts CAR T Cell Signaling

CARs combine antigen-binding and signaling domains into one receptor, and structural design choices can influence membrane localization and cellular function[7,12].

Receptor clustering shapes function

While some studies link CAR clustering to tonic signaling and dysfunction, others show enhanced synapse formation and cytotoxicity — underscoring the need to understand what regulates CAR membrane organization[6,7,13].

Trogocytosis alters tumor recognition

CAR T cells can acquire target antigens from tumor cells via trogocytosis, reducing antigen availability and promoting immune escape[14,15].

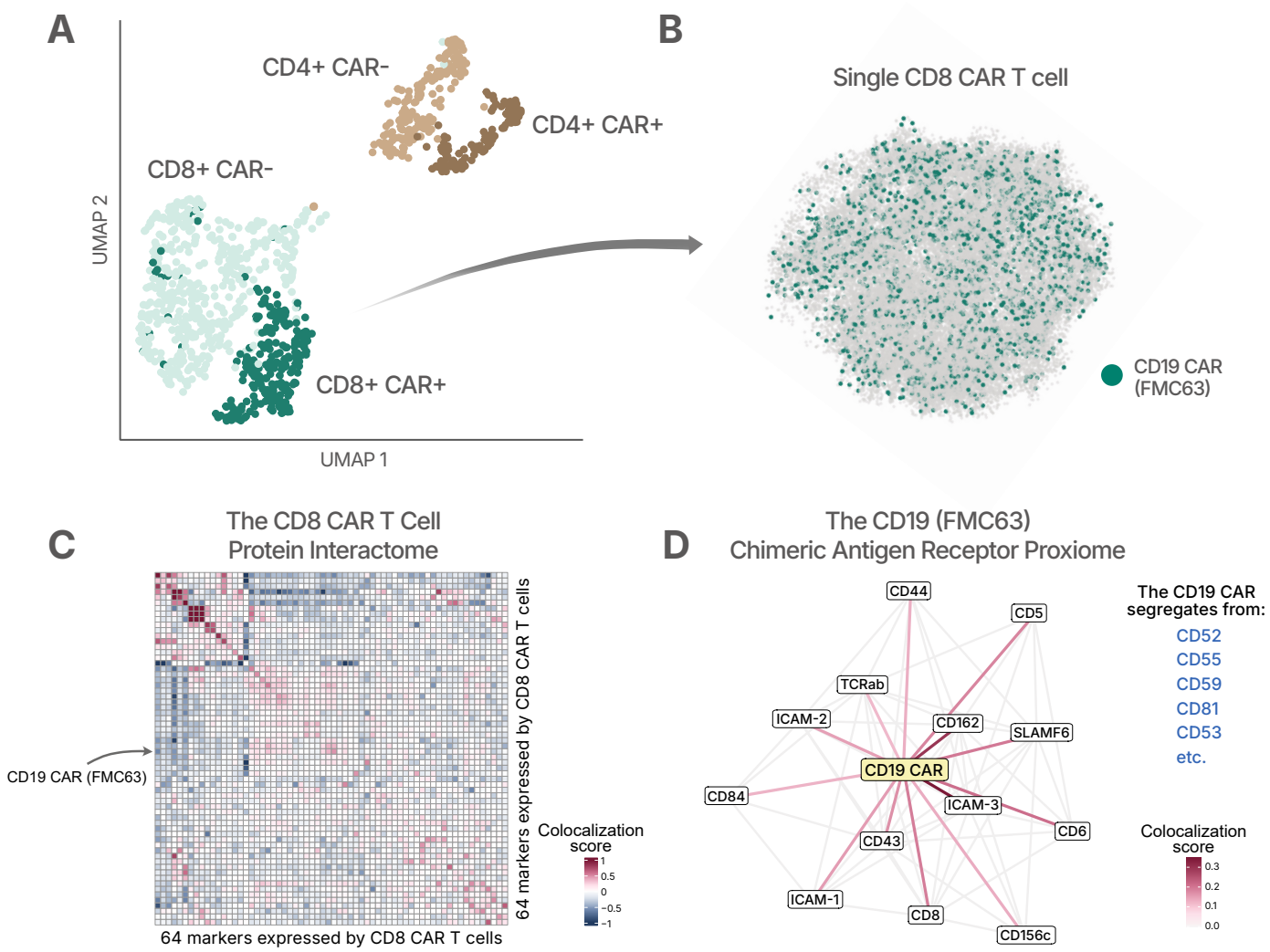

Figure 3. The Proximity Network Assay reveals the interactome of CD19 CAR T cells. (A) The T cell product consisted of a mix of CD4 and CD8 cells, with around 40 % of cells being CD19 CAR positive. (B) Individual cells can be visualized in 3D, and the FMC63-based chimeric antigen receptor was broadly distributed on the surface of positive cells. (C) Average pairwise colocalization scores were calculated for 64 proteins expressed on CD8+ CAR+ cells. (D) Proteins displaying positive colocalization (average score ≥0.1) with the CD19 CAR were plotted in a network (the CD19 CAR proxiome). Positive colocalization scores for protein pairs other than the CD19 CAR have been grayed out for clarity.

DYNAMIC PROTEOMIC REMODELING DURING TUMOR ENCOUNTER

To investigate dynamic proteomic changes during CAR T cell responses, CD19 CAR T cells were cocultured with CD19⁺ Raji tumor cells for either 4 or 24 hours and subsequently fixed for analysis using the Proximity Network Assay (Fig. 4). Large-scale shifts in protein abundance were observed in CD8⁺ T cells at both time points (Fig. 5). After 4 hours, early activation markers such as CD69 and 4-1BB (CD137) were upregulated, while at 24 hours, markers associated with sustained activation and regulatory signaling, PD-1 (CD279), GITR (CD357), and OX40 (CD134) became more prominent. In contrast, a reduction in 2B4 (CD244), CD28, and TCRαβ levels suggested altered costimulatory capacity and TCR involvement. Additionally, the FMC63 CAR signal was diminished following coculture, likely due to antigen masking, internalization, or receptor shedding. These findings highlight functional shifts that occur during tumor engagement and offer insight into CAR T cell adaptation over time.

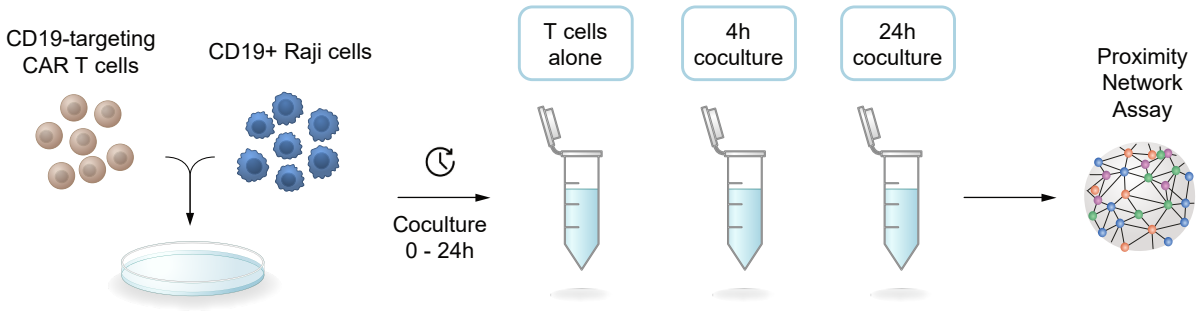

Figure 4. Workflow for functional characterization of CAR T cells. CAR T cells were either kept separate, or were mixed with CD19-positive Raji cells at an 1:1 E:T ratio. Cells were cocultured for either 4h or 24h, after which they were retrieved and fixed using 1 % PFA. Each sample was split into two duplicates and processed with the Proximity Network Assay according to the DP001 CAR Barcoded Antibody spike-in Proxiome kit (v1.00) manual.

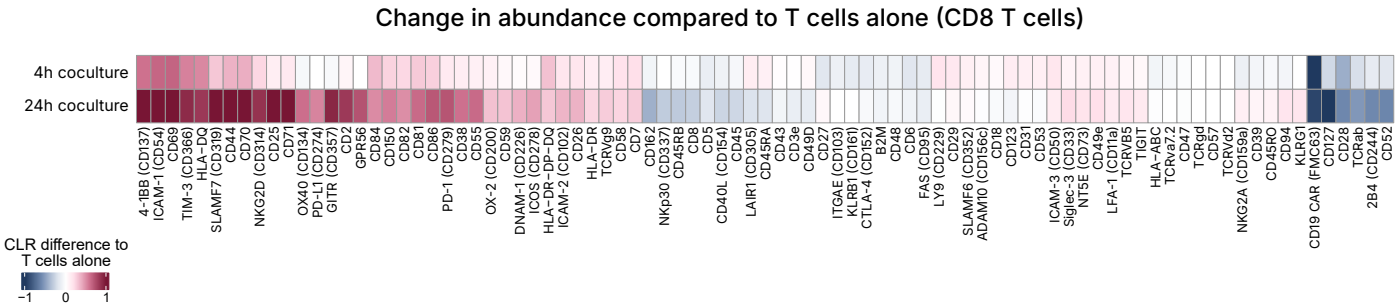

Figure 5. Tumor interaction alters the expression profile of CAR T cells. Cell types were identified in the Proximity Network Assay data and CD8 T cells were selected for further analysis. Average protein abundance levels were compared between CD8 T cells cultured alone and those cocultured for 4 and 24 h. A large number of proteins were up and downregulated including activation markers CD69 and CD25, checkpoint proteins PD-1 and TIM-3.

PROTEIN INTERACTOMICS BY PROXIMITY NETWORKS

- 155-plex panel containing antibodies recognizing all major cell types in PBMCs, granulocytes, stem cells, hematological cancer cells etc.
- By physically linking neighboring proteins, the method provides information about abundance and spatial location of all panel proteins within each single cell.
- Possibility to detect and characterize CAR T cells using an anti-FMC63 barcoded antibody spike-in.
- High spatial resolution - on average 50 000 unique molecular locations on each cell. Spatial relationships are assessed for all 155×155 protein pairs, generating up to 12 090 spatial scores for each single cell.

TROGOCYTOSIS DRIVES A PHENOTYPIC SHIFT IN CAR T CELLS

During the proteomic analysis, we observed that ICAM-1 (CD54) was significantly increased on CD8 T cells following coculture with Raji cells (Fig 5, Fig 6A). Upon visualizing the cells in 3D, we noted that ICAM-1 exhibited a highly patchy distribution, with only part of the proteins being evenly distributed while another protein pool was located in patches on the T cell membrane (Fig 6B). Accordingly, the clustering score, a metrics which estimates protein clustering, reported increased ICAM-1 clustering in cocultured cells (data not shown). Importantly, the ICAM-1-rich patches were colocalized with other B cell markers such as CD40 and CD21, indicating that these areas of high ICAM-1 content were not upregulated, but in fact membrane patches acquired through trogocytosis, a process where T cells capture membrane fragments from interacting cells (Fig 6B,C).

Thanks to the ability to visualize and measure the organization of individual proteins, The Proximity Network Assay allows for the distinction between protein upregulation and protein acquisition via trogocytosis.

To quantify the extent of trogocytosis, we developed a method to count the number of tumor-derived patches on each T cell (Fig 6D). Activated CD25+ cells acquired more patches than non-activated cells (Fig 6E). Interestingly, PD-1+ cells exhibited the highest number of membrane patches, suggesting a potential link between trogocytosis and T cell exhaustion. This observation is consistent with previous studies, which have associated trogocytosis with the impairment of anti-tumor activity over time. These findings underscore the importance of understanding the dynamics of trogocytosis in regulating CAR T cell function and efficacy.

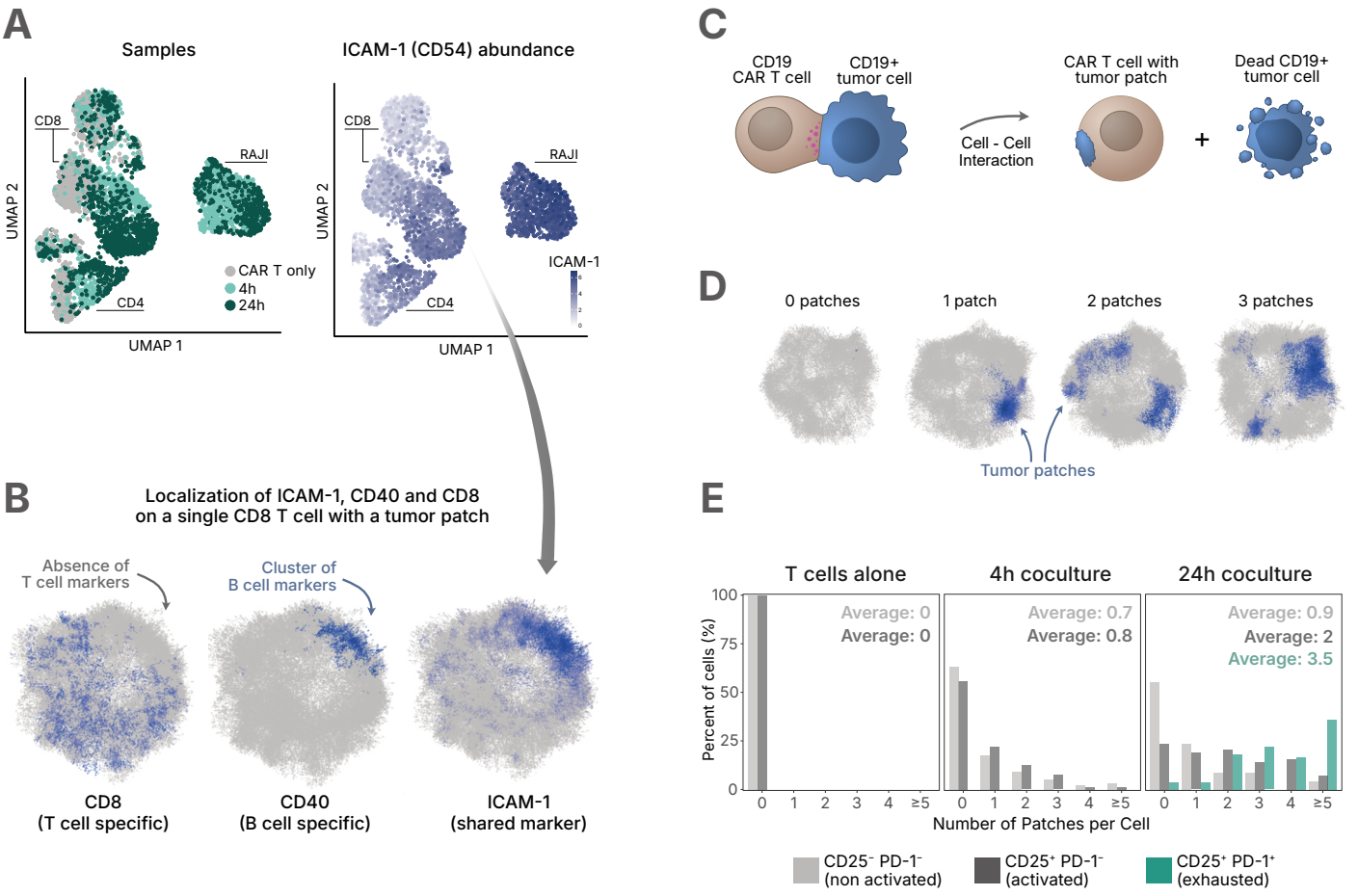

Figure 6. Activated and exhausted CAR T cells present distinct patterns of trogocytosis. (A) Left: High-dimensional clustering based on protein abundance data for CAR T cells alone, 4h cocultured cells, and 24h cocultured cells efficiently separating CD4, CD8 and Raji cells. Right: Cocultured T cells displayed increased levels of ICAM-1. (B) 3D visualization of a single CD8 T cell from the 24h cocultured sample. The cell displays a patchy ICAM-1 distribution where the patch colocalize with B cell markers like CD40, while anti-colocalizing with T cell marker CD8. (C) Illustration showcasing the process of trogocytosis, exchange of plasma membrane between cells, in CAR T cells. (D) Examples of 4 cells displaying varying numbers of tumor patches (in blue) on their surface (0-3 patches). (E) Quantification of tumor patches on the surface of CD8+ non-activated ($CD25^-$, $PD\text{-}1^-$), activated ($CD25^+$, $PD\text{-}1^-$) and exhausted ($CD25^+$, $PD\text{-}1^+$) T cells.

VISUALIZING T CELL-TUMOR CELL CONJUGATES AT SINGLE CELL LEVEL

Interactions between CAR T cells and their target cells are central to therapeutic efficacy, yet these events are difficult to capture and characterize in commonly used assays. Using the Proximity Network Assay, we were able to detect and visualize physical conjugates between CD8+ CAR T cells and CD19+ Raji tumor cells within fixed coculture samples (Fig 7). These conjugates were detected by identifying tightly apposed T cell–tumor cell pairs based on physical morphology and distinct lineage marker distribution.

By evaluating the number and location of the lineage-specific molecules, the assay enables differentiation between true cell–cell conjugates and individual T cells with extensive trogocytosis. While both scenarios may display tumor-derived proteins on the T cell surface, only conjugates show clear membrane juxtaposition with a single, large tumor cell, preventing misinterpretation of high antigen acquisition as presence of a cell conjugate.

In future applications, such data could support the exploration of how tumor cell phenotype influences conjugate formation, or how activation and exhaustion markers vary between engaging and non-engaging CAR T cells. This opens opportunities for understanding cell interaction dynamics at scale in therapeutic development.

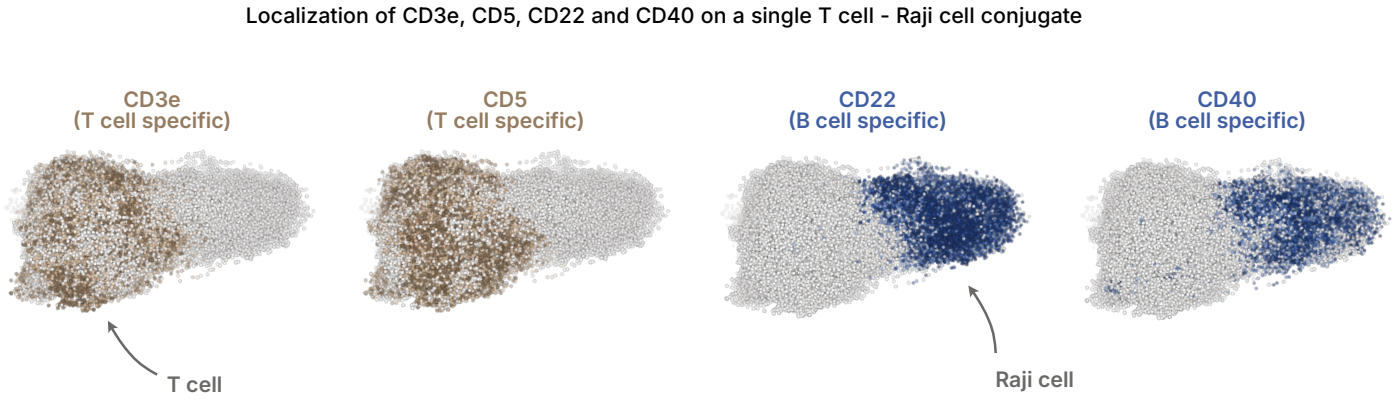

Figure 7. The Proximity Network Assay identifies CAR T : Tumor cell conjugates for deeper characterization. An example of a CD8 T: Raji cell conjugate. The T cell (left) displays expression of T cell specific markers like CD3e and CD5, while the Raji cell (right) expresses B cell markers CD22 and CD40. The assay makes it possible to visualize the conjugates in 3D and to characterize the expression profile and organization of cell type specific, as well as shared surface proteins.

SUMMARY

CAR T cell phenotyping has traditionally focused on bulk or single cell measurements of protein abundance, using tools like flow cytometry or CITE-seq. In this Application Note, we demonstrate how the Proximity Network Assay offers a deeper view of CAR T cell biology. We showcase how the assay uniquely characterizes CAR T cells—not only by quantifying surface protein levels, but also by mapping how CARs and other key receptors are organized and interact on the cell membrane. This enables detailed analysis of receptor clustering, immune synapse formation, and trogocytosis, as well as the identification of T cell–tumor cell conjugates. The result is a powerful tool for dissecting CAR T cell behavior which could provide powerful insights from basic to clinical research.

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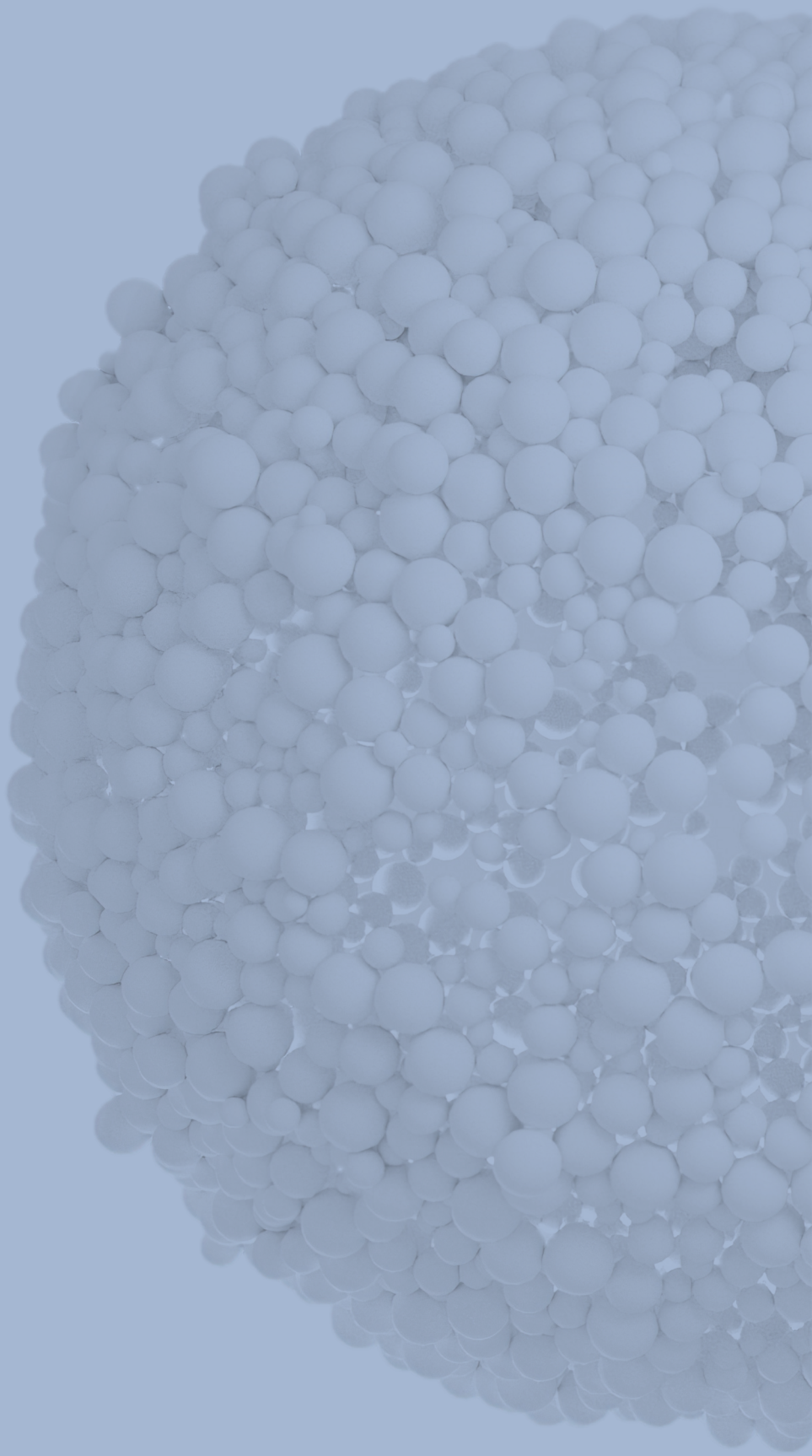

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