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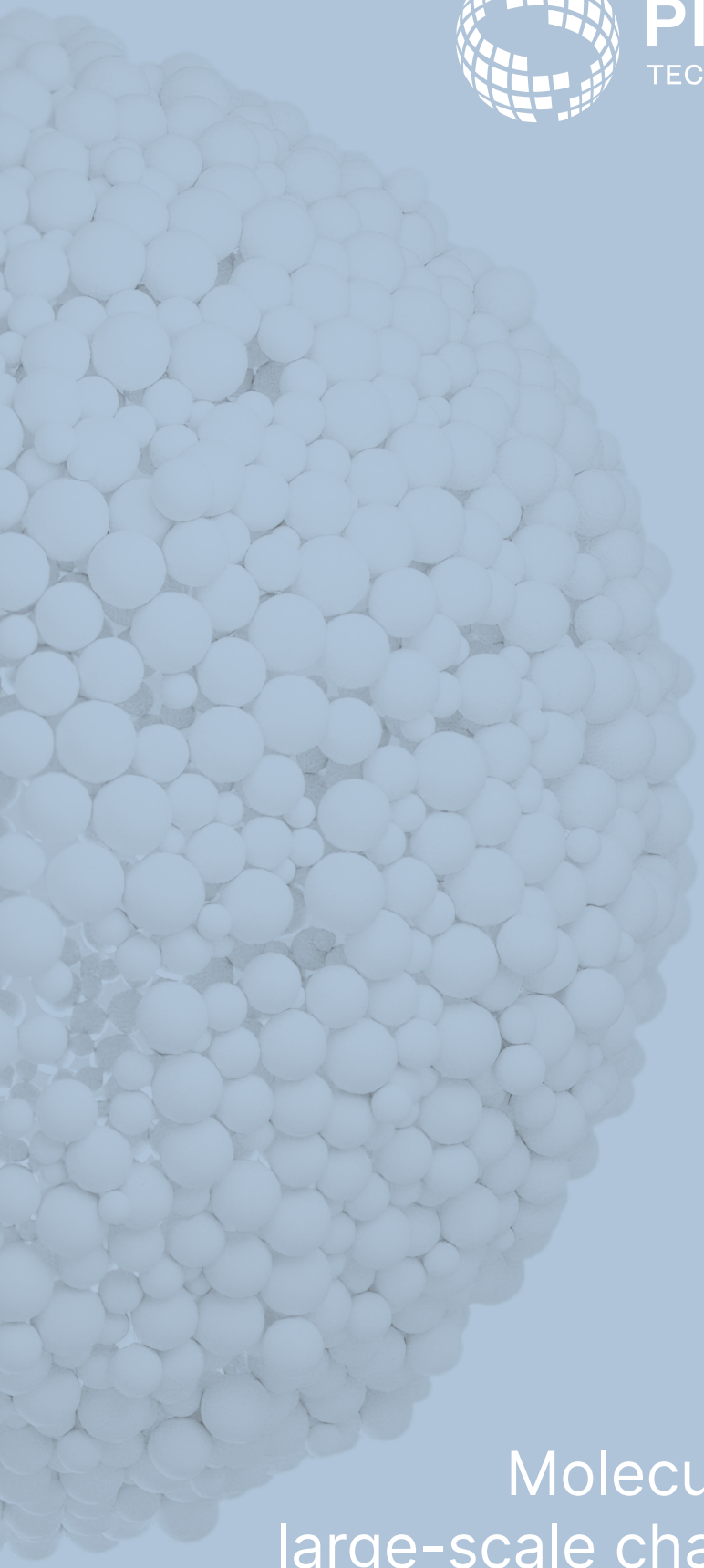

Molecular Pixelation uncovers large-scale changes in the membrane protein architecture of activated T cells

APPLICATION NOTE

AN00001D

NEW MEANS OF STUDYING T CELL ACTIVATION

Molecular Pixelation identifies activation-induced changes in protein abundance, clustering and colocalization

T cell activation is traditionally measured through the detection of cytokine production or the expression of activation markers including CD69 and CD25. In this Application Note, we harnessed the high multiplexing capability and the spatial resolving power of the Molecular Pixelation (MPX) technology, to show that T cell activation is associated with not only large-scale alterations in protein abundance levels, but also with a profound reorganization of membrane proteins. Using the 80-plex Single Cell Spatial Proteomics Kit, Immunology Panel I, Human, we detected T cell subsets in PBMCs, and revealed activation-induced differential expression of more than 30 proteins, including the upregulation of activation markers CD69, CD25 and CD38. Importantly, MPX also uncovered dramatic activation-induced changes in the organization of T cell surface proteins. For instance, we found that the tetraspanin CD82 reorganized and displayed a clustered phenotype in activated cells. Interestingly, this protein reorganization was seen only in a fraction of cells and was not correlated with the protein abundance level, demonstrating how spatial protein analysis can be used for deeper phenotyping and improved characterization of cellular states. By exploring both the abundance and spatial organization of a multitude of surface proteins simultaneously, MPX thus offers a unique angle as a novel discovery tool when studying immune cell responses.

HIGHLIGHTS

- **The 80-plex panel allows cell type characterization, and more than 30 markers display differential abundance in activated compared to resting T cells**
- **MPX demonstrates the existence of a distinct T cell state presenting a high degree of CD82 clustering**
- **MPX unravels multi-protein colocalization networks, including the enrichment of CD55 and CD48 in nanoclusters at the surface of activated T cells**
- **By simultaneously mapping the abundance and spatial organization of membrane proteins in single cells, MPX offers a unique angle to study the complex phenotypes of immune cells**

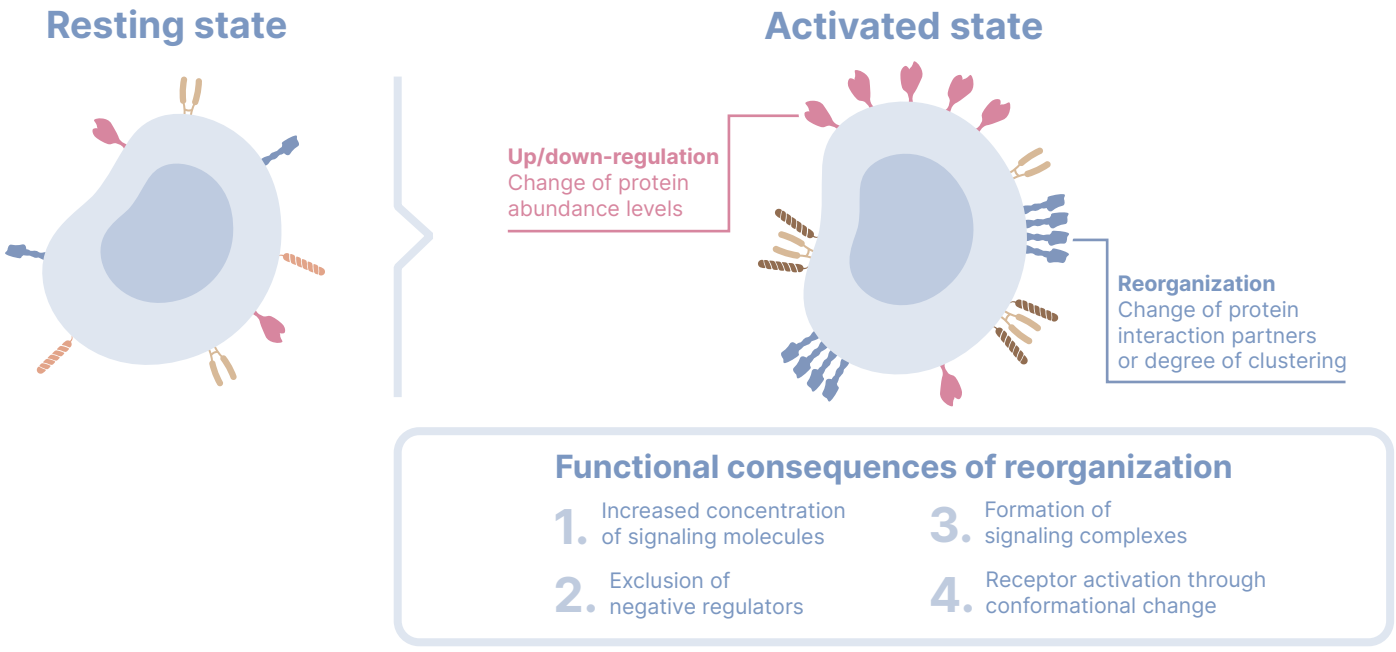

Figure 1. T cell activation is associated with alterations in the membrane protein organization. Activation results in a profound reshaping of the surface landscape of T cells, including changes in the spatial organization of the proteins. Although critical for cellular function, the spatial organization of membrane proteins is typically overlooked by most traditional T cell analysis methods.

BACKGROUND

T cells are key mediators of immunity, and provide strong targeted responses against pathogens (1). Their functions are diverse and include the ability to induce inflammation, aid in the regulation of other immune cells, and to directly kill infected and transformed cells. In order to carry out many of these effector functions, T cells require activation mediated by antigen-presenting cells (APCs) (2). This requirement acts as a safety mechanism to ensure a response targeted towards the threat, and to avoid harmful autoimmune reactions. Apart from the process of natural activation seen *in vivo*, efficient T cell activation can also be achieved *in vitro*, for example when generating T cell products for cell therapy. Protocols for *in vitro* T cell activation include the use of antigen-coated beads, CD3/CD28-stimulating antibodies, or the mitogen Phytohaemagglutinin P (PHA) (3–5). PHA induces activation through cross-linking of the T cell receptor, and the agent is commonly used to assess T cell fitness and function (6,7).

The activation of T cells is accompanied by dramatic changes in protein expression patterns (8). This process ensures that the cell is adapted to new functional and metabolic demands. The expression of markers associated with activation, for example CD69 and CD25, can be used to identify activated T cells in a heterogeneous population. Additionally, it is getting increasingly appreciated that T cell function is not only affected by protein abundance levels but also by the organization of the surface proteins. For example, enrichment of CD3, and depletion of CD45 molecules on the tip of microvilli has been suggested to facilitate efficient T cell mediated probing of antigen on APCs (9). Thus, analyzing both the abundance and the organization of surface proteins will provide a deeper understanding of what factors influence the function of T cells.

MOLECULAR PIXELATION TO STUDY T CELL ACTIVATION

In this Application Note, we demonstrate how Molecular Pixelation can be applied to study T cell activation (Fig 2). Specifically, we show that the multifaceted capacity of MPX to simultaneously measure membrane protein abundance, while also untangling the spatial organization of membrane proteins, can aid in discovering new features of activation.

In three independent experiments, PBMCs were isolated from buffy coats or whole blood of three healthy donors using density gradient centrifugation followed by red blood cell lysis. Part of the isolated resting cells were fixed using 1% PFA and frozen, whereas the other part was subjected to a 72 hours-long activation culture using cell medium supplemented with PHA. After 72 hours, the activated cells were harvested, fixed and frozen for subsequent analysis. MPX was carried out simultaneously on resting and activated cells using the Single Cell Spatial Proteomics Kit, Immunology Panel I, Human, following the Molecular Pixelation User Manual Version 1.02. PCR products were sequenced using a NextSeq 2000 with a P2 or P3 flow cell.

The resulting sequencing reads were preprocessed by the best-practice data processing pipeline Pixelator, available as part of the nf-core project ('nf-core/pixelator v1.0.3'). This analysis workflow outputs the protein abundances as well as the protein polarity and colocalization scores presented in this application note. Further downstream analysis of these metrics was performed using the open-source R package 'PixelatorR' (also available in Python as 'Pixelgen-pixelator') and other publicly available packages.

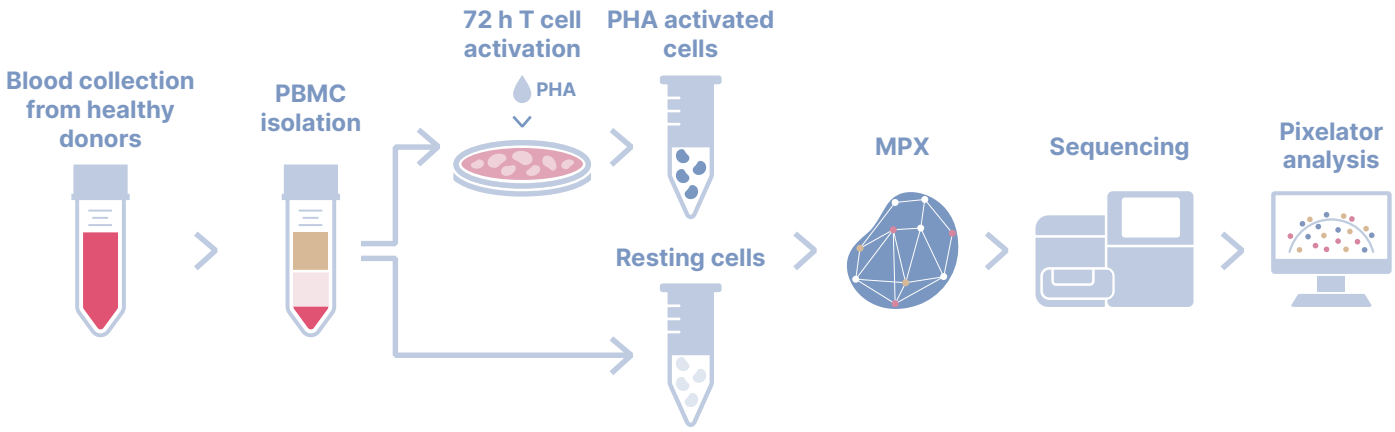

Figure 2. Experimental workflow for MPX-mediated analysis of T cell activation. PBMCs isolated from three healthy donors were either fixed as resting, or activated for 72 hours with PHA before fixation. We performed Molecular Pixelation (MPX) on all samples using the Immunology Panel I, Human, and analyzed the results using the Pixelator pipeline (number of analyzed cells n = 1000, 1000, 500 respectively).

MOLECULAR PIXELATION IDENTIFIES MAJOR PBMC SUBSETS

The 80-plex Immunology Panel I, Human, contains antibodies that enable the detection of key cell types in human PBMCs, including CD4 T cells, CD8 T cells, B cells, Monocytes, and NK cells. To segregate T cell subsets for downstream analysis, we performed multi-dimensional cell clustering of the PBMC samples based on the abundance of all surface markers. Through manual annotation, all major cell types could be efficiently detected in PBMCs of the three donors (Fig 3A), and the abundance levels of cell type-defining markers, including CD3, CD19 and CD14, displayed the expected distribution profile (Fig 3B). As commonly seen in heterogeneous human populations, the relative proportion of each cell type varied between the individual donors (Fig 3C), but for all donors, PHA activation led to an enrichment of the T cell fraction.

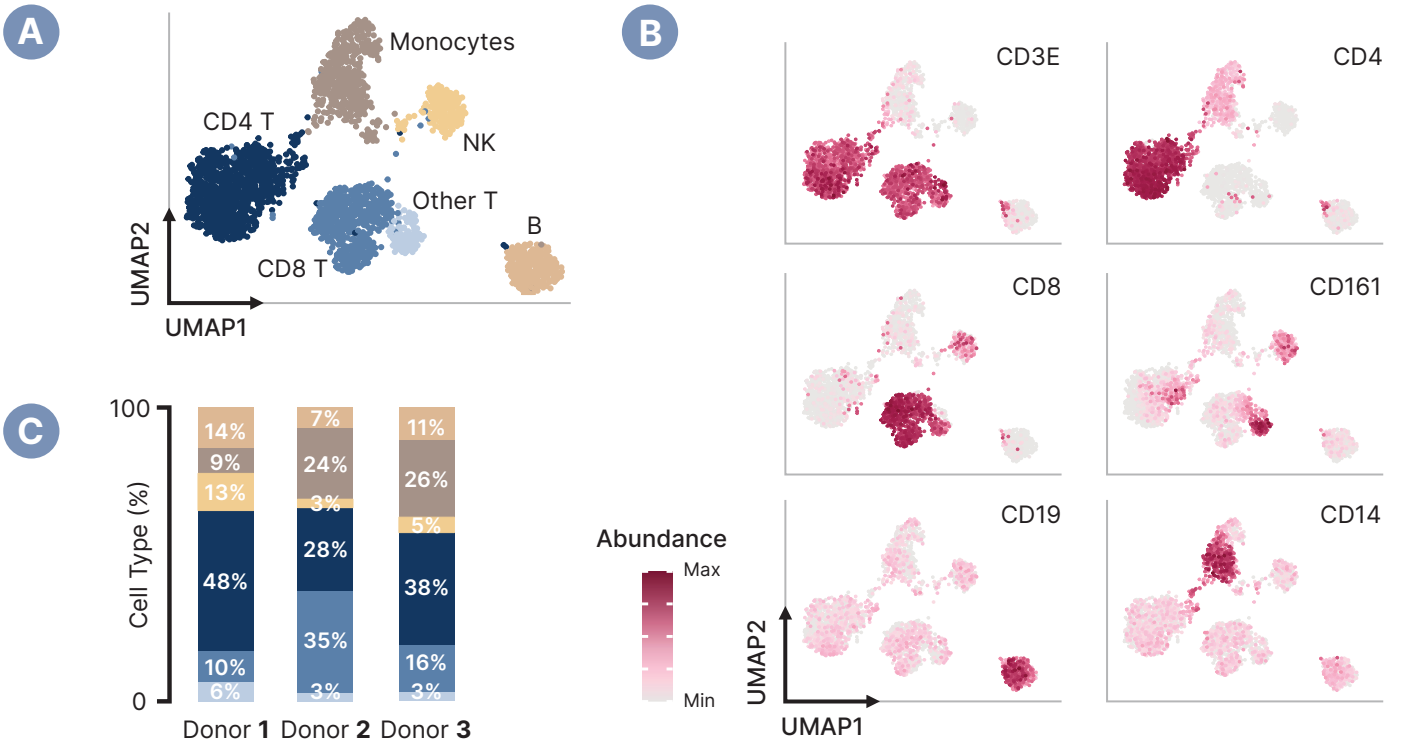

Figure 3. MPX detects key cell types of PBMCs. Cell type clustering based on the abundance levels of the 80 panel markers in resting PBMCs from the three healthy donors. (A) UMAP representation of the major cell types segregated into distinct clusters. (B) Example expression of cell type-defining markers. (C) Relative proportion of different cell types in the resting PBMC population of the three donors.

IMMUNOLOGY PANEL I, HUMAN

- 80-plex panel containing antibodies targeting 76 surface proteins, 1 intracellular control protein, and 3 isotype controls
- The panel targets major cell types of PBMCs including CD8 T cells, CD4 T cells, B cells, Monocytes, and NK cells
- 30+ of the targets can be found in T cells
- A vast number of protein target types including activating receptors, inhibitory receptors, adhesion molecules, nutrient receptors etc

Single Cell Spatial Proteomics Kit, Immunology Panel I, Human

Product number: PXGIMM001

PHA ACTIVATION INDUCES CHANGES IN T CELL MEMBRANE PROTEIN LEVELS

As PHA is a T cell mitogen, we were interested in investigating how PHA-mediated activation affects protein abundance levels in CD4 and CD8 T cells. As many as 34 of the panel markers could be detected in T cells and the activation led to an altered expression of a large number of these proteins, including the upregulation of common activation-associated proteins such as CD69, CD71, CD38, and CD25 (Fig 4A). Interestingly, the MPX assay detected variations in the degree of response between the three donors, with donor 2 displaying less efficient activation as compared to the other two donors.

T cell activation is commonly studied using flow cytometry. Similarly to MPX, flow cytometry is a single cell technology, and provides information about protein levels in individual cells within a sample. Using MPX and flow cytometry in parallel, we analyzed the expression of CD69 in CD8 positive T cells. Both technologies provided similar CD69 expression patterns, with an increased CD69 expression in PHA-activated cells (Fig 4B). Importantly, the fraction of CD69-positive CD8 T cells was very similar for the two technologies.

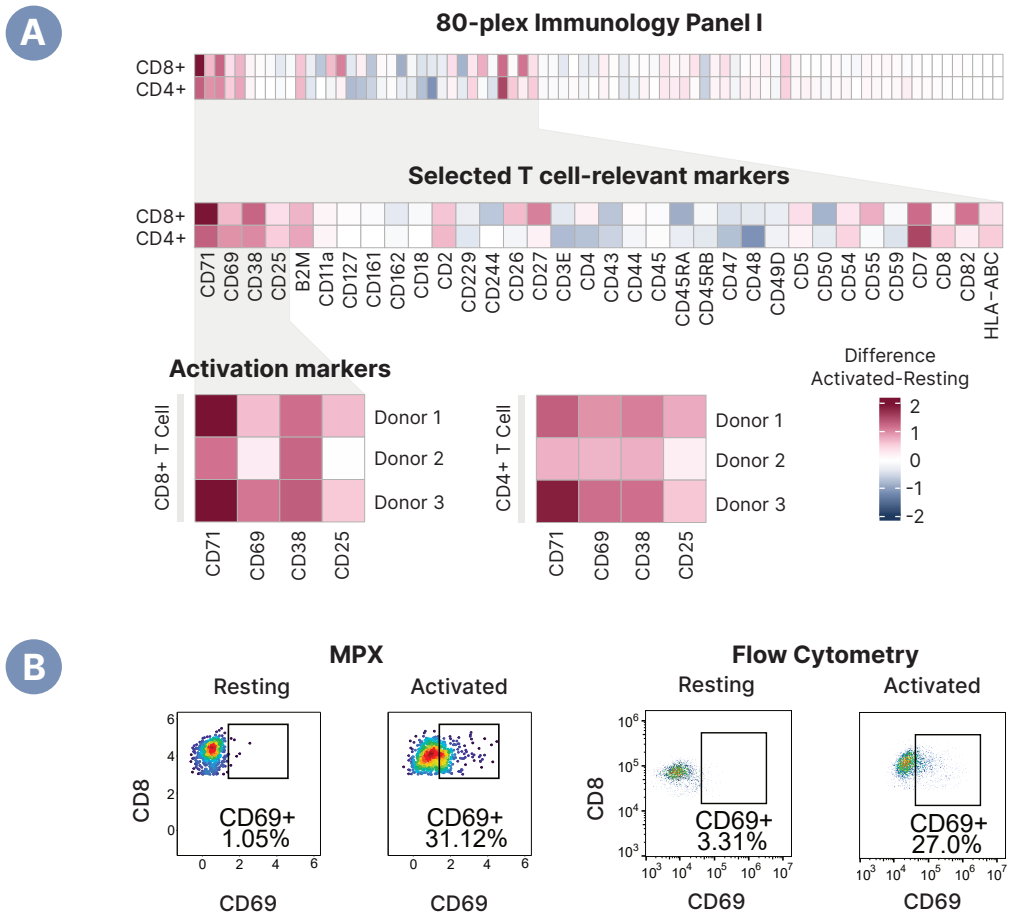

Figure 4. MPX elucidates activation induced changes in T cell surface protein abundance. (A) Differential protein abundance between resting and PHA-activated CD4 and CD8 T cells, among all panel markers (top), T cell-relevant markers (middle), or selected activation markers (bottom). Top and middle: average for the three donors. (B) Proportion of CD69-positive cells among resting or activated CD8 T cells, as measured by MPX (left) or flow cytometry (right).

MOLECULAR PIXELATION REVEALS THE RELOCATION OF T CELL MEMBRANE PROTEINS DURING ACTIVATION

Beyond the analysis of protein abundance levels, Molecular Pixelation also uniquely assesses the spatial organization of all membrane proteins included in the panel. One way to measure organization is using the **MPX Polarity Score**. This score reports how spatially clustered a particular protein is, measuring every protein marker, in every single cell. Focusing our analysis on T cells, we discovered that PHA-mediated activation in fact resulted in the reorganization of membrane proteins (Fig 5). One protein displaying a dramatic increase in spatial clustering was CD82, a tetraspanin known to form nanoclusters, and to be important for immune cell function including immune synapse formation (10, 11). PHA activation led to a small population-wide increase in the abundance levels of CD82 in T cells (Fig 5A),

but a subpopulation of activated T cells displayed a large increase in their CD82 polarity score (Fig 5B), indicating increased clustering. However, this change in clustering was not correlated with CD82 abundance, suggesting a separate activation signature for this subset of T cells (Fig 5C). To visualize the distribution of CD82 in the two samples, we generated 3D reconstructions of the cells from the MPX data (Fig 5D). In agreement with this MPX data, immuno-fluorescence microscopy clearly displayed how CD82 polarized into large-scale clusters in activated CD8 T cells (Fig 5E). These results suggest that the spatial organization of membrane proteins can be used as a distinct signature of T cell activation, providing new insights into the single cell heterogeneity of T cell functional responses.

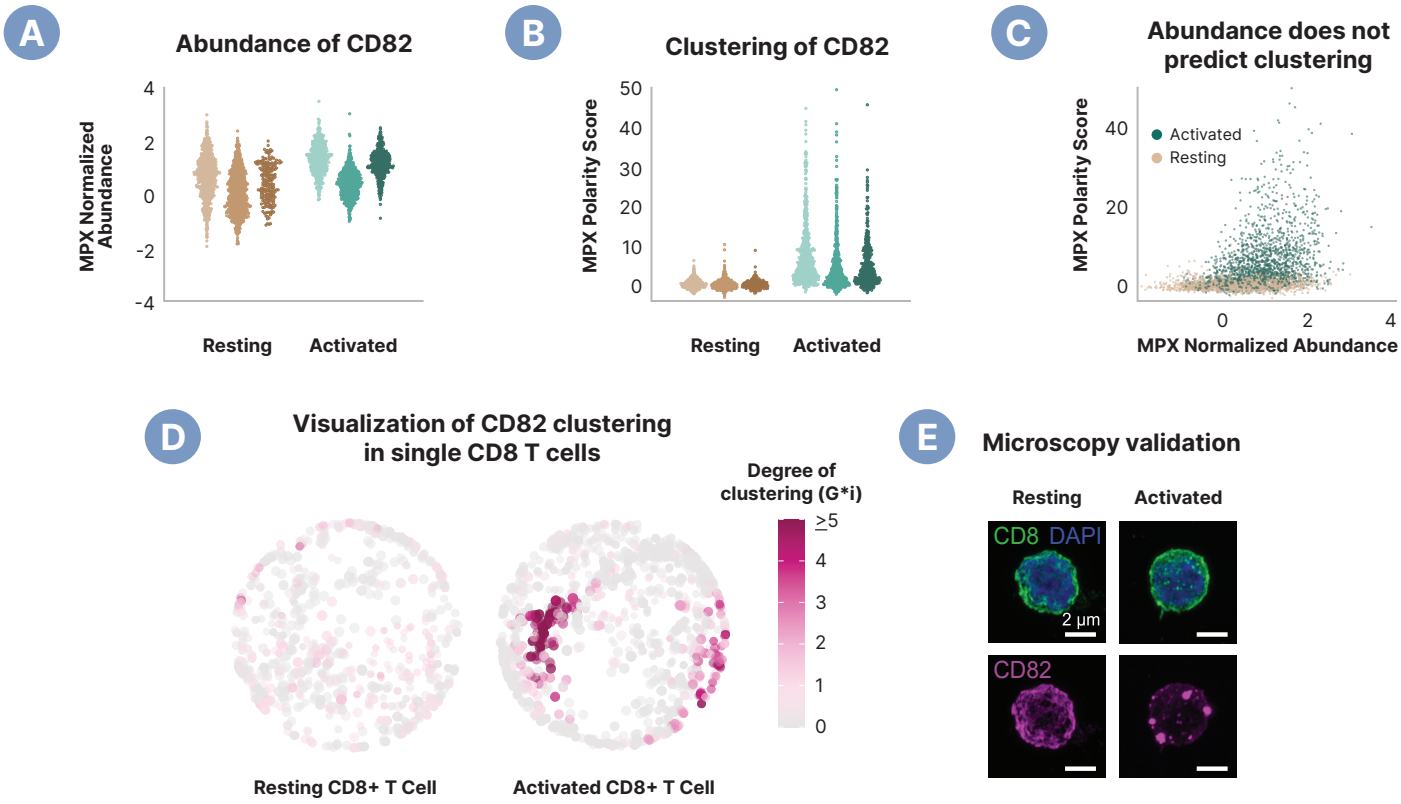

Figure 5. MPX reveals the existence of a distinct activated T cell population with clustered CD82 at the cell surface. (A) Beeswarm plot of normalized abundance of CD82 on single resting and activated T cells. (B) MPX Polarity Score of CD82 on resting and activated T cells, showing a subset of activated T cells with highly polarized CD82. (C) The relation between CD82 Polarity Score and abundance on single T cells. (D) Spatial visualization of CD82 molecules on the surface of an example resting (left) or PHA-activated (right) CD8 T cell. Each point corresponds to a uniquely measured spatial zone, DNA pixel, with the color scale indicating the degree of protein clustering in that location. (E) Airyscanning superresolution micrographs confirming CD82 clustering on PHA-activated, but not on resting CD8 T cells.

WHAT IS THE MPX POLARITY SCORE?

- The MPX Polarity Score describes the degree of clustering or polarization of a given surface protein
- It is calculated per marker in each individual cell as Moran's I, a measure of spatial autocorrelation which ranges from -1 (perfectly dispersed) to 1 (perfectly clustered)
- To compare cells, it is transformed into Moran's z-score, to reflect its deviation from the degree of clustering expected by random chance

WHAT IS THE MPX COLOCALIZATION SCORE?

- The MPX colocalization score measures the co-occurrence of a pair of proteins within small neighborhoods on the cell surface
- It is calculated in single cells as the Pearson correlation between two markers, and often presented as a Pearson z-score, to reflect its deviation from the degree of colocalization expected by random chance
- A z-score above zero indicates that the colocalization is higher than expected by random chance. A colocalization score below zero can indicate a segregation of the two markers

MPX ENABLES THE IDENTIFICATION OF MULTIPROTEIN COLOCALIZATION NETWORKS

The colocalization of specific proteins can be pivotal for their function, by for example providing stabilization, enabling co-signaling or even inducing direct signaling through conformational change or ligation in cis (12-14). Hence, studying the colocalization of membrane proteins can yield valuable information regarding both the function of individual proteins and the existence of multi-protein domains. Using MPX, protein colocalization can be revealed by calculating the **MPX Colocalization Score.** The score estimates the colocalization of individual protein pairs, by measuring the relative enrichment or depletion of two proteins in proximity to each other. Focusing on PHA-activated T cells, we calculated the colocalization scores of all 34×34 protein pairs (Fig 6A). To further investigate the intricate structure of the membrane of activated T cells, we generated colocalization networks from protein pairs with scores higher than 0.5 (Fig 6B).

Two proteins displaying a central position in the colocalization network were the GPI-coupled proteins, CD55 and CD48, both previously shown to associate with lipid rafts. In contrast, the T cell associated protein CD7 displayed no colocalization with neither CD48 nor CD55. We validated the findings using immunofluorescent co-staining of the three proteins followed by super-resolution Airyscanning microscopy, which showed that both CD48 and CD55 displayed a high degree of nanoclustering and a large fraction of the clusters colocalized. CD7 on the other hand displayed a smooth expression profile with uniform distribution and no enrichment in the CD48/CD55 clusters (Fig 6C). These findings highlight how MPX can be applied to untangle complex protein colocalization networks at the surface of immune cells, offering a unique opportunity to reveal novel mechanisms of cellular function.

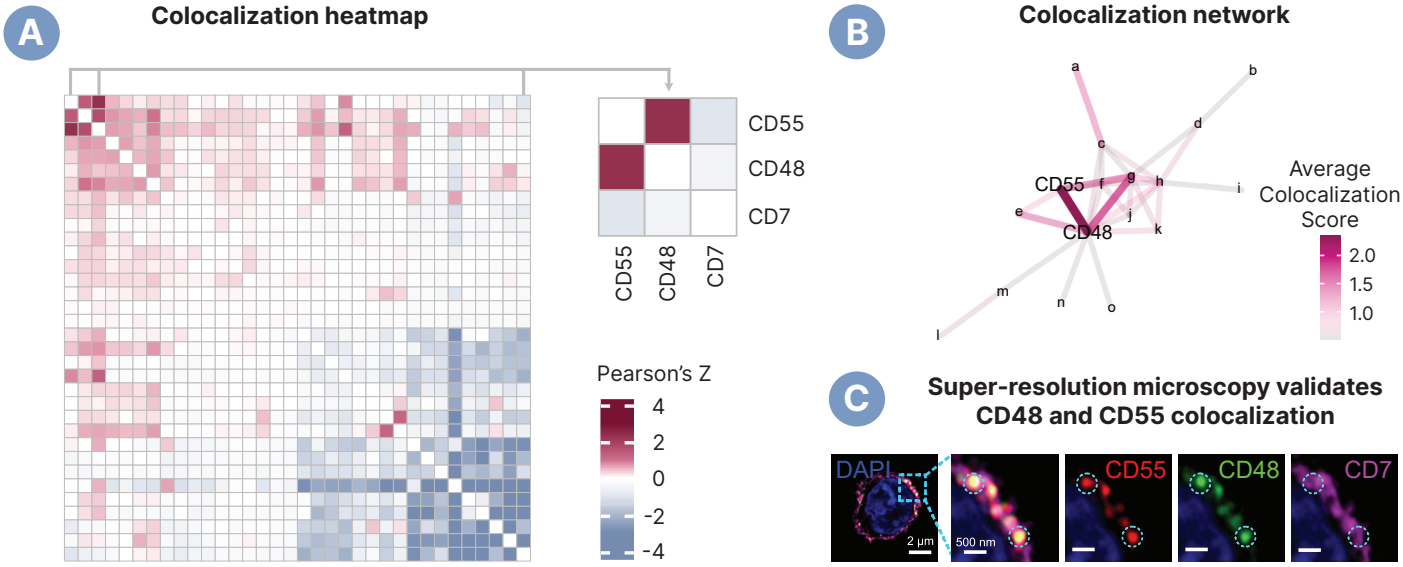

Figure 6. The MPX Colocalization Score reveals multiprotein colocalization networks. (A) Pearson's Z colocalization scores for all protein pairs expressed by PHA-activated T cells. (B) Colocalization network constructed using positive colocalization scores (mean score>0.5) from the matrix shown in (A). (C) Airyscanning superresolution micrographs confirming the colocalization of CD55 and CD48 in nanoclusters at the surface of activated T cells, compared with the even distribution of CD7 as a negative control.

SUMMARY

T cell activation is traditionally studied by directly or indirectly investigating protein abundance, for instance using flow cytometry or RNA sequencing. In this Application Note, we highlight how Molecular Pixelation (MPX) offers a broader view of activation-induced changes of the T cell surface proteome. By simultaneously mapping the abundance and the spatial organization of a wide range of surface proteins, MPX uniquely reveals the abundance, clustering and colocalization signature of PHA-induced T cell activation. This multiplexed analysis enables the detection of new cellular states and the identification of potential receptor interaction partners.

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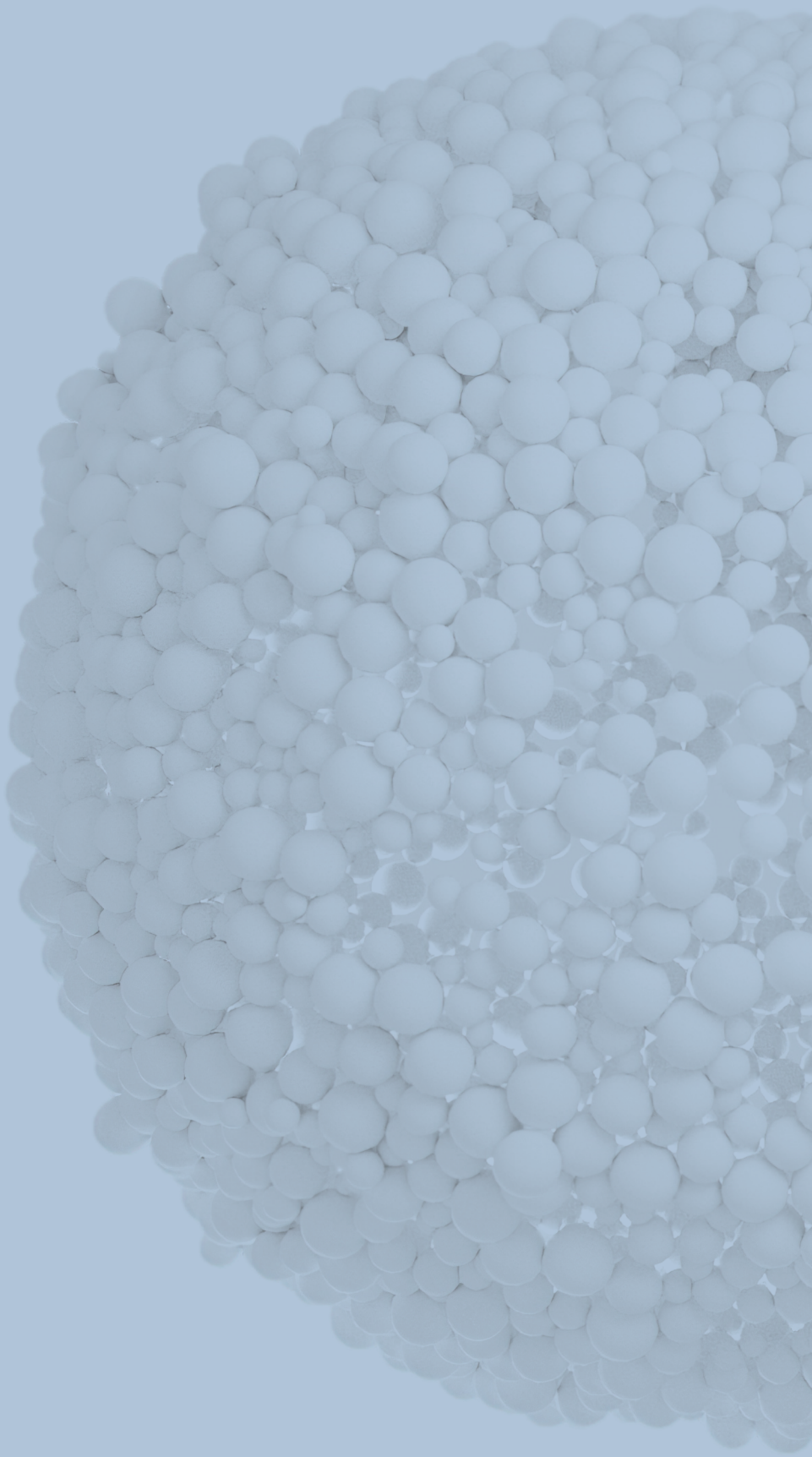

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