APPLICATION NOTE

Molecular Pixelation reveals cancer-specific protein patterns of individual cells

- Leverage Molecular Pixelation and Illumina sequencing to discover new mechanisms of immune cell behavior
- Measure the abundance and spatial organization of 80 surface proteins on 1000 single cells per sample
- Perform single-cell proteomics analysis and visualize cell surface maps using the Pixelator pipeline and analysis package

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Introduction

The spatial organization of membrane proteins is crucial for immune cell function, influencing ligand binding and facilitating precise and directed communication. For instance, the clustering of T-cell receptors enhances antigen recognition and mediates directed killing of virus-infected and transformed cells,[1–3] while the spatial rearrangement of integrins potentiates cell adhesion and migration.[4,5] As with many other cellular processes, the organization of membrane proteins can be exploited by cancer cells, as seen with increased B-cell receptor clustering being a cancer-driving mechanism in some B-cell malignancies.[6–8] While these examples demonstrate the importance of examining protein organization when studying disease processes, current proteomics techniques have notable limitations in resolving the functional complexity at the cell surface.

Most high-throughput proteomics methods (eg, flow cytometry, mass cytometry, and cellular indexing of transcriptomes and epitopes) only provide a readout of protein abundance. Meanwhile, protein organization at the single-cell level has only been accessible using low-plexity, imaging-based approaches (eg, microscopy, and imaging flow cytometry). Molecular Pixelation (MPX) is a cutting-edge single-cell proteomics technique that enables simultaneous quantification and spatial mapping of 80 membrane proteins by biochemically partitioning the cell surface into spatial zones and applying next-generation sequencing (NGS). Following Pixelation steps and NGS, data are computationally assembled into quantitative, single-cell surface maps displaying the arrangement of individual protein molecules (Figure 1).[9]

In this application note, we highlight how MPX can be easily deployed on Illumina sequencing systems, offering a wealth of new information for understanding immune cell function and diversity.

Methods

MPX is primarily designed for the analysis of immune cells, including fresh or frozen peripheral blood mononuclear cells (PBMCs), cell therapy products, and hematological cancer cells. The entire protocol is carried out in a single reaction tube for each sample and cells can be pooled and sequenced together without any need for single cell isolation. The method requires cells to be put in suspension and the input number of cells can be as little as 50,000. MPX is compatible with Illumina sequencing platforms, demonstrated here using the NextSeq™ 2000 (Illumina, Catalog no. 20038897), NovaSeq™ 6000 (Illumina, Catalog no. 20012850), and NovaSeq X Plus (Illumina, Catalog no. 20084804) Systems.

Sample preparation

For this study, we compared healthy PBMCs to immune-cell cancer lines from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Table 1). Pixelation was performed using the Single Cell Spatial Proteomics Kit, Immunology Panel 2, Human v2 (Pixelgen, Catalog no. PXGIMM002) following the manufacturer protocol (Figure 2). To preserve the organization of the membrane proteome, cells were put in solution and fixed with 1% paraformaldehyde.

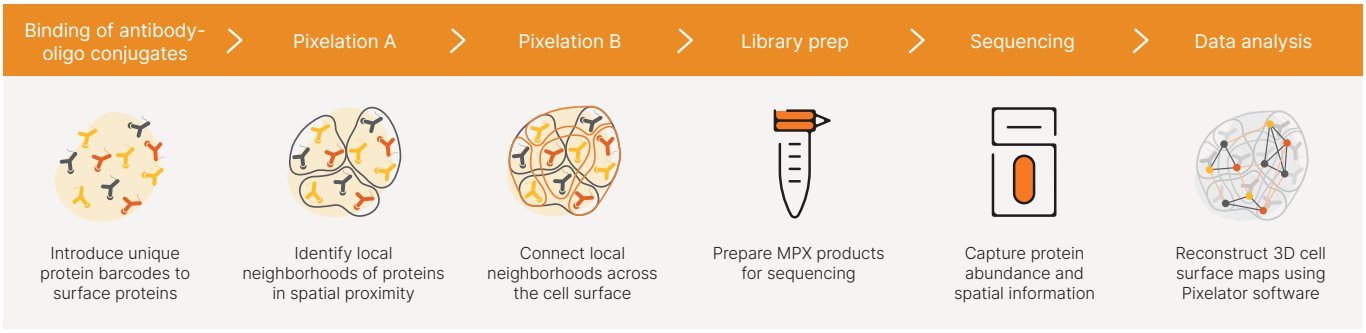

Figure 1: Overview of MPX—Cells are fixed and labeled with antibody-oligonucleotide conjugates targeting 80 surface markers and introducing unique protein-specific barcodes. DNA A pixels hybridize to the antibodies, adding barcodes to create zone A local neighborhoods, while B pixels add barcodes spatially relating adjacent zone A neighborhoods. Following sequencing, the interconnected zones are informatically joined into a quantitative and spatial representation of proteins at the individual cell surface.

Table 1: Cancer cell lines used for MPX experiments

Cancer type	Cell line	DSMZ Catalog no.
Burkitt lymphoma	Daudi	ACC 78
	Raji	ACC 319
	Ramos (RA1)	ACC 603
T-cell lymphoblastic lymphoma	SUP-T1 [VB]	ACC 140
Acute monocytic leukemia	THP-1	ACC 16

Library preparation

Following the MPX protocol, cells were suspended in solution and stained with the included panel of oligo-conjugated antibodies (AOCs) targeting 80 cell surface proteins. DNA pixels, rolling-circle DNA amplification products approximately 100 nm in size and each containing multiple copies of a unique barcode, were added to the suspended cells. Individual DNA pixels were hybridized to a limited number of AOCs located in close proximity on the cell surface and a gap-fill ligation reaction was used to incorporate the DNA pixel barcode onto the ligated antibody oligos. These steps partitioned the AOCs bound to the cell surface into thousands of local zones defined by their shared DNA pixel barcode sequence. This process was repeated using a second set of DNA pixels, creating two sets of overlapping zones, which allowed for *in silico* reconstruction of the relative positions of each zone across the cell surface. The resulting antibody and Pixelation barcodes were amplified by PCR, during which sample-specific Illumina sequencing adapters were incorporated. Before sequencing, the amplicons were purified using AMPure XP beads (Beckman Coulter, Catalog no. A63880).

Sequencing

MPX libraries require paired-end sequencing. Pixelgen Technologies recommends a minimum of 120,000 read pairs per cell, while the number of samples and cells per sample can be adjusted depending on the application and sequencing equipment available (Table 2).

Libraries were sequenced on NextSeq 2000, NovaSeq 6000, and NovaSeq X Plus Systems. The method required minimum paired-end reads of 44 cycles for read one and 78 cycles for read two. On the NextSeq 2000 System, a NextSeq 2000 P4 XLEAP-SBS™ Reagent Kit (100 cycles) (Illumina, Catalog no. 20100994) was used and libraries

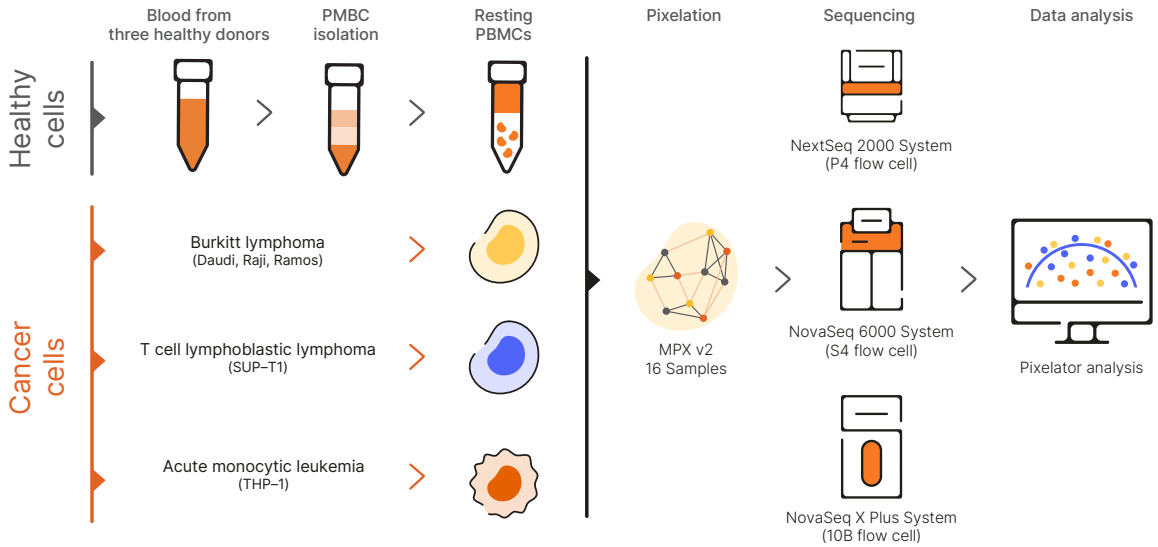

Figure 2: MPX workflow for comparing healthy PMBCs and immune cell–derived cancer lines—Cell suspensions, containing PBMCs from one of three healthy donors or one of five immune cell-derived cancer cell lines were collected and processed in duplicates with the Pixelgen Single Cell Spatial Proteomics Kit, Immunology Panel 2, Human v2. After MPX, the 16 samples were sequenced in parallel on the NextSeq 2000, NovaSeq 6000, and NovaSeq X Plus Systems. Data processing and visualization were performed using Pixelator 0.18 with the nf-core/pixelator pipeline 1.3.0.

Table 2: Sample throughput for MPX assay on selected Illumina sequencing systems

	NextSeq 2000 System	NovaSeq 6000 System	NovaSeq X Plus System
Flow cell	P4 XLEAP-SBS™ Reagent Kit (100 cycles)	Xp 4-lane Kit v1.5 (300 cycles)	10B Reagent Kit (300 cycles)
Total single reads	1.8B	8–10B	10B
Output cells/reaction	1000	1000	1000
Recommended max number of reactions per flow cell[a]	15[b]	66	83
Recommended max number of cells per flow cell[a]	15,000[b]	66,000	83,000

a. Calculated using 1000 cells per sample and ≥ 120,000 single reads per cell.
b. Sixteen samples (16,000 cells) were sequenced in this experiment, generating a total of 2.13B reads on the P4 flow cell.

were sequenced using 44 × 78 paired-end reads. For the NovaSeq 6000 System, a NovaSeq Xp 4-lane Kit v1.5 (300 cycles) (Illumina, Catalog no. 20043131) was used and libraries were sequenced using 150 × 150 paired-end reads at the National Genomics Infrastructure (NGI) in Stockholm, Sweden. For the NovaSeq X Plus System, a NovaSeq X Series 10B Reagent Kit (300 cycles) (Illumina, Catalog no. 20085594) was used and libraries were sequenced using 150 × 150 paired-end reads at the Clinical Genomics Platform in Stockholm, Sweden. Additional recommendations for read configuration and PhiX spike-in are available on the Pixelgen Technologies website.

Data analysis and visualization

Sequencing reads were processed using the open-source Pixelator pipeline, available as part of the nf-core initiative (nf-core/pixelator). Pixelator was used to reconstruct surface protein maps of individual cells and generate output files for quality control and analysis. Along with the PXL files containing the processed data for downstream analysis, statistics, and figure generation, Pixelator also generated a web report summarizing key quality metrics for the experiment.

PXL files were easily processed by common single-cell omics analysis tools, including Scanpy and Seurat, and contained four primary elements: protein abundance counts for each protein and cell, MPX polarity scores for each protein and cell, MPX colocalization scores for the pairwise comparison of all panel proteins in each cell, and an edge list containing the data to build graphical representations of individual cells as well as the information to calculate spatial statistics. MPX analysis support and tutorials are readily available on the Pixelgen Technologies website.

Results

MPX is compatible with current mid- to large throughput Illumina sequencing systems. To demonstrate this, a PBMC sample was prepared using MPX, as described, and sequenced on three different Illumina platforms (ie, NextSeq 2000, NovaSeq 6000, and NovaSeq X Plus Systems). The PXL data files were analyzed and their quality was assessed using the R package, pixelatorR (v0.10.2). Components with extreme sizes and suspected aggregates were removed before the downstream analysis. Raw counts were normalized using the Centered Log-Ratio (CLR) transformation[10] and summarized as the mean CLR value per marker. A comparison of the MPX abundance outputs confirmed that similar high-quality results can be obtained across Illumina sequencing platforms (Figure 3).

The combination of protein abundance and spatial analysis at the single-cell level offers the potential for deep phenotyping and detailed characterization of immune cell behavior. We demonstrated this capacity by comparing hematological cancer cell lines to healthy immune cells in terms of protein abundance (Figure 4), protein polarization (Figure 5), and protein colocalization (Figure 6).

For example, in Burkitt lymphoma cell lines we show that the content of multiprotein domains changes, with proteins such as the therapeutic target CD40 being segregated from tetraspanin-rich domains, while CD38 is enriched. Interestingly, ligation of CD40 with antibody drugs has been found to induce opposite responses in healthy B-cells and Burkitt lymphoma cells[11] and our MPX findings suggest that CD40 organization could be a driver for such differential responses (Figure 6).

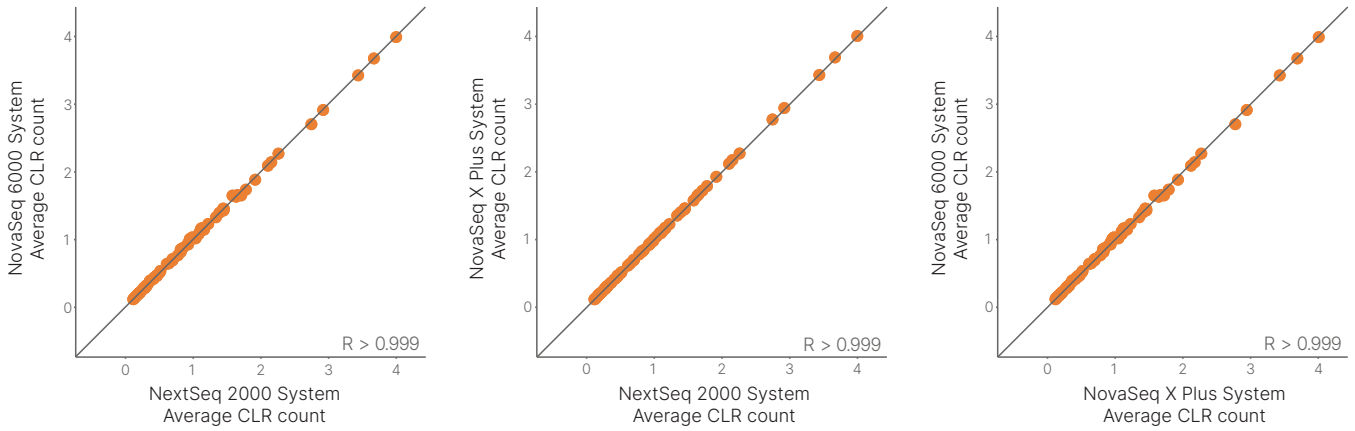

Figure 3: Correlation between MPX outputs across Illumina sequencing systems—MPX data from a single PBMC sample sequenced on three Illumina platforms (NextSeq 2000, NovaSeq 6000, and NovaSeq X Plus Systems) were analyzed using the R package, pixelatorR (v0.10.2). Raw counts were normalized using the CLR transformation[10] and summarized as the mean CLR value per marker. The linear correlation between all three systems, across all markers, shows significant correlation (P value ≤ 0.0001).

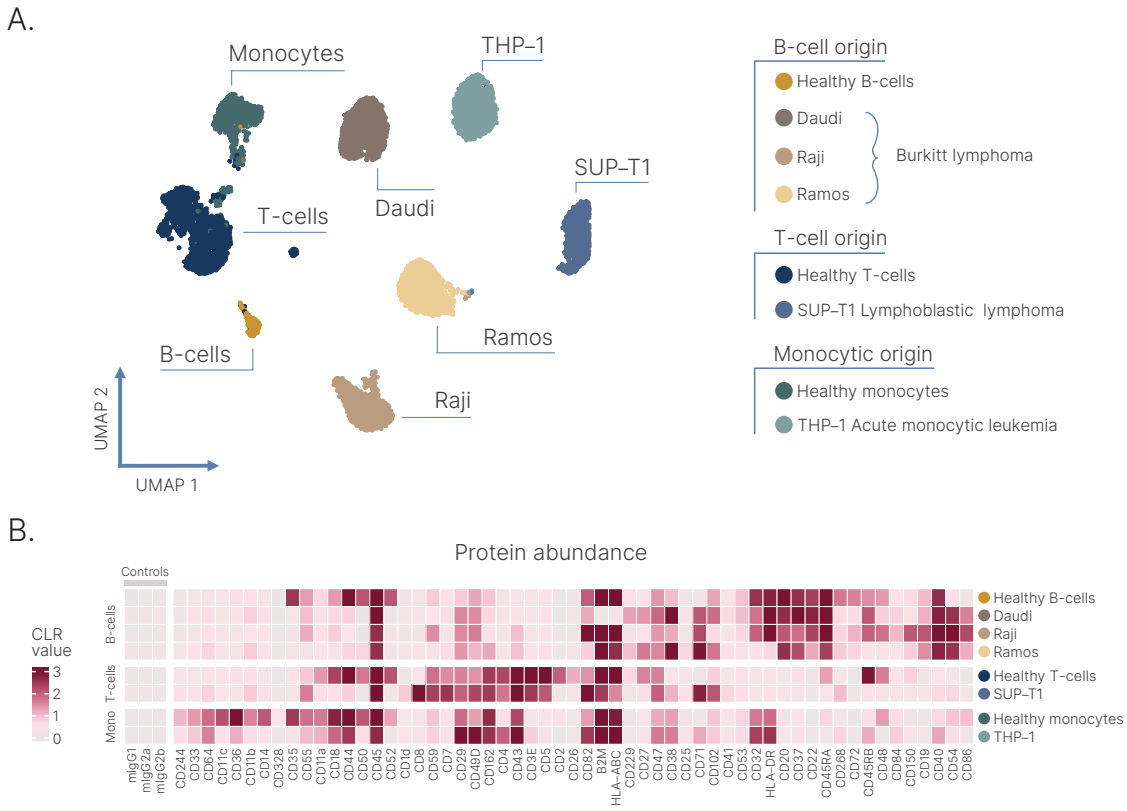

Figure 4: MPX highlights differential surface protein abundance in PBMCs and immune cell–derived cancer lines—(A) Multidimensional clustering of PBMC T-cells, B-cells, monocytes, and the five cancer cell lines, based on the expression of all immune panel markers. (B) Heat map of median CLR-adjusted abundance levels of all expressed panel markers in the cell clusters identified. CLR values are capped at three.

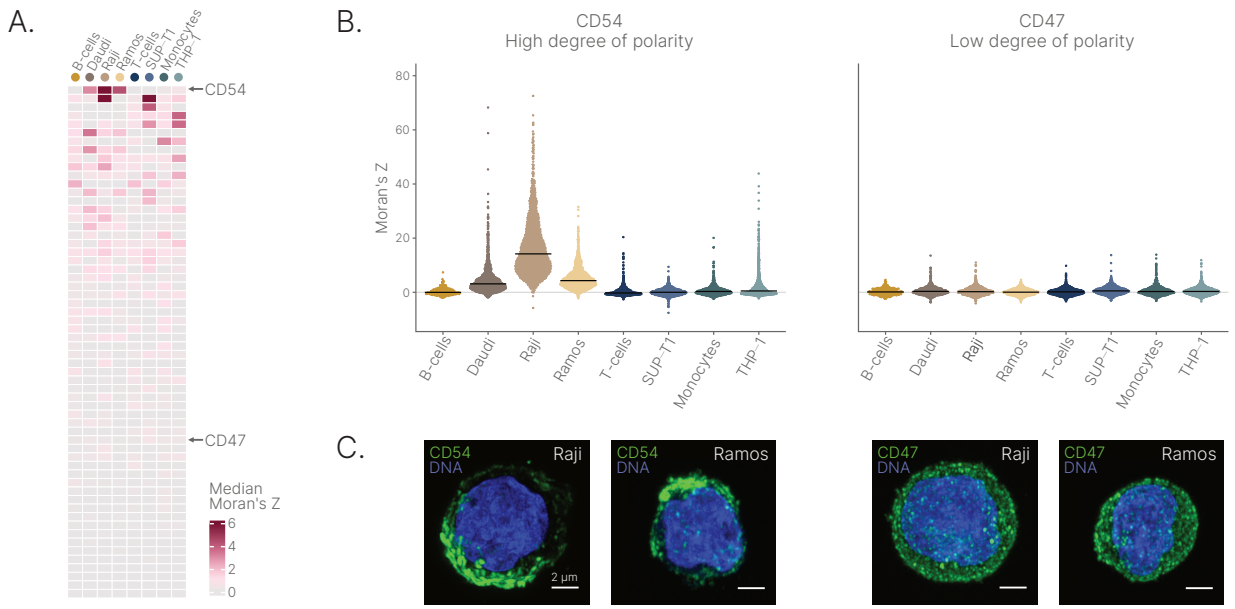

Figure 5: MPX reveals distinct protein polarity between PBMCs and immune cell–derived cancer cell lines—(A) Heat map of the median Moran's Z scores indicating spatial clustering for all expressed panel markers in each cell type. Moran's Z values are capped at six. (B) Single-cell distributions of MPX polarity scores for CD54 and CD47 with the line representing the median polarity score. (C) Airyscan immunofluorescence micrographs highlighting the polarized profile of CD54 in Raji and Ramos cells (left), CD47 shows even distribution in the same cell lines (right).

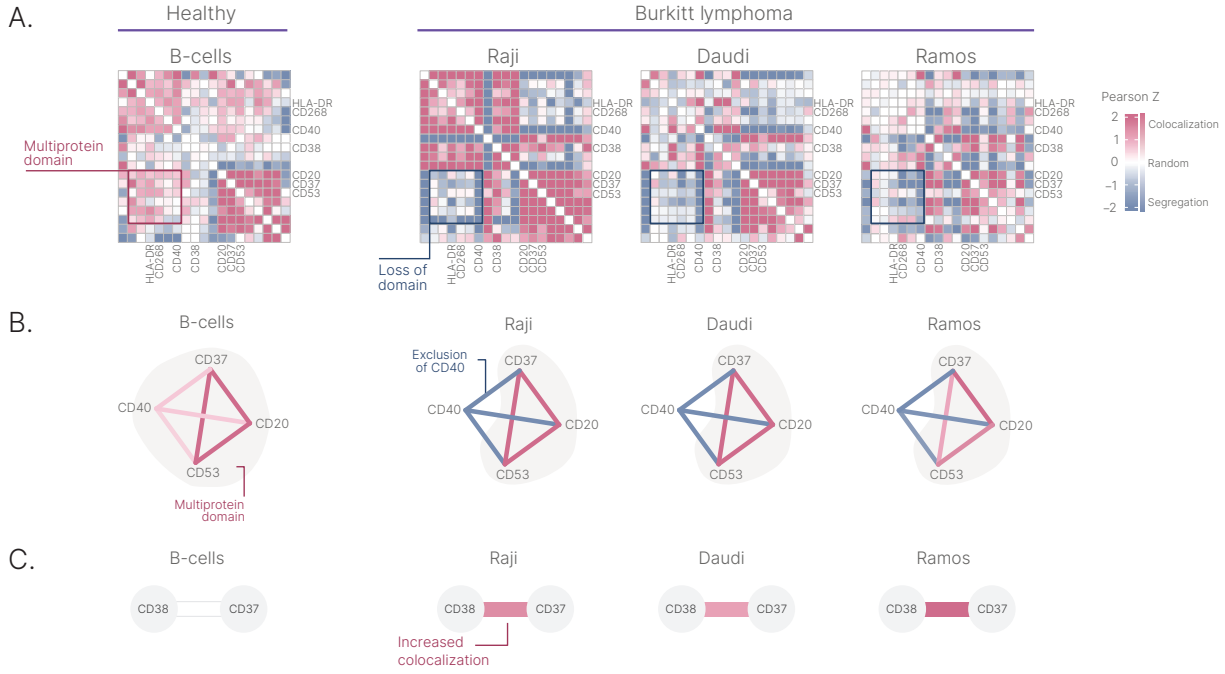

Figure 6: Reorganization of the membrane proteome in Burkitt lymphoma cell lines—(A) Median pairwise MPX colocalization scores for 19 markers commonly expressed on healthy B-cells and Daudi, Raji, and Ramos Burkitt lymphoma cell lines. Each cell type displays a unique large-scale membrane organization, while all cancer cell lines show a specific loss of the multiprotein domain containing proteins such as CD40, BAFF-R (CD268), and HLA-DR. (B) Plotted network representations of selected pairwise protein colocalization scores for the different cell types. CD40 was found to segregate from the CD20-associated, tetraspanin-rich domain in all Burkitt lymphoma cell lines but not in healthy B-cells. (C) Focusing on single protein-protein interactions reveals increased colocalization between CD38 and CD37 in Burkitt lymphoma cell lines.

Focusing on single protein-protein pairs, we also identified an increased colocalization between CD38 and CD37 in Burkitt lymphoma cell lines (Figure 6). CD38 is a therapeutic target in B-cell malignancies, yet the protein is expressed by many different immune cells, causing off-target effects during treatment.[12] Designing bispecific drugs targeting colocalizing protein pairs, such as CD38 and CD37, could provide higher tumor specificity and improved treatment efficiency. These findings can aid in identifying novel drug targets and diagnostic biomarkers, ultimately improving our understanding of signaling processes in cancer.

Summary

This application note demonstrates the advantages of Molecular Pixelation with Illumina sequencing systems, for simultaneously assessing the abundance and spatial organization of 80 surface proteins on 1000 individual immune cells per sample. With a multidimensional view of the surface protein landscape on single cells, researchers can now unravel complex mechanisms of immune cell function in health and disease that were previously inaccessible with existing methods.

Illumina and Pixelgen Technologies support teams are available to assist throughout the Molecular Pixelation workflow. Contact Pixelgen Technologies Support (support@pixelgen.com) for assay and analysis questions, and Illumina Support (techsupport@illumina.com) for sequencing questions.

Learn more

Illumina sequencing platforms

Molecular Pixelation technology

Molecular Pixelation full protocol

Pixelator software for processing and analysis of Molecular Pixelation data

References

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