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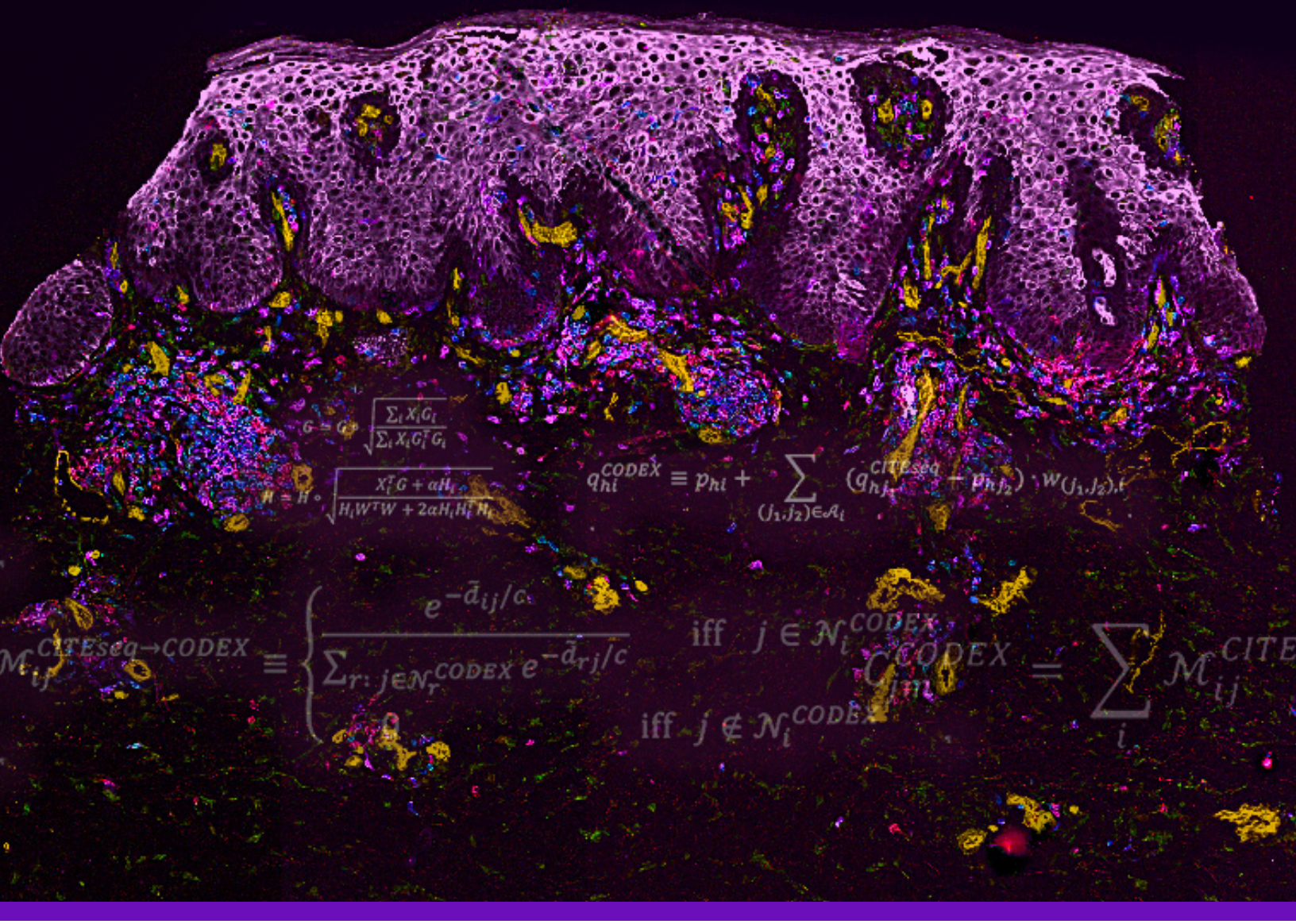
Analysis Frameworks for Putting Single-Cell Analytics in Context: A Spatial Multiomics Approach



From CyTOF to CITE-seq, single-cell omics continue to transform biology, generating new insights into cellular heterogeneity.

While these technologies have been instrumental in automated single-cell analysis, a gap remains for many researchers between the data and the tissue from which the cells arise. Cell behavior, state, and fate are tremendously affected by spatial location, surrounding components, and more. One can even go further and interpret that cell fate and behavior are the sum of both a cell's biological blueprint (DNA, RNA, and proteins) and the surrounding microenvironment. Thus, to truly understand cells, one needs to study cells within the context of their blueprint and neighborhoods.

That is the goal of the new generation of spatial technologies. One such approach, spatially resolved transcriptomics, was named Method of the Year 2020 by *Nature*.[1] In making the announcement, the editorial board observed that "this maintenance of spatial context is crucial for understanding key aspects of cell biology, developmental biology, neurobiology, tumor biology, and more, as specialized cell types and their specific organization are crucially tied to biological activity and remain poorly explored on the scale of whole tissues and organisms."[1]

Current spatial transcriptomic methods are powerful tools for unbiased discovery, enabling analysis of thousands of genes simultaneously within the spatial context of the intact tissue sample. However, they achieve this ultra-high level of multiplexing at the expense of single-cell resolution, relying on region-of-interest or spot-based capture methods. Future imaging-based approaches may be able to bridge this gap.

Proteins are the functional molecules of all cells, and ultimately the effectors of almost all biological processes. Accordingly, the vast majority of drug targets are proteins, which makes protein biomarkers especially useful for developing therapies and diagnostic tools. Transcriptional information, while critical, provides a projected cellular phenotype that should be confirmed by protein-based phenotyping. Imaging-based spatial proteomics allows researchers to quantify spatial expression of 40+ protein markers across a whole tissue section at single-cell resolution, enabling spatial phenotyping of cells within tissue context.

To unravel cellular complexity, scientists aren't limiting themselves to one mode of analysis. This is where spatial multiomics comes in.

Spatial multiomics is the bridge between single-cell omics, spatial transcriptomics, and spatial phenotyping. Integrating findings from these technologies provides a systems-biology view of the tissue microenvironment, allowing researchers to translate their transcriptomic findings and hypotheses to whole tissue and observe their role in the interplay between cells in their native states. It is the step that links RNA to protein to cellular organization and behavior.

1. Method of the Year 2020: spatially resolved transcriptomics. *Nat Methods*. 2021;18(1). https://doi.org/10.1038/s41592-020-01042-x

THE POWER OF SPATIAL PHENOTYPING: PUTTING CELLS AND NEIGHBORHOODS INTO CONTEXT

Spatial phenotyping enables a researcher to view, characterize, and quantify cells by lineage and variant with single-cell resolution in the context of an intact tissue. This is possible because of a technological breakthrough called multiplexed imaging, which allows a single tissue sample to be labeled with dozens of biomarkers—40 or more in many analyses—at single-cell resolution.

Different combinations of markers reveal distinct cell clusters with unique protein expression patterns. These cell clusters, or neighborhoods, provide insights into healthy tissue function, disease progression, and response to therapy. Schürch et al.[2] describe cellular neighborhoods as the different cellular structures and patterns around the tumor that can help predict the course of disease in some patients.

They demonstrated this in a study of two different types of colorectal cancer (CRC), in which different neighborhood structures clearly distinguish the more lethal form of the disease—characterized by diffuse inflammatory infiltration (DII)—from the Crohn's-like reaction (CLR), which is associated with much longer overall survival.

FIGURE 1. shows their results with the multiplexed images on the top and the cell maps based on biomarker expression patterns on the bottom. The side-by-side images reveal obvious *spatial phenotypes* for the two forms of the disease. The relatively ordered clustering of CD20-expressing B cells and CD4-expressing T cells characterizes the more benign CRC phenotype on the left, while the diffuse mix of CD8-expressing T cells, macrophages, and non-immune cells characterizes the more lethal DII seen in the tissue on the right.

Spatial phenotyping uses multiplex antibody panels to characterize cells by function and state in the context of an intact tissue sample. The resulting annotated cell-by-cell maps of the cellular ecosystem reveal patterns in localization, density, organization, and spatial proximity or "cellular neighborhoods"—the spatial phenotypes that unravel insights into development, differentiation, and response. In immuno-oncology studies, these spatial phenotypes have been shown to be highly predictive of disease outcome and response to immunotherapy.

2. Schürch CM, et al. Coordinated cellular neighborhoods orchestrate antitumoral immunity at the colorectal cancer invasive front. *Cell*. 2020;182(5):1341-1359.e19.

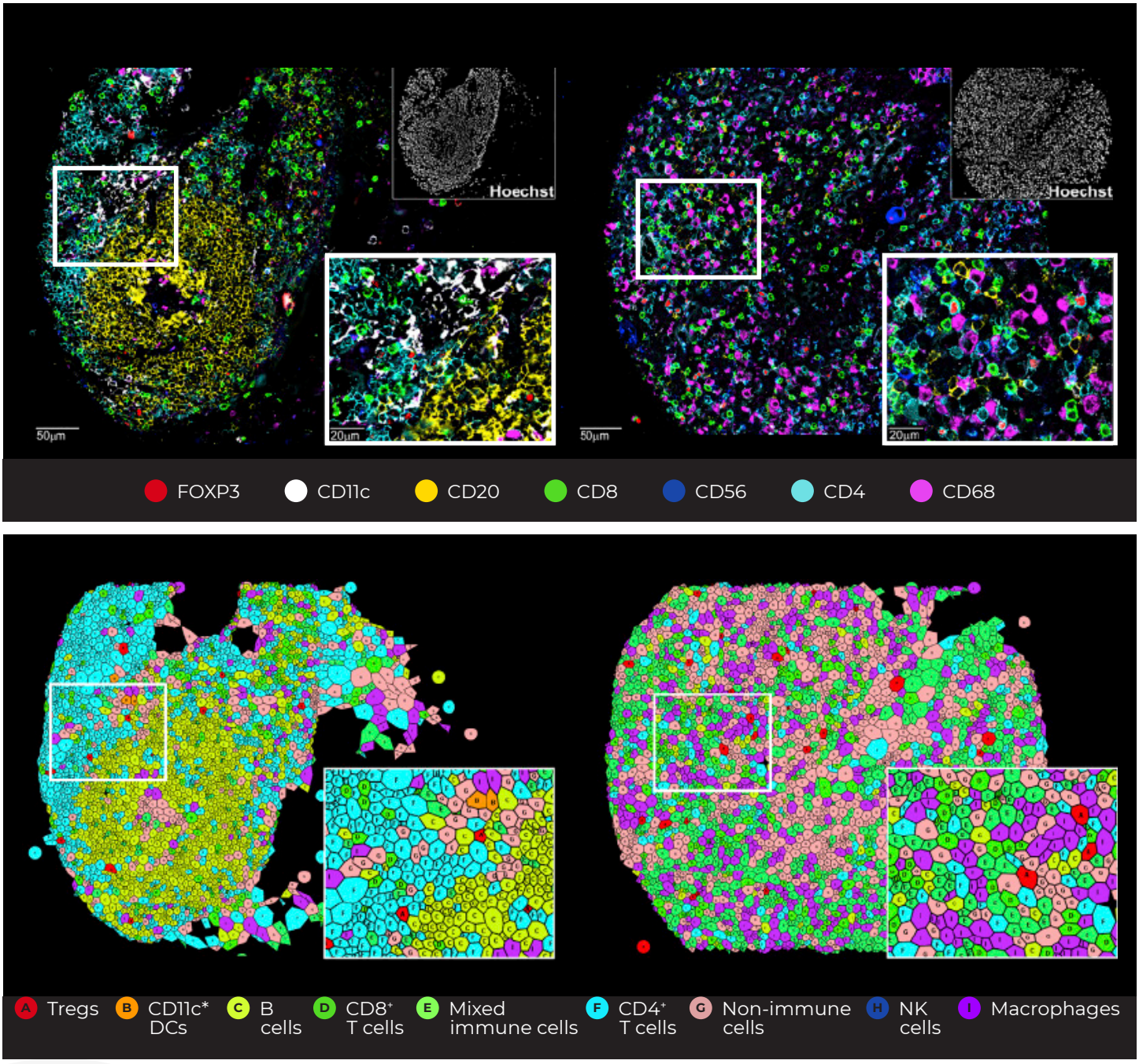


FIGURE 1:
In this study of different types of CRC, the relatively ordered clustering of T- and B-cells on the left distinguishes the CLR phenotype from the diffuse pattern of T-cells, macrophages, and non-immune cells that characterizes the more lethal DII form of the disease.[3]

3. Schürch CM, et al., 2020.

SPATIAL MULTIOMICS: REDEFINING OUR UNDERSTANDING OF BIOLOGY

Spatial multiomics integrates information from various modalities to connect expression levels with cell phenotype. This allows one to go beyond transcriptomic and protein data to truly understand cell-to-cell signaling networks, the reasons behind cell activation state, and more.

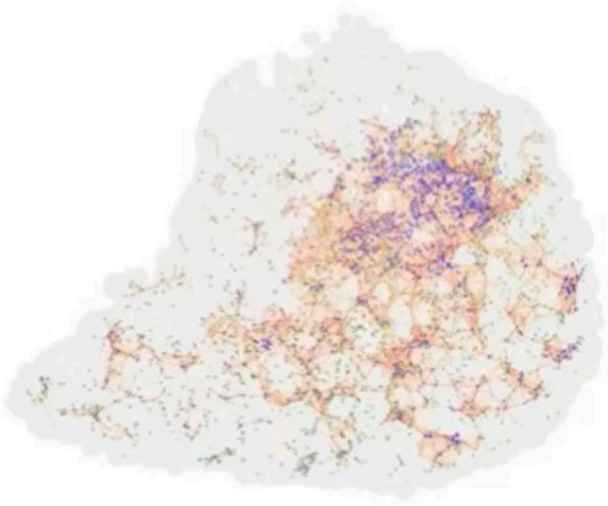
Wang et al.[4] employed an integrated spatial multiomics approach to study tissue regeneration and aging using two complementary approaches: spatial phenotyping via CODEX and CITE-seq. Combining the data obtained from CODEX and CITE-seq, using a common protein space allowed the authors to project RNA expression data back to individual cells within the tissue sample.

Using this approach, they were able to identify cell-to-cell signaling networks and determine whether the communication between cells was spatially restricted. This showed that not all cells with complementary ligands and receptors organize in a context that makes communication possible. "Such cells may never see each other due to spatial constraints," they note.[4]

In one example, they compared signaling patterns for the proinflammatory cytokine IL-1β and the corresponding ILR2 receptor to those for osteogenic BMP2 and BMPR1A receptors in damaged muscle tissue. The results show that the inflammatory signaling is spatially restricted, localized to the area in and around the injury site, while the BMP2 signaling is more diffuse, occurring throughout the periphery of the muscle tissue. **FIGURE 2.**

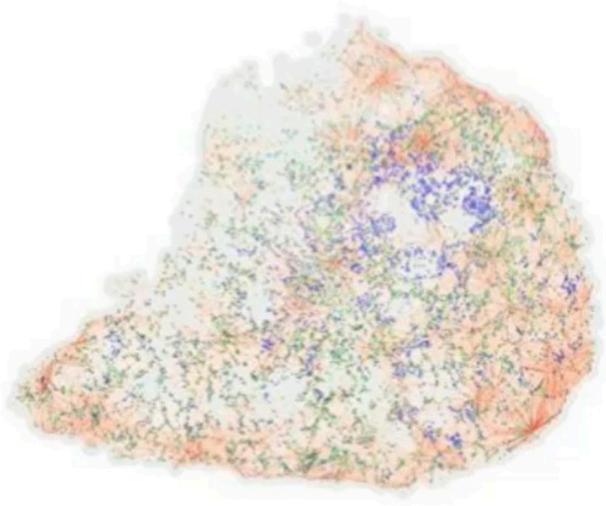
This is just one example of how a combination of CITE-seq and CODEX multiplex imaging capabilities is revealing new insights into the spatial component of cell-to-cell signaling.

INJURY-SITE SPECIFIC SIGNALING



IL1B-IL1r2

MUSCLE PERIPHERY SIGNALING



BMP2-BMPR1A

FIGURE 2:
A spatial analysis of cell-signaling pioneered at Stanford University shows that signaling between proinflammatory IL-1β cytokines and corresponding ILR2 receptors is spatially constrained to the area of injury (blue), whereas signaling from a protein like BMP2 (green) is more diffuse in peripheral tissue.

4. Wang W, et al. Elucidating multiomics changes in skeletal muscle aging. Stanford University. Webinar. https://www.akoyabio.com/webinar/spatial-multiomics-webinar-series/

GLUER: AN ANALYTICAL FRAMEWORK TO INTEGRATE scRNA-SEQ AND SPATIAL PHENOTYPING DATA

Peng et al.[5] have proposed a new methodology for aligning single-cell transcriptomic data to CODEX-based whole tissue cell maps, which they call GLUER (inteGrative anaLysis of mUlti-omics at single-cEll Resolution). GLUER integrates the data obtained via the two analytical approaches using joint non-negative matrix factorization, a mutual nearest neighbor algorithm, and deep learning neural network.

By way of demonstration, they profiled 7,097 murine spleen cells using scRNA-seq and 9,186 murine spleen cells using a 30-antibody multiplexed imaging panel. The initial resulting UMAP cell cluster plots created using Seurat[6] and LIGER[7] algorithms showed nominal concordance and little delineation between cell types. **FIGURE 3.**

When those same data were assessed using the GLUER methodology, the cell-type alignment and clustering became much tighter, and the plots showed improved ability to distinguish between different cell types. Since all cells captured using the CODEX system were indexed to x-y coordinates, these findings could be tracked to specific areas of the tissue sample, allowing new understanding of the link between transcriptomic data, cellular structures, and functions.

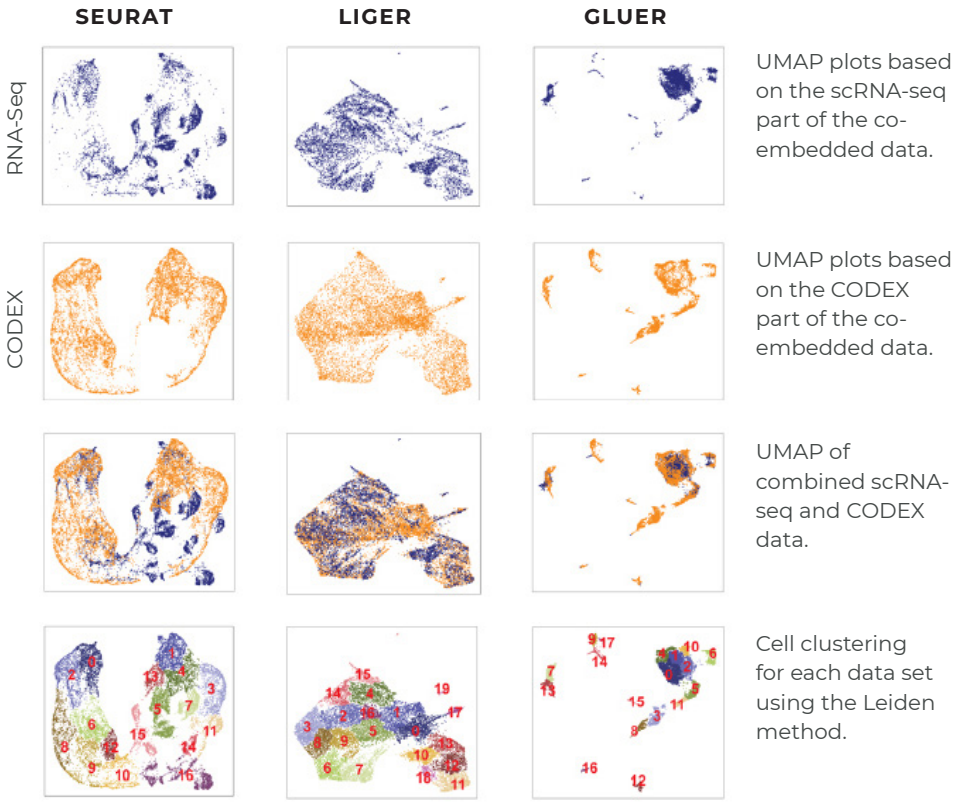


FIGURE 3:
The GLUER methodology produced much tighter alignment between the two data sets (scRNA-seq, top; multiplexed imaging, row two) and more clearly distinguished different cell types such as the NK cells and red pulp macrophages.

5. Peng T, et al. GLUER: integrative analysis of single-cell omics and imaging data by deep neural network. *bioRxiv*. 2021;1(25):427845.
6. Stuart T, et al. Comprehensive integration of single-cell data. *Cell*. 2019; 177(7):1888-1902.e21.
7. Welch JD, et al. Single-cell multi-omic integration compares and contrasts features of brain cell identity. *Cell*. 2019;177(7):1873-1887.e17.

STvEA: ANALYSIS FRAMEWORK TO INTEGRATE CITE-SEQ AND SPATIAL PHENOTYPING DATA

Govek et al. developed STvEA (Spatially resolved transcriptomics via epitope anchoring) to enrich the output of CODEX whole-tissue, single-cell analyses with CITE-seq-generated RNA sequencing data. This framework allows the authors not only to annotate cells by type and states, but also to identify spatial gene expression and study interactions between cell population.

STvEA computationally consolidates the protein expression spaces of the corresponding multiplexed images via CODEX and CITE-seq data sets based on a shared antibody panel. Then it determines the optimal clustering of the CITE-seq mRNA expression data, so that different mRNA sequences can be mapped to the CODEX images with single-cell resolution.

In one analysis of murine spleen cells, Govek et al. showed how this methodology was able to refine the cell clustering pattern to almost double the distinct, identifiable cell populations based on subtle gradients in gene expression within cell clusters and highlighted these variations in epitope levels as a way to distinguish cells with similar protein expression levels. **FIGURES 4, 5.**

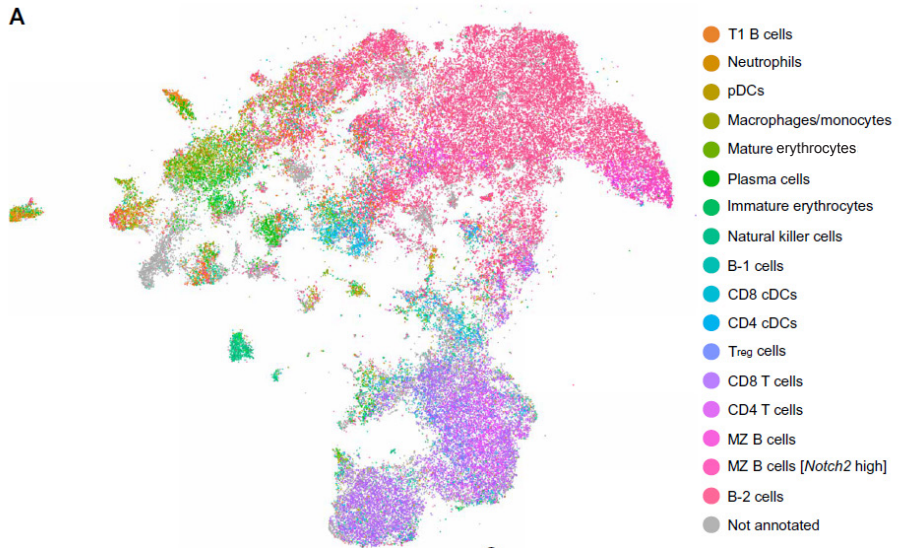


FIGURE 4:
This UMAP representation shows the 17 different color-coded splenic cell types profiled using multiplexed imaging and annotated based on CITE-seq-derived transcriptome data.

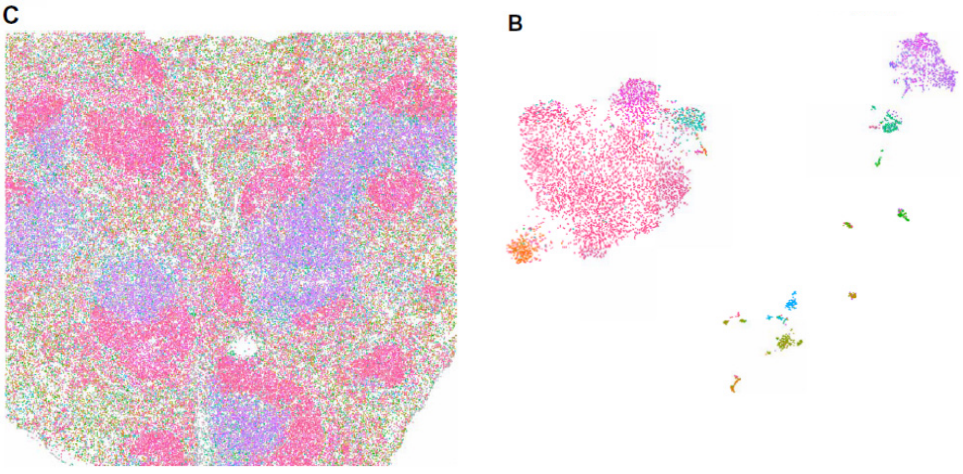


FIGURE 5:
The image on the left shows the same labels applied to the CITE-seq gene expression space as annotated by STvEA, while the image on the right shows the different cell types mapped back to the actual tissue based on the CODEX multiplexed image.[8]

8. Govek KW, et al. Single-cell transcriptomic analysis of mIHC images via antigen mapping. *Sci Adv*. 2021;7(10): eabc5464.

SPATIAL PHENOTYPING AND YOUR RESEARCH

GLUER and STvEA are a few examples of how researchers are anchoring gene expression data to multiplexed imaging to study cell behavior and function in intact tissue, with context.

This process is revealing what most researchers already know, namely that what is sequenced is not always what is expressed; RNA only matters to the extent it impacts protein synthesis; and protein expression defines the phenotype. Bringing spatial phenotyping and single-cell omics together provides the thread that links biological research from genome to transcriptome to proteome, and ultimately to living tissue.

Spatial multiomics makes that link possible, allowing researchers to translate their transcriptomic findings and hypotheses to whole tissue and observe their role in the interplay between cells in their native states.

That said, not all spatial biology tools outlined in the *Nature* article are equivalent. Jay Shendure of the University of Washington, who is quoted in that article, points out that many of the methodologies it describes "are still somewhat bespoke and really only operational in one or a few labs."

By contrast, the CODEX spatial phenotyping system used in all protocols outlined above works with existing microscopy equipment and employs standard biomarkers that everyone in your lab knows and can use with confidence. The process for labeling and imaging, even with dozens of biomarkers, is largely automated. It also applies labels directly to cells in microdissected tissue and does not involve additional oligo tags or indirect labeling. You are always working from the content of the original slide.