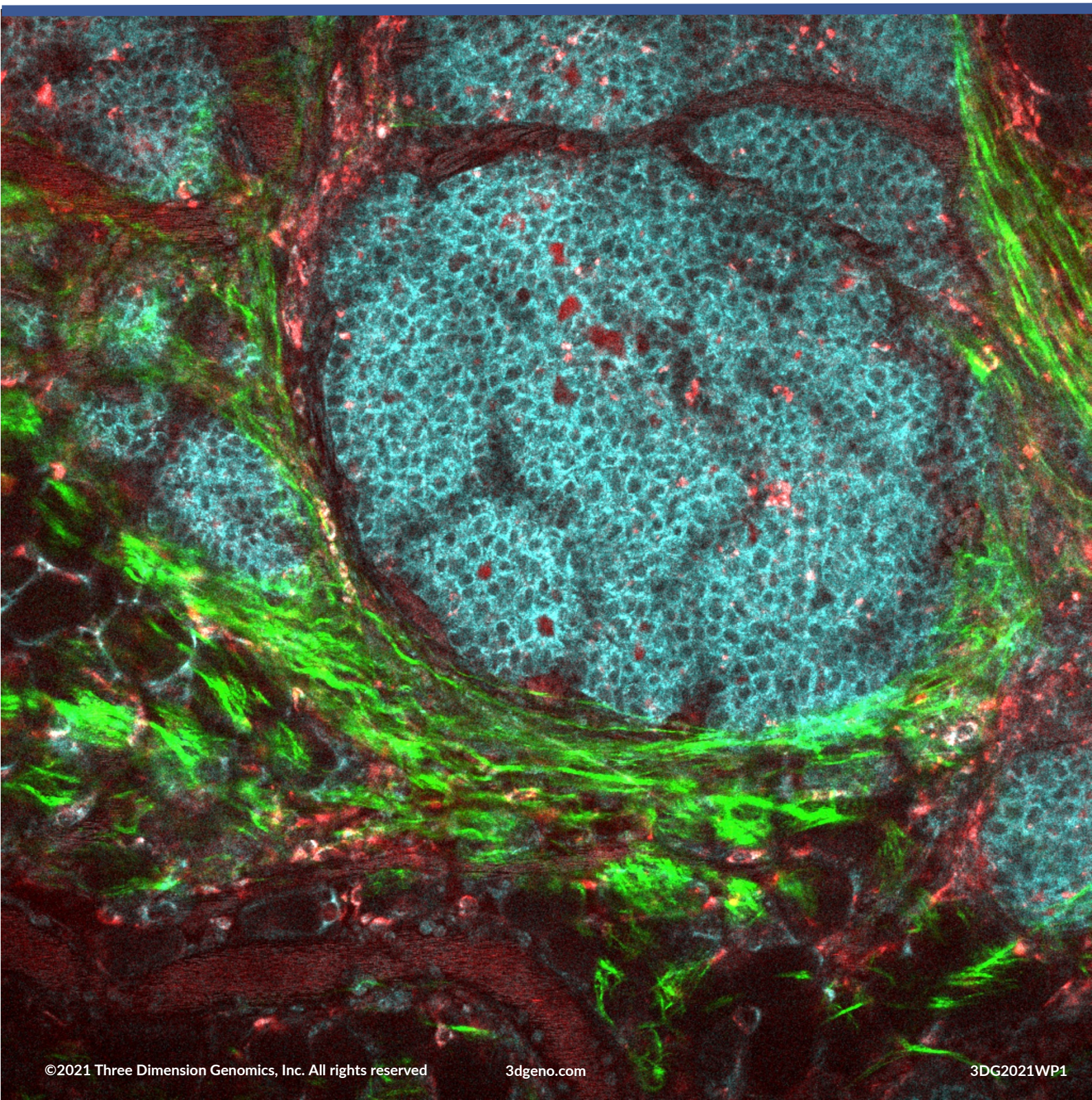
The tumor microenvironment:

Applications of spatial transcriptomics and single-cell RNAseq



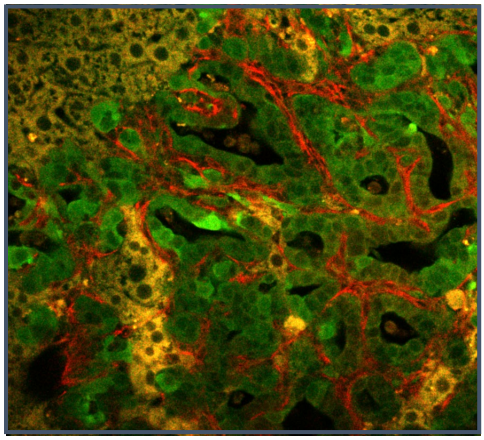
Understanding how a tumor's microenvironment impacts pathology will pave the way for novel therapies, patient stratification, and clinical benefit. Initially considered a disease of rare, malignant cell populations, it is now appreciated that the tissue surrounding malignant cells plays a crucial role in determining cancer development and progression. It will also require new technical approaches. This white paper provides an overview of the importance of the tumor microenvironment and applications of single-cell sequencing and spatial transcriptomics to the field.

Introduction

Cancer remains the leading cause of death worldwide and a significant market for therapeutics **(1)**. In 2019 the global oncology drug market was valued at $128B and is expected to increase to $222B by 2027 **(2)**. The last two decades have seen an explosion of therapy options targeting cancers, including immunotherapies, gene therapies, and novel targeted therapies. Many of these treatments do not aim to kill malignant cells directly but rather target non-malignant cells that are also present in tumors. These include drugs that encourage immune destruction of cancer cells and target non-malignant cells that support tumor survival, growth, and spread. In one way or another, these therapies all aim to alter the **tumor microenvironment (TME)** to make it less hospitable for cancer cells.

Defining the TME

A tumor mass does not consist solely of cancer cells – they also contain **non-malignant cells** that are either resident in the healthy tissue or have infiltrated into the tumor. Additionally, cancer cells will be exposed to **secreted soluble factors** and **extracellular matrix proteins**. Together, this heterogeneous mix of cells, signaling and scaffolding proteins make up the **tumor microenvironment (TME)**. The exact composition of the TME depends on the tumor type, and there can be significant heterogeneity in TME between patients with the same cancers. However, there are some common cell types that often play crucial roles in the TME:



Pancreatic cancer cells in the liver in a mouse model of metastasis. Cancer cells in green, cancer-associated fibroblasts in red, liver hepatocytes can be seen as yellow cells with large, central nuclei.

- **Endothelial cells** comprise the vasculature of tumors, and play a key role in oxygen and nutrient delivery to sustain tumor growth
- **Cancer-associated fibroblasts (CAFs)** refer to mesenchymal cells that have been transformed by the tumor. CAFs are major source of extracellular matrix and play an important role in drug resistance and metastasis of tumors
- **Immune cells** have various critical roles in tumor growth and treatment. Tumors contain many different immune cell populations (e.g. macrophages neutrophils, T-cells, B-cells) – all with

The TME plays a role in disease progression throughout cancer development. Though cancers arise via mutations in single cells that allow them to escape normal controls on cell growth, **malignant cells require interactions with surrounding cells to survive**. Early on in this process, malignant cells alter the environment around them to generate a TME that supports cancer progression. The tumor mass can rapidly become hypoxic in solid tumors and starved of nutrients if new blood vessels are not formed to deliver oxygen and metabolites. Abnormal cell surface proteins on malignant cells can induce a "non-self" immune response that will kill the cancer if left unchecked.

The TME limits the ability of T- and B-cells to respond through a combination of physical barriers and immune suppression **(3)**. Physical constraints and signals from neighboring cells also promote invasive phenotypes in tumor cells and drive metastasis to distant organs **(4)**. Changes in the TME also play a vital role in the development of treatment resistance **(5)**.

A new frontier for drug development

The concept of targeting non-malignant cells to treat cancer is not new. Immunotherapies that stimulate anti-cancer immune response represent a significant proportion of oncology treatments and are a rapidly growing market. As the understanding of the broader role of the TME grows, there is increasing interest in developing therapies targeting the microenvironment. There are several potential opportunities for this:

- **Combination therapy** – treatments that target both malignant cells and TME are making their way to the clinic. Examples include targeting **tumor vasculature** (e.g., anti-VEGF therapies) and inhibiting **macrophage recruitment** (e.g., CSF1R)

- **Patient stratification –** determining treatment based on **molecular classification** of an individual's cancer can provide more targeted therapy options.

- **Preventing metastatic disease** – while most cancer patients die of metastatic disease, **therapies targeting metastasis remain limited**. Pathways impacting the ability of malignant cells to escape from primary tumors, seed in distant organs, or awaken from dormancy, are likely to become critical therapeutic targets in the coming decades

- **Drug response** – immunotherapies have shown remarkable promise but are **ineffective in many patients (6)**. The TME is key to understanding what prevents immune cell infiltration or anti-cancer activity in these patients.

A double-edged sword

Therapies targeting the TME often have unexpected consequences, highlighting the complex interactions between cancer cells and different components of the microenvironment.

An illustration comes from preclinical studies targeting cancer-associated fibroblasts (CAFs) in pancreatic cancer. Pancreatic cancer is characterized by a dense, extracellular matrix-rich stroma that drives malignant cells to be more invasive and impairs immune cell infiltration **(7)**. Studies using genetic ablation of CAFs reduced life-expectancy of mice with pancreatic cancer due to increased invasion of cancer cells and myeloid-derived suppressor cells **(8)**.

Subsequent studies have used single cell sequencing to uncover heterogeneity within the CAF population and could provide routes to target tumor-promoting CAFs whilst retaining the tumor-restricting cells **(9)**.

Techniques to dissect the TME

Understanding the changes that occur within the TME requires **high cellular and spatial resolution**. Traditionally bulk RNA sequencing has been used to characterize the transcriptional profile of tumors. Still, these data are a biased average of transcriptional signatures from cells in the tissue and contain no spatial information about *where* genes are expressed. Immunohistochemistry and in situ hybridization are standard techniques to capture spatial information, but both are limited to a few proteins or mRNAs. Advanced next-generation sequencing methods allow researchers to combine **comprehensive gene expression profiling** with **spatial information** (Figure 1).

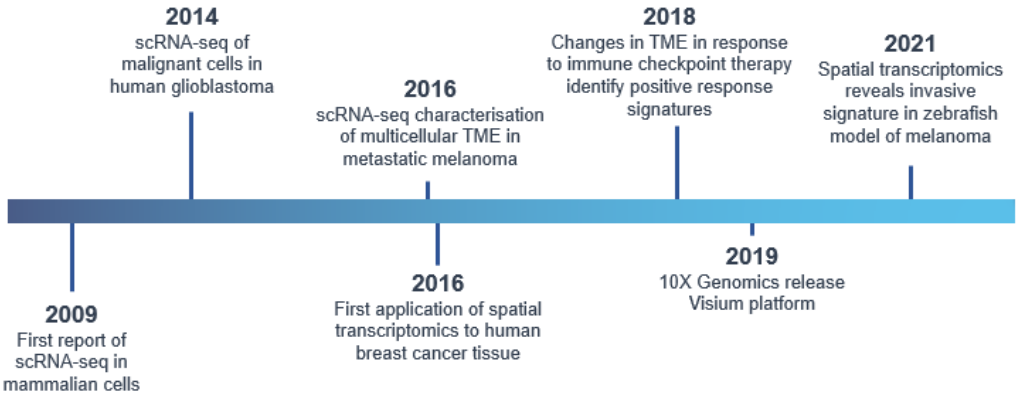


Figure 1: Timeline of milestones in the development and application of scRNA-seq and spatial transcriptomics to the TME. References: **(10-12, 16)**

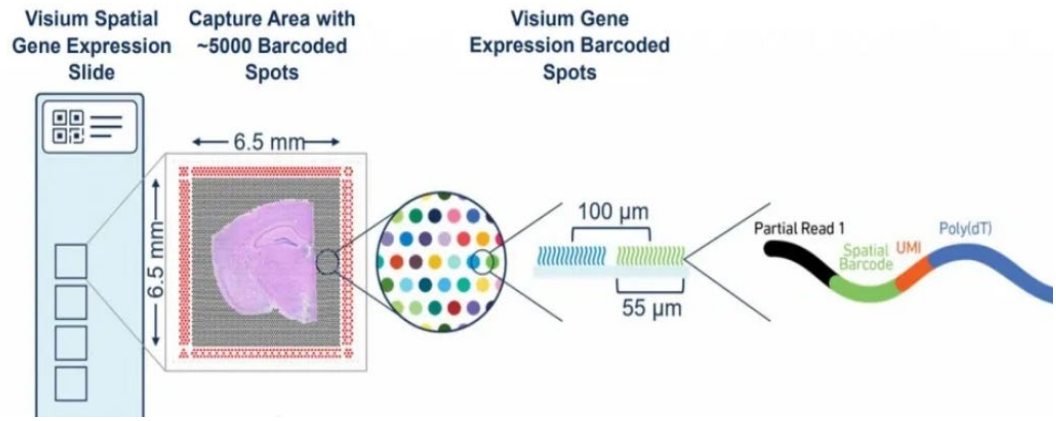
Single-cell RNA sequencing (scRNA-seq) enables gene expression profiling of individual cells. Though approaches differ (plate-based, droplet-based, microwell-based) – all require dissociating tissue into a single cell suspension, from which individual cells are isolated and sequenced. ScRNA-seq has provided unprecedented insight into tumors' cellularity and heterogeneity within cell populations in the TME. Since its first application in tumors, the technique has been applied to numerous cancer types, including prostate cancer, breast cancer, glioma, and pancreatic adenocarcinoma **(10)**. First applied to the TME of melanoma in 2016, scRNA-seq has since become a critical tool for studying TME cellularity **(11)**.

ScRNA-seq involves tissue dissociation and, therefore, loses all spatial information about where cells are located in the tumor. To address this, **spatial transcriptomics** uses barcoding of mRNA from small areas of tissue prior to sequencing to map transcripts to specific locations within the tissue. Since its first application in breast cancer in 2016, spatial transcriptomics has been widely applied to a range of cancers **(12)**. This approach has been particularly instructive for studying the TME of solid tumors, for which spatial organization and interactions between cells are essential.

Visium technology

The **Visium platform** from 10X Genomics utilizes a spatial barcoding approach to map transcriptomic data to regions of tissue. A Visium Spatial Gene Expression slide has 4 captures areas that each span **6.5mm x 6.5 mm** and hold **~5,000 barcoded spots**. Since an individual spot is **55µm**, you can expect to capture between 1-10 cells per spot depending on the tissue type.

Tissue is mounted onto the capture area and imaged prior to mRNA extraction. Barcoded sequencing data can then be linked back to the specific region of tissue the mRNA originated from, providing a spatial map of gene expression across the full tissue section.



At Three Dimension Genomics, we use 10X Genomics' Visium platform for spatial transcriptomics (see **Visium technology**). This approach is at the cutting edge of ST technologies and has key advantages over earlier methods:

- **Tissue compatibility –** Visium is compatible with both fresh-frozen and formaldehyde-fixed, paraffin-embedded (FFPE) tissues
- **High resolution –** 55µm barcode spots enable 1-10 cell resolution depending on tissue type
- **Process entire sections –** while earlier microdissection techniques required the selection of regions of interest, 10X Visium enables profiling of entire sections

In dense tissues, **individual barcoded spots may contain multiple cells** and thus do not match the single-cell resolution of scRNA-seq approaches. To address this, signals from multiple cells within a spot can be deconvoluted into constituent cell type transcriptomes by combining with a single-cell or single-nuclei transcriptome dataset. This **multiomic data integration** approach provides a powerful way to limit the weaknesses of any individual approach.

A new frontier for cancer therapies

The application of spatial transcriptomics and scRNA-seq to studies of the TME have huge potential to provide deeper insight into the biology of tumors. The table below outlines some of the major outstanding questions facing cancer research in the coming decades and how these technologies are essential to tackling them.

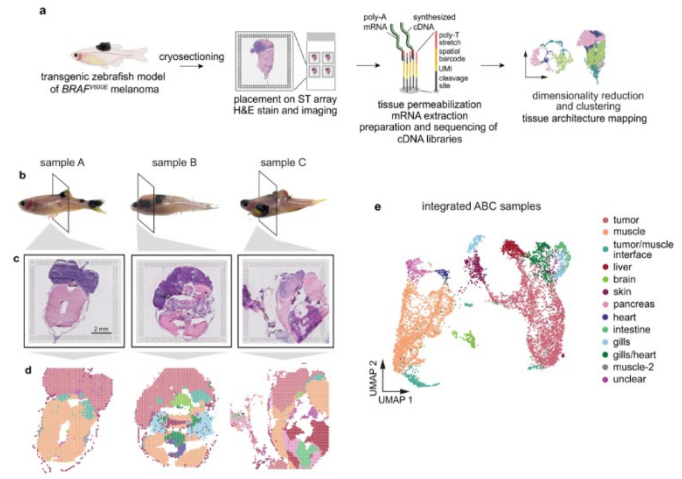
Question	Opportunity
How can we better stratify patients for treatment?	scRNA-seq can identify molecular profiles of the TME to determine optimal therapeutic strategies
How different cell types in the tumor interact?	Cell-cell interactions can be estimated from scRNA-seq data and directly observed using spatial transcriptomics
Are cancer cells in different parts of the tumor functionally different?	Spatial transcriptomics can compare expression profiles of malignant cell areas at different locations in the tumor

To give a more tangible sense of the applications of the 10X Visium platform to TME research, we highlight two recent studies that use the approach in different contexts. In both cases, an ST-based approach provides unprecedented insight into TME biology.

Hunter et al. 2021: Uncovering heterogeneity at cancer's leading edge

Hunter et al. (2021) used spatial transcriptomics in a zebrafish model of melanoma **(13)**. Whole-animal tissue sections were processed with 10X Visium to study gene expression of the tumors and surrounding tissue **(Figure 2)**.

Most of the tissue was composed of two categories, one large group of tumor spots (pink) and another group of muscle spots (orange). Transcriptome data revealed a unique group of spots (teal) clustered separately and **spatially located at the boundary between muscle and tumor**. The authors termed this area the "interface" between muscle and tumor. Although the "interface" spots are transcriptionally distinct, histological analysis reveals nothing visibly unique in the tissue in this area. Instead, these cells appear mostly indistinguishable from muscle cells.



Source: Hunter et al. 2021

Hunter et al. (2021) also performed single-nuclei RNA-seq on these tissues to deeply characterize the transcriptome. Looking at the "interface" area, the authors found an **enrichment of cilia genes in the single-nuclei RNA-seq data**. They also confirmed the presence of cilia proteins at the front of the tumor, where it invades muscle tissue. Although the muscle and tumor themselves are not ciliated, this "interface" is, and the authors are now studying **how cilia could drive the tumors' growth into the neighboring tissue.**

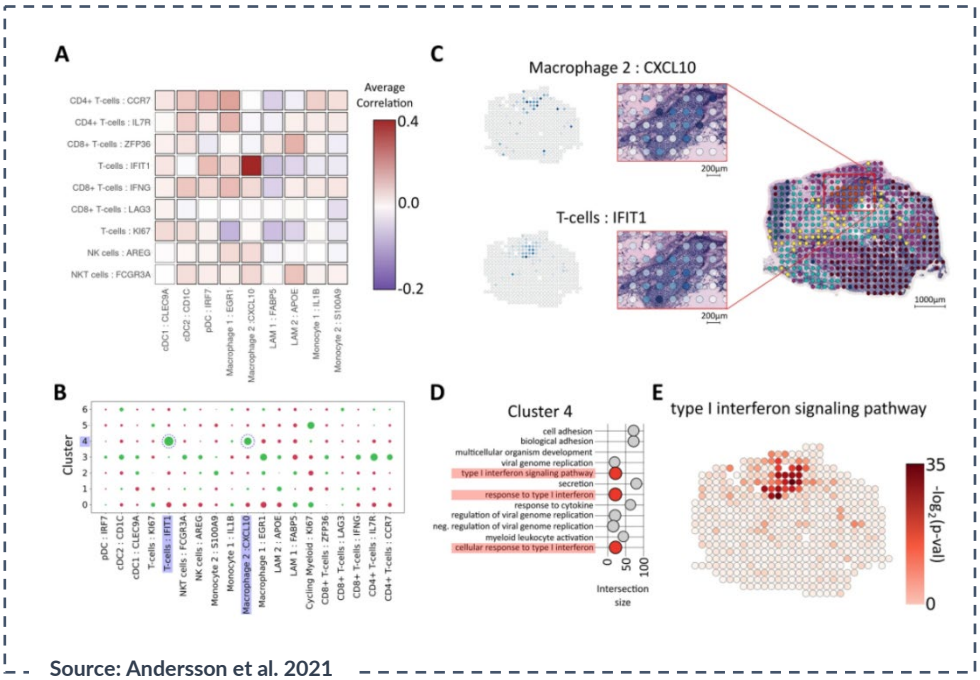
Andersson et al. 2021:

Anderson et al. 2021 applied a spatial transcriptomic approach to uncover heterogeneity between and within the HER2 positive breast cancer tumors of 8 individuals **(14)**. The study is a useful illustration of how these datasets can be applied to human tissue samples and the analysis integrated with traditional histopathological assessments.

The authors began by clustering the ST spots and using differential expression and geneset enrichment analysis to classify the tumor into distinct regions (e.g., cancer, immune-rich). They then compared this classification with a pathologist's annotation and found a strong concordance between the two. Interestingly, ST spots did identify some areas of the tumor that were molecularly distinct but had similar morphology, highlighting the potential for this approach to derive further insight.

To deconvolute the cell composition of ST spots, the authors used an existing scRNA-seq dataset from HER2+ breast cancer**.** As well as clarifying the cell types responsible for the ST regions identified previously, this approach allowed the authors to compare how different cell types "co-localized" together in the same regions. This approach revealed a strong colocalization between myeloid cells (macrophages) and T-cells. The authors then returned to the original ST spot expression data to show that areas of the tumor which had both macrophages and T-cells present had a potent type I interferon signal.

Finally, the authors used a similar approach to identify regions of the tumor rich in both T and B-cells. These regions are associated with the formation of tertiary lymphoid structures (TLSs) – a histological feature of breast cancers associated with improved survival. Gene expression of the TLSs was used to generate a TLS score that accurately predicted survival in a validation melanoma dataset.



Source: Andersson et al. 2021

Summary

Tumor microenvironments are heterogeneous both in **cellular content** and **spatial organization**. This heterogeneity poses a challenge to our understanding of cancer progression and the development of effective therapies. Modern sequencing techniques able to resolve single cell and spatially-mapped transcriptomes provide methods with which to tackle these challenges. This paves the way for a holistic understanding of how cancers develop *in situ*, how they respond to and resist treatment, and where novel therapeutics targets may be found.

About 3D Genomics

We are a group of Ph.D. scientists dedicated to providing the **highest quality research services** to help you in your biomedical and pharmaceutical research projects. We believe that every experiment is unique and deserves the **best custom research,** from design to execution to data analysis.

We specialize in providing **end-to-end services for single-cell multiomics**. We typically receive tissue samples for spatial projects and return transcriptome data with cluster analyses, H&E images, unstained frozen section slides, or images from fluorescent antibody imaging of client-specified antigens. We can isolate nuclei from tissues for single-cell RNAseq and integrate the transcriptomes with spatial transcriptomes from serial sections.

Areas of expertise

Single-cell multiomics technologies

- Spatial transcriptomics (10X Visium, typically integrated with 10X Genomics single nuclei RNAseq)
- Single-cell transcriptomics with large and small numbers of cells (10X Genomics and SmartSeq, respectively).
- Single-nuclei RNAseq from fresh-frozen and FFPE embedded tissues.
- IHC imaging (widefield/confocal, chromogenic/fluorescent)
- High Content Imaging (Cell Painting, HCS)
- Single-cell immune repertoire analysis, B-cell/T-cell clonotype studies, with cell type isolation by FACS and MACS.

Bulk cell technologies

- 3D organoid models for gene expression and drug response studies in multiple therapeutic areas using patient-derived and control cell lines
- Custom cell-based assay development and compound studies with multiple modality readouts.
- Capillary westerns (ProteinSimple)

Tissues for spatial transcriptomics

- Brain, kidney, lung, heart, etc.
- FFPE samples coming soon

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