

# Enabling pathway analysis of RNA expression in formalin-fixed paraffin embedded tissues with the GeoMx<sup>®</sup> DSP Platform

## Summary

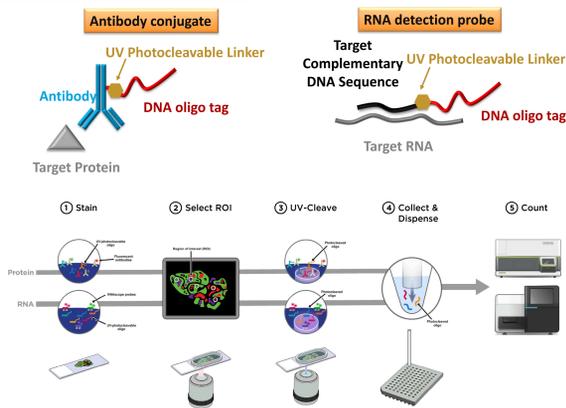
The GeoMx Digital Spatial Profiling (DSP) Platform enables robust detection of high-plex protein and RNA expression from user-defined compartments within formalin-fixed paraffin-embedded tissues. As the number of targets detected within such tissues increases, it becomes imperative to apply systems biology strategies in order to interpret the complex biology of the tumor microenvironment.

In this study, we profiled expression of greater than 1600 genes by utilizing more than 10,000 DSP-specific ISH probes on FFPE samples, including colorectal cancer patient samples from which we have matching bulk RNA sequencing and nCounter<sup>®</sup> analysis, as a platform for developing tools to move beyond single-gene profiling into understanding pathway-based expression profiles. The panel of genes profiled focused around capturing biological signaling along key canonical signaling pathways and cell-intrinsic signaling from immune cells and other cell types. We demonstrate the ability to leverage foundational pathway interrogation tools, including Reactome, with the data to capture spatially-resolved pathway interactions and signaling within FFPE tissues.

As we look towards the future of the GeoMx<sup>™</sup> platform and high-plex RNA profiling of tissue samples, these experiments highlight not only the need but the capacity for this platform to derive deep understanding of the biology within and across a single slide of tissue. These experiments are being used to drive development of the software features within the GeoMx<sup>™</sup> ecosystem, which will provide native support for pathway-level exploration of expression when working with highly multiplexed reagents in future platform offerings.

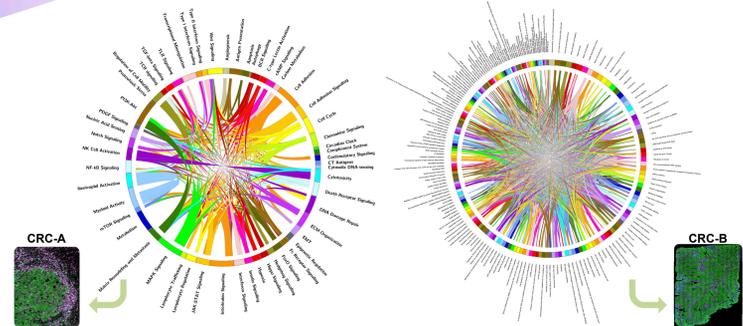
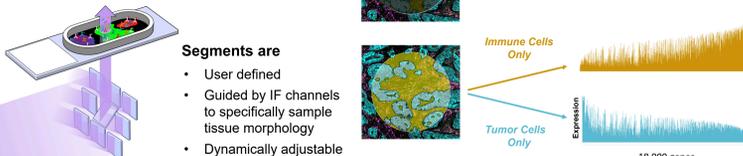
## Introduction

### GeoMx DSP chemistry and workflow



### Flexible Illumination Paths Enable Complex Interrogation of TME

The GeoMx<sup>™</sup> DSP contains a tunable micromirror array which enables profiling of distinct tissue compartments and complex morphological structures.



The Cancer Transcriptome Atlas (CTA) contains over 1,800 genes, representing comprehensive coverage of the tumor, immune system, and microenvironment in cancer.

The Whole Transcriptome Atlas contains over 18,000 genes, covering an optimized set of protein coding genes in the human transcriptome.

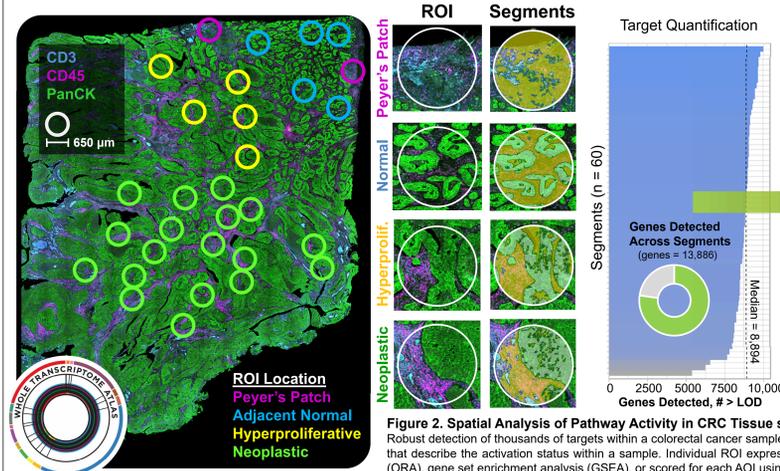
**Figure 1. Circos plots of the Cancer and Whole Transcriptome Atlas Content by Pathway**  
Annotations are sourced from Reactome (reactome.org) and associated with the genes from each panel. Connections between annotations represent shared gene content exceeding 15 genes.

## Conclusion

- Pathway analysis methods, including ORA, GSEA, and ssGSEA, can be employed with spatial analysis using the Cancer and Whole Transcriptome Atlas products
- Differential pathway activation is evident within samples, and both across segments and amongst them
- Spatial pathway analysis reveals key immunological activation as cells transit across the invasive margin



## GeoMx Transcriptome Profiling of Spatially-resolved Colorectal Cancer Samples Enables Pathway Analysis



**Figure 2. Spatial Analysis of Pathway Activity in CRC Tissue section using Whole Transcriptome Profiling**  
Robust detection of thousands of targets within a colorectal cancer sample enables spatially resolved pathway signaling using distinct methods that describe the activation status within a sample. Individual ROI expression is aggregated and explored using over-representation analysis (ORA), gene set enrichment analysis (GSEA), or scored for each AOI using single sample gene set enrichment analysis (ssGSEA)

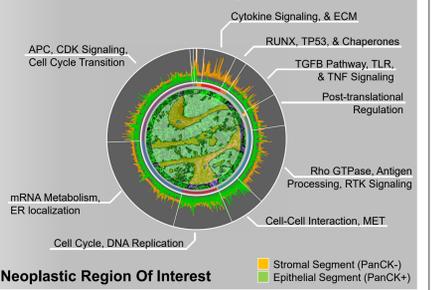
## Results

**Over-Representation Analysis (ORA)** for functional exploration of gene sets enriched across samples

**Gene Set Enrichment Analysis (GSEA)** extends differential expression testing into pathways

**Single sample GSEA** quantifies pathway status of individual compartments within ROIs

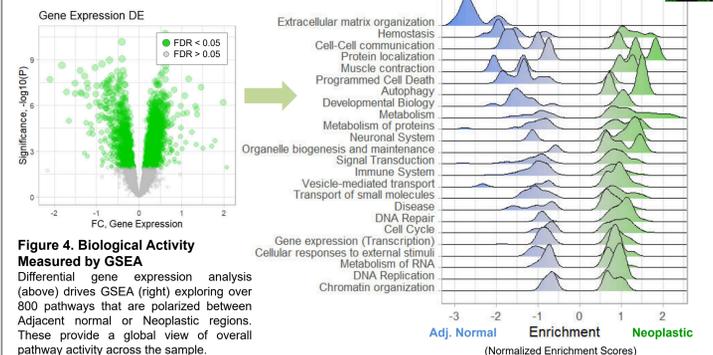
## Over-Representation Analysis Classification of genes expressed within distinct tissue compartments



**Figure 3. Spatial Overlays and ORA**  
The top 50% of genes detected based on median expression are clustered around the original ROI. Expression is shown as for each compartment within a tissue based on the segmentation strategy. Gene expression is colored by associated tissue segment.

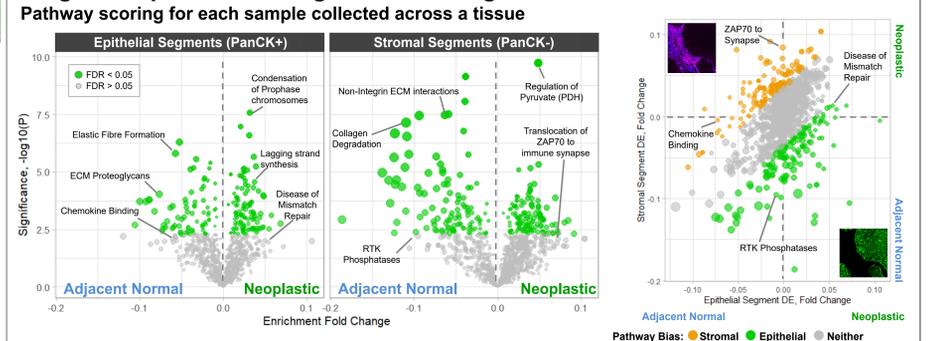
## Pathway Analysis Drives Contextual Insight into Spatial Gene Expression Profiles

### Gene Set Enrichment Analysis Explore Localized Enrichment of Pathways

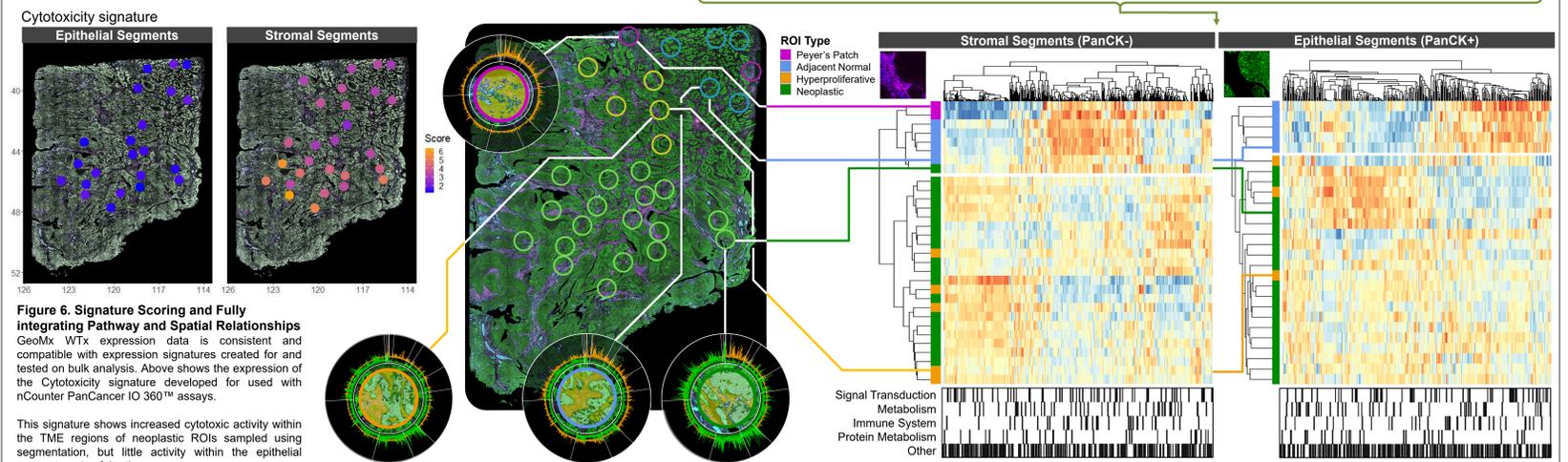


**Figure 4. Biological Activity Measured by GSEA**  
Differential gene expression analysis (above) drives GSEA (right) exploring over 800 pathways that are polarized between Adjacent normal or Neoplastic regions. These provide a global view of overall pathway activity across the sample.

### Single Sample GSEA & Signature Scoring

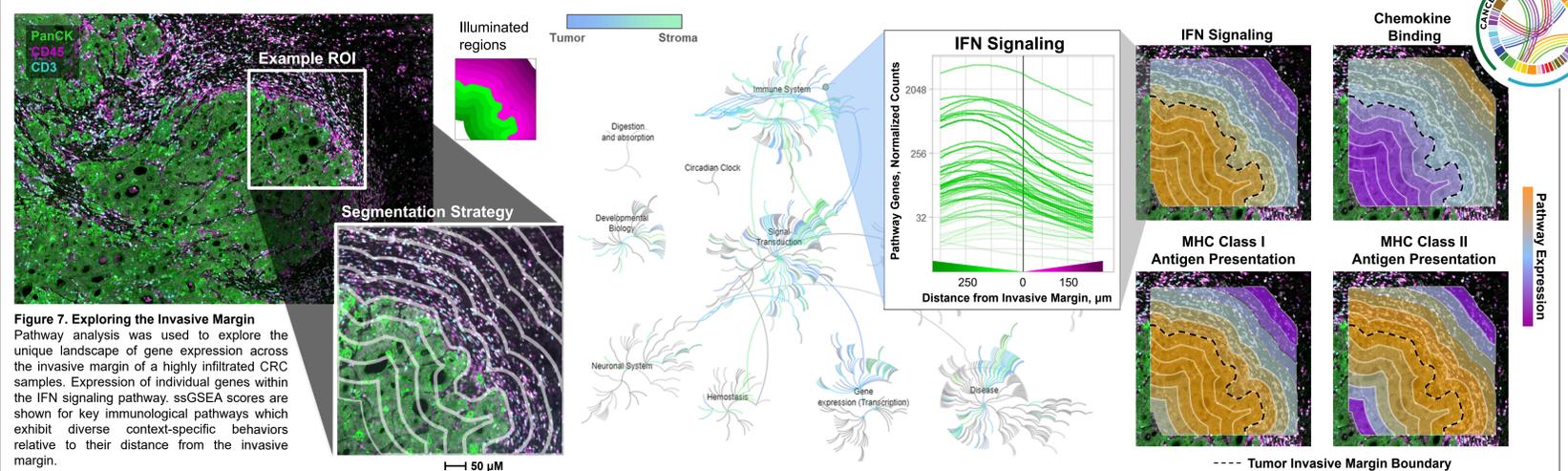


**Figure 5. ssGSEA Enables In-depth Quantitative Analysis within ROIs and Segments**  
ssGSEA provides a framework for exploration of the individual activity level of pathways within each segment to quantitatively assess individual sample pathway dysregulation. By having segment-level pathway scores, differential expression testing of (above left), exploration of segment pathway activation (top right), as well as coordinated expression analysis (below).



**Figure 6. Signature Scoring and Fully Integrating Pathway and Spatial Relationships**  
GeoMx WTX expression data is consistent and compatible with expression signatures created for and tested on bulk analysis. Above shows the expression of the Cytotoxicity signature developed for use with nCounter PanCancer IO 360<sup>™</sup> assays. This signature shows increased cytotoxic activity within the TME regions of neoplastic ROIs sampled using segmentation, but little activity within the epithelial compartments of the tissue.

## Analysis of the Tumor Invasive Margin Demonstrates Dynamic Activation of the Immune System



**Figure 7. Exploring the Invasive Margin**  
Pathway analysis was used to explore the unique landscape of gene expression across the invasive margin of a highly infiltrated CRC sample. Expression of individual genes within the IFN signaling pathway. ssGSEA scores are shown for key immunological pathways which exhibit diverse context-specific behaviors relative to their distance from the invasive margin.