PRODUCT GUIDE

GeoMx® Digital Spatial Profiler (DSP)

Project Design Guide

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nanoString®

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This guide provides an overview of planning, designing and analysis for spatial biology experiments for GeoMx Digital Spatial Profiler (DSP), as well as case studies of highlighted research.

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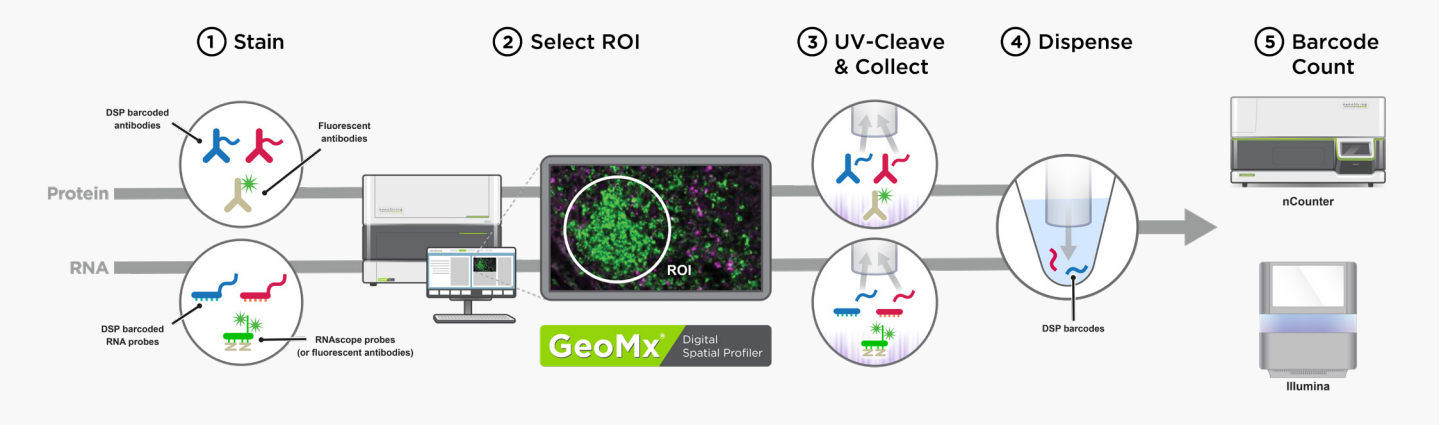
Part 1: The Spatial Biology Solution

Historically, immunohistochemistry and immunofluorescence have been used to assess spatial heterogeneity of proteins and nucleic acids in tissue slices. However, these techniques are of limited plexity due to restricted dynamic range, difficult quantitation, and limited multiplexing capability.

NanoString's GeoMx® Digital Spatial Profiler (DSP) combines standard immunofluorescence techniques with digital optical barcoding technology to perform highly multiplexed, spatially resolved profiling experiments. In a single slide, the GeoMx DSP performs whole slide imaging with up to four fluorescent stains to capture tissue morphology and select regions of interest for high plex profiling. The ability to perform tissue morphology guided profiling experiments increases the likelihood of capturing rare events often missed by bulk or single cell experiments. This chemistry also provides high-plex profiling of RNA and protein targets on just two serial sample sections, enabling deep characterization of the sample.

GeoMx DSP specifications include:

- Throughput: 4-8 slides per day
- Multi-analyte: RNA and Protein
- Imaging Resolution: > 1 cell
- Multiplexing capability: up to 96 targets with nCounter readout or 100's-to-Whole Transcriptome with NGS readout
- Quantitative: 6 logs of dynamic range



GeoMx DSP has been applied to research in diverse fields, including oncology, immunology, neuroscience, and infectious disease. Select case studies demonstrating the utility of spatial analysis can be found in Part 6 of this guide, highlighting research from:

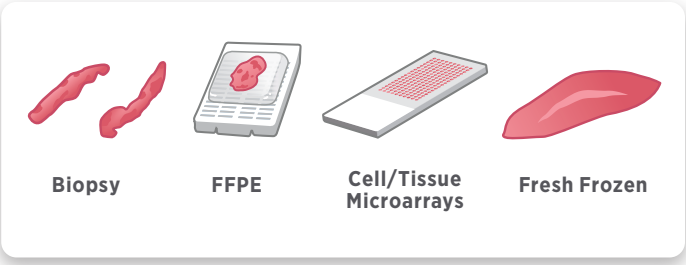
- David Rimm, M.D., Ph.D., Yale University
- Stefan Prokop, M.D., University of Florida
- Pete Nelson, Ph.D., Fred Hutchinson Cancer Research Center
- Alex Swarbrick, Ph.D., Garvan Institute of Medical Research

In addition to these case studies, the most recent publications can be found **here**.

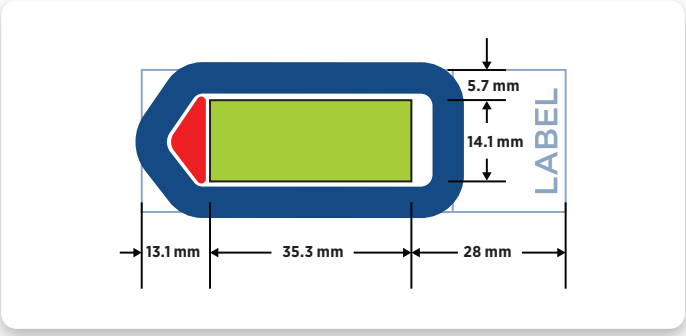
This guide is intended to provide an overview of GeoMx DSP, cover the basics to designing a spatial biology study, details on considerations for planning an experiment, an overview of the workflow and data analysis features. It is intended to serve as a primer to help initiate projects for GeoMx DSP, The Spatial Biology Solution™.

Part 2: Starting a Spatial Biology Study

When designing a spatial biology study, considerations should be taken into the tissue source, sample type and assay to be performed for the study. For spatial biology experiments conducted on GeoMx DSP, a range of tissue types from both human and mouse have been extensively used. Commonly used sample types in spatial biology studies include tissue biopsies, formalin fixed paraffin embedded (FFPE) tissue, fresh frozen (FF) tissue, fixed frozen tissue, cell microarrays, tissue microarrays and whole tissue.



Even the most difficult tissue types have yielded significant results on GeoMx DSP, including skin, adipose, FFPE brain tumors and high-grade lymphomas. Spatial biology experiments on GeoMx DSP only require that they are slide mounted within the following dimensions:



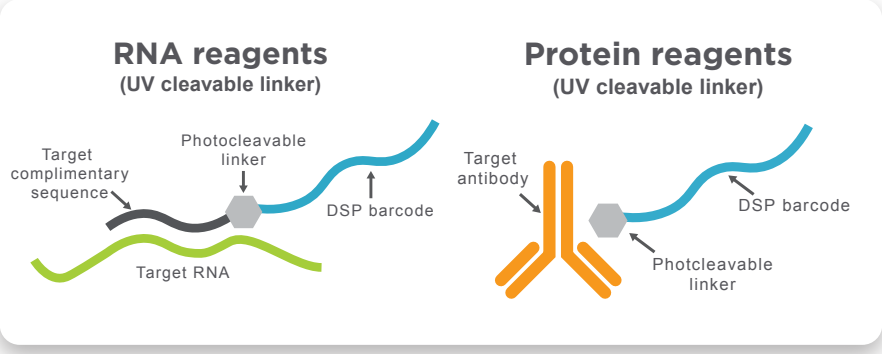
Additional considerations for slide mounted samples include:

- Blocks should be sectioned with 4-6 µm thickness and then mounted on adhesive/positively charged slides.
- Tissue sections should be placed in the center of the slide and be no larger than 35.3 mm by 14.1 mm.
- Tissue less than 3 years old is preferred but GeoMx DSP has performed well with a wide range of sample ages. We recommend cutting sections fresh for best performance with RNA. Protein samples can be fresh cut or previously slide mounted.
- After slide mounting tissue, samples used for protein assays are stable at room temperature while sections for RNA assays are stored at 4°C in desiccator.

Selecting Assay Content

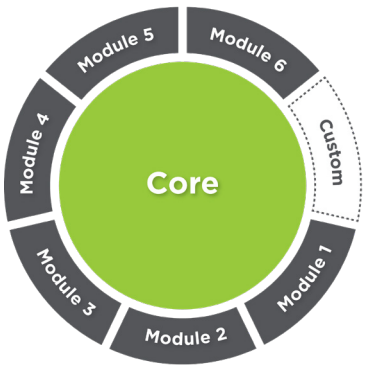
The GeoMx RNA and Protein assays allow you to quantitatively profile 10's-20,000's of RNA or protein targets from a single FFPE or Fresh Frozen tissue section on the GeoMx DSP. GeoMx Assays are pre-validated, modular, and available for nCounter or NGS-based readout to provide flexibility and support a range of research needs.

Validated content covers immunology, immuno-oncology, neurodegeneration, and neuroinflammation with a rapidly growing pipeline. Additionally, GeoMx Assays can be customized through the Protein Barcoding Service or RNA Barcoding Service.



GeoMx Assays are designed for readout by the NanoString nCounter platform or Illumina NGS platforms. The table below describes the benefits of each readout type. Please refer to product pages linked below to ensure your content of interest is compatible with your readout method of choice.

	nCounter	NGS
Description	Direct digital readout on nCounter via NSTG barcodes.	Spatial analysis using existing NGS infrastructure
Plex capacity	96	20,000+
Instrument Required	nCounter	Illumina Sequencer (NextSeq, NovaSeq)
Readout Chemistry	Direct readout on nCounter via NSTG barcodes.	PCR based library prep to introduce barcodes for sequencing.
RNA content	<96	Whole Transcriptome
Protein Content	<96	96+
Data analysis	Integrated analysis with GeoMx DSP software to map profiling data to imaging data.	NGS data is converted to counts then reimported back to the GeoMx DSP software to map profiling data to imaging data.
Turnaround Time	<4 days	<5 days



GeoMx Protein Panels

All GeoMx® Protein Assays undergo extensive validation to ensure high quality GeoMx DSP data and are offered for nCounter or NGS readout. Additionally, antibodies can be customized for use with GeoMx DSP through the Protein Barcoding Service.

Protein content is modular to meet your research needs. **Visit our website** to explore off-the-shelf content in immune-oncology, immunology, and neuroscience.

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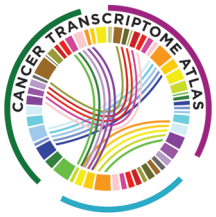
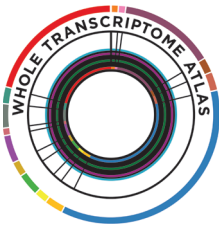
GeoMx RNA Panels

GeoMx RNA assays are offered in formats for either nCounter or NGS readout methods.

- The GeoMx Immune Pathways Panel is designed for targeted profiling of the tumor, tumor microenvironment, and tumor immune status. Profile up to 96 RNA targets with spatial resolution from a single tissue section using the **GeoMx Digital Spatial Profiler (DSP)**.



- The **GeoMx Cancer Transcriptome Atlas (CTA)** is designed for comprehensive profiling of the tumor biology, tumor microenvironment, and the immune response. Profile the RNA expression over 1,800 genes simultaneously with spatial resolution in any region of interest from a single tissue section using the GeoMx Digital Spatial Profiler.



- The **GeoMx Whole Transcriptome Atlas (WTA)** and **GeoMx Mouse Whole Transcriptome Atlas** is designed for comprehensive profiling of spatial biology. With full coverage of protein coding genes, WTA delivers spatial analysis of any target in any tissue in the biological regions that matter most.

Part 3: Designing GeoMx DSP Experiments

Once a spatial biology study is outlined, identifying desired tissue source, sampling type and analyte to assay, individual experiments are ready to be designed. This portion of the guide will walk researchers through specifying key components of a GeoMx DSP experiment:

- Tissue types and number
- Content Selection
- Guiding ROI/AOI Selection
- ROI Selection Strategies

Although this portion of the guide is designed to provide an overview of the different components of a GeoMx DSP experiment, the table displayed in this section is intended to be reviewed with your GeoMx DSP Project Specialist.

Tissue Type and Number					
Tissue Type	☐ Fixed Frozen	☐ FFPE	☐ Fresh Frozen	☐ Other:	GeoMx DSP has been validated with FFPE and fresh frozen samples.
Species	☐ Human		☐ Mouse	☐ Other:	GeoMx DSP Assays have been validated for human and mouse
Number of Slides	☐ Whole Mount		☐ TMA		Typical experiments range from a minimum of 12 to up to 100s of samples. Ensure proper group sizes for statistical analysis.
Number of ROIs/ AOIs Slide					Typical experiments include 12-24 AOI to ensure statistical comparisons between region types. For TMA analysis, each "spot" typically consists of a single ROI (region of interest) and one or more AOI (areas of illumination). Sample heterogeneity may also determine the appropriate number of ROIs with Homogeneous = fewer ROIs needed and Heterogeneous = more ROIs needed

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GeoMx Assays are available for RNA and Protein covering Immuno-Oncology, Immunology, Neuroscience, and Infectious Disease applications. Serial sections can be used for multi-analyte analysis. Please refer to the current probe lists to select the content most applicable to your research.

Content Selection				
Species	☐ Human	☐ Mouse	☐ Other:	
Protein	nCounter Readout	☐ Immuno-Oncology and Immunology	☐ Neuroscience	
	NGS Readout	☐ Immuno-Oncology and Immunology	☐ Neuroscience	
	☐ Custom Targets			
RNA	nCounter Readout	☐ Immune Pathways		
	NGS Readout	☐ Cancer Transcriptome Atlas **Human Only*	☐ Human WTA	☐ Mouse WTA
	☐ Custom Targets			

To provide direction for selecting regions of interest (ROI) and areas of interest (AOI), appropriate fluorescent morphology markers must be used. These markers are meant to provide a visual roadmap for selecting discrete regions/areas to profile the selected content above.

ROI / AOI Selection		
GeoMx® Morphology Reagents	☐ GeoMx Solid Tumor TME Morphology Kit ☐ GeoMx Melanoma TME Morphology Kit ☐ GeoMx Alzheimer's Morphology Kit ☐ GeoMx Parkinson's Morphology Kit ☐ Additional Qualified Antibody Morphology Marker(s) Download Here	NanoString provides off the shelf morphology kits to define ROI selection for common use cases. All kits contain a nuclear stain and two fluorescently labeled antibodies for ROI selection. Users may add an additional custom target for use in the 666nm channel. NanoString has also tested several other fluorescent markers for guidance on additional markers of interest. Refer to the Morphology Markers Reference List for more information.
Custom Morphology Reagents	☐ Custom Antibody Morphology Marker(s) ☐ Custom RNAScope Morphology Marker(s)	Users may design their own morphology marker set for ROI selection with up to three targets plus a nuclear stain. Antibodies used for IHC in other applications are likely to perform well as are RNAscope probes. Additional guidance can be found below.

Choosing suitable custom morphological markers

- For RNAScope Markers: Obtain prelabeled probes from ACD
- For Antibody Markers
  - o Step 1: Select a vendor
  - o Step 2: Identify pre-labeled primary antibodies. If none, explore labeling options with vendor.

- Step 3: Match fluorophore to available channels*

Channel	Recommended Dye
525 nm	FITC
568 nm	Cy3
615 nm	Texas Red
666 nm	Cy5, DyLight™ 650**

- ◆ Tip: When working with tissues known to be auto-fluorescent, consider choosing fluorophores in longer wavelengths.
- ◆ Tip: If your morphology marker matches a protein target in the GeoMx Protein Assay being used, we suggest staining with the morphology markers first and doing additional analysis to assess competition in the GeoMx Protein Assay for that particular target.

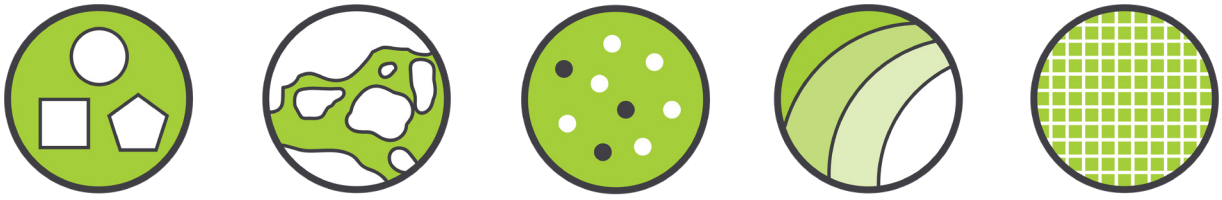
*One channel needs to be reserved for DNA Stain

**If using DyLight 650 and Alexa Fluor® 594, be aware of bleed-over.

- Step 4: Test against control tissue if not already pre-validated

Region of Interest Selection Strategies

ROI Selection Modality	☐ Geometric ☐ Segmentation ☐ Contour ☐ Cell Type Profiling ☐ Gridded	The Morphology Reagents selected will guide ROI section, which can be done using one of 5 selection modalities. See below for more details as to when each modality may be most applicable.



Modality	Geometric	Segmentation	Cell Type	Contour	Gridded
Utility	Assess tissue heterogeneity with standardized geometric shapes or unique polygons across distinct tissue regions	Maximize cellularity and profile distinct biological compartments within a ROI based on morphology marker characterization	Reveal the function of distinct cell populations within a ROI based on morphology marker characterization	Evaluate how proximity affects biological response and the local microenvironment around a central structure with radiating ROIs	Perform deep spatial mapping of a tissue region using a tunable gridding pattern
Example Questions	How does the expression of tumor and immune markers differ across a sample? What is the expression pattern in distinct cortical layers?	How does the tumor differ from the tumor microenvironment?	How do rare immune cells impact tumor biology and therapeutic response? How do specific neural cell populations impact neurobiology and disease progression?	How does proximity to the tumor or an immune cell population alter biological response? What is the expression profile of the amyloid-beta plaque microenvironment?	What novel biology is uncovered with deep spatial mapping of tissue?
Sample Morphology	Homogeneous or measuring change over a long distance	Heterogeneous, Continuous	Heterogeneous, Non-Continuous or overlapping cell types	Measuring change over short distance	N/A - Deep spatial mapping of a portion of the tissue independent of morphology

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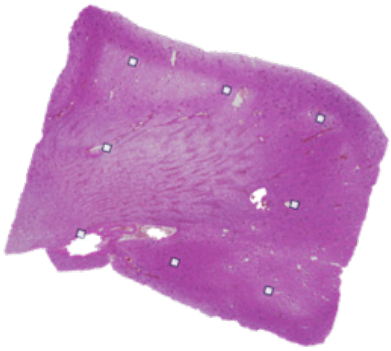
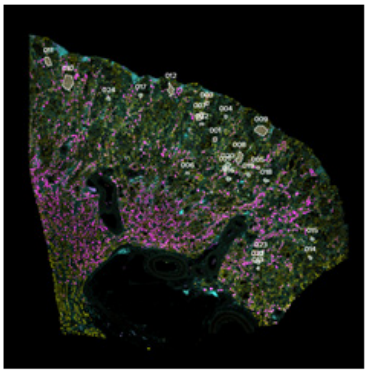
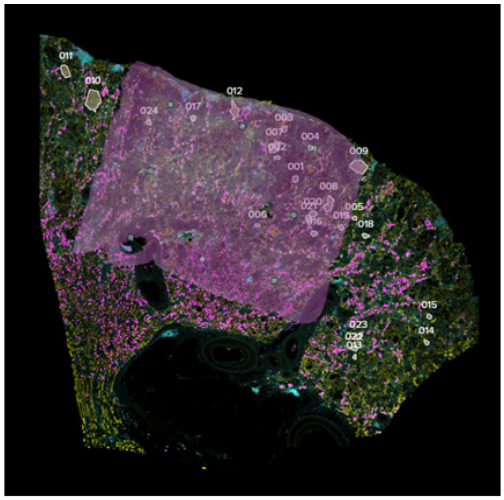


H&E stained tissue



GeoMx serial section



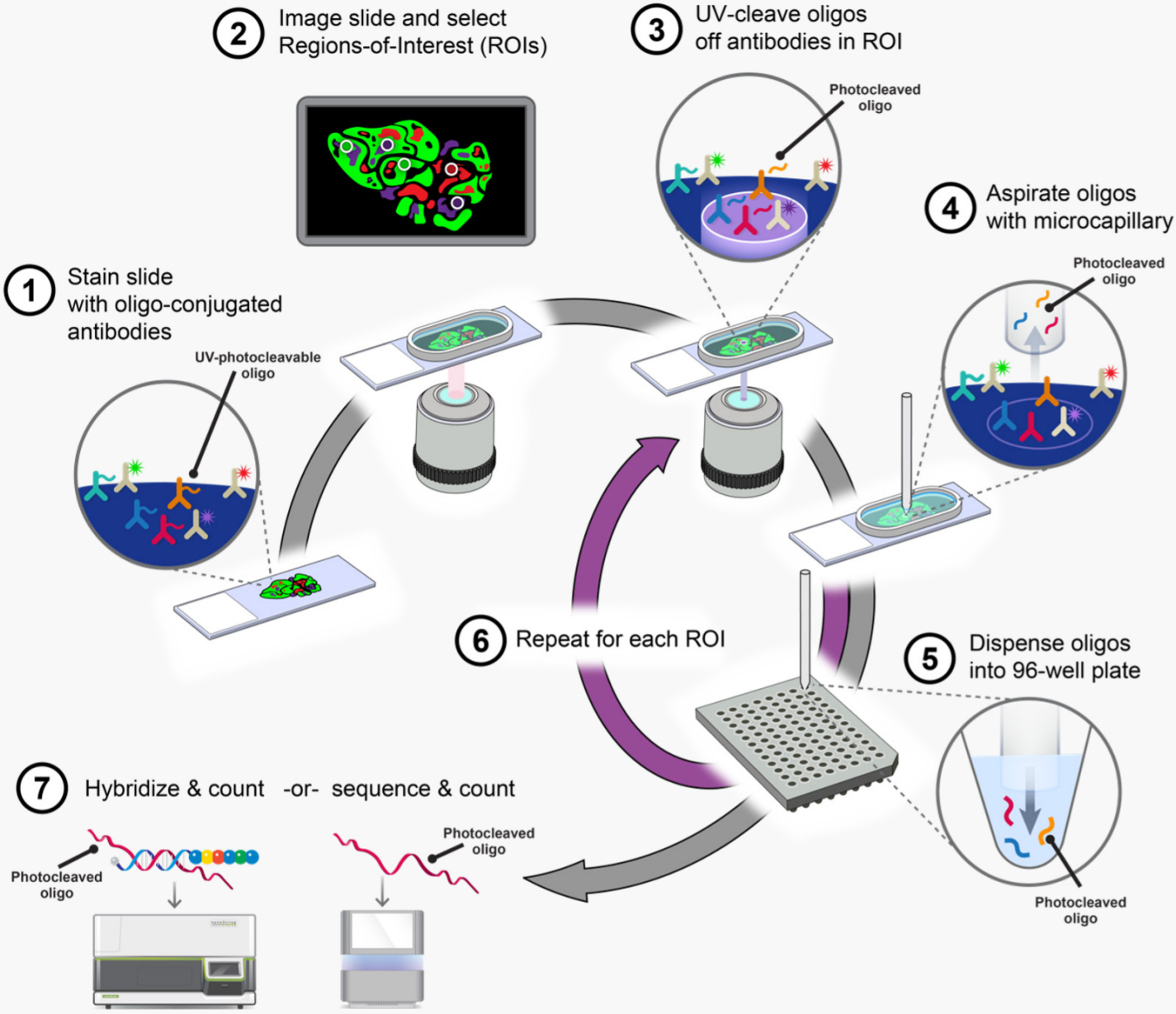
H&E overlaid on GeoMx scan

An intuitive interface allows users to select and highlight ROIs with these modalities. To further support the ROI selection process, the Image Overlay feature in GeoMx DSP can be utilized. Here, one section is stained with H&E, then used to mark up regions. A serial section is prepared for GeoMx DSP and after scanning, an image of the edited H&E stain can be uploaded and laid over the GeoMx DSP experiment slide. Once this is done, ROIs can be easily transferred from the H&E stained slide.

Part 4: Workflow

GeoMx DSP is designed to require minimal hands on time and is compatible with the Leica Bond autostainer for further automation of the workflow. Slides are prepared and co-stained with fluorescent morphological markers plus either oligo-conjugated antibodies (for protein assays) or oligo-conjugated in situ hybridization probes (for RNA assays) (1). Samples are then fluorescently imaged in GeoMx DSP to visualize fluorescent morphological markers to allow for region-of-interest (ROI) selection (2). Once selected, each ROI is collected one-at-a-time by discretely illuminating UV light over a specific region (3) and released photocleaved oligos aspirated with microcapillary (4). Once collected, oligos are dispensed into a unique well in a collected plate (5) and the process is then repeated for each ROI (6). Once completed, photocleaved oligos are counted by an nCounter or NGS system (7).

For more workflow detail, please see courses and documents in **NanoString University**.



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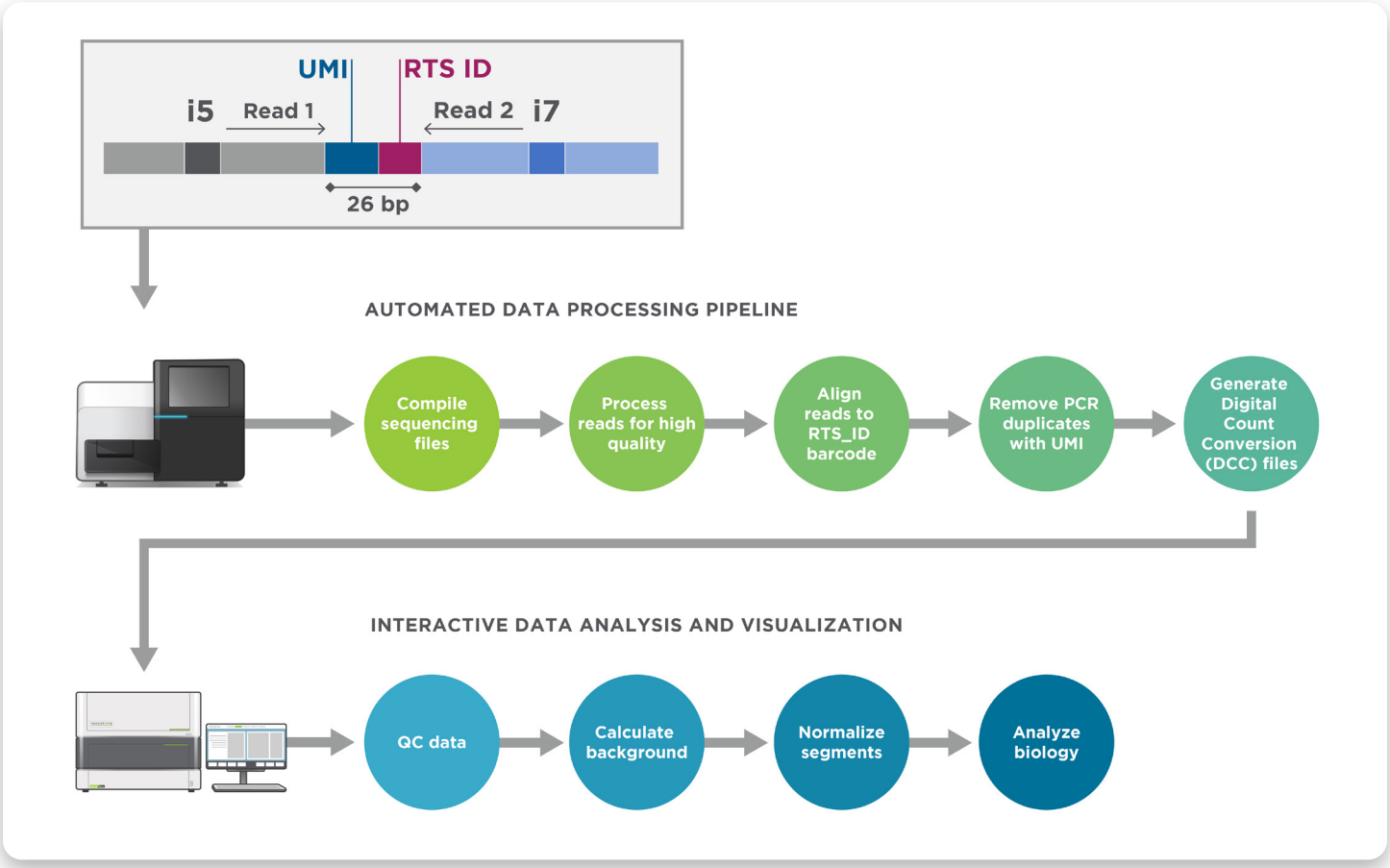
Part 5: Data Analysis

GeoMx DSP includes sophisticated tools for end-to-end analysis of either nCounter or NGS readout assays from image acquisition, region of interest selection to a fully integrated data analysis experience.

For NGS readout assays an integrated workflow takes FASTQ files from the Illumina sequencer and processes them to Digital Count files (DCC) for further analysis in the DSP Data Analysis Suite.

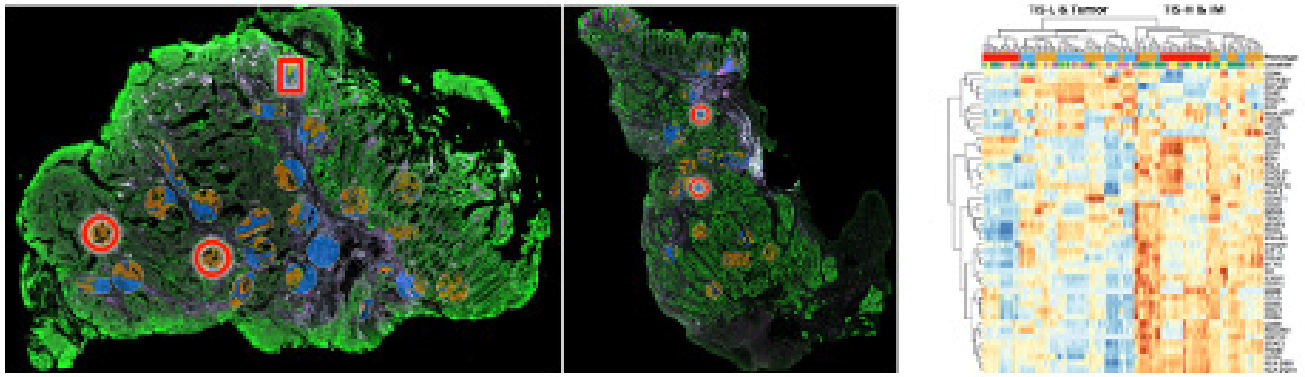


The GeoMx NGS Pipeline is also now available on Illumina BaseSpace Sequence Hub cloud ecosystem. Simply sign-up for a BaseSpace account and search for the GeoMx NGS Pipeline application to run the pipeline on the DRAGEN Bio-IT platform for increased performance and ease of use.

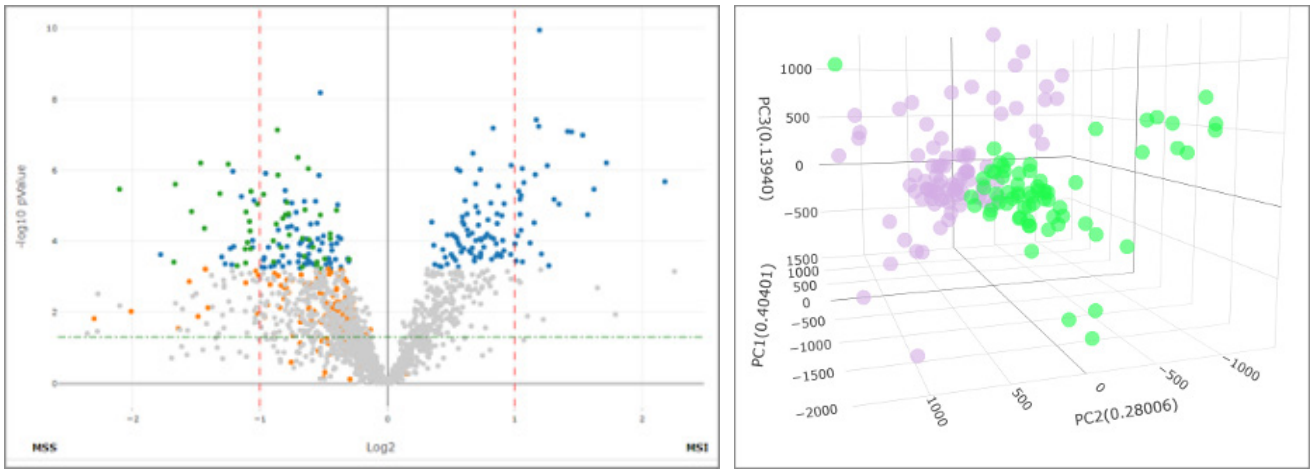
Analyzing GeoMx DSP Data

Once in the DSP Data Analysis suite, integrated nCounter and NGS data QC provide seamless data quality assessment. Normalization by cell density (automated cell counting) or area-based normalization can be used.

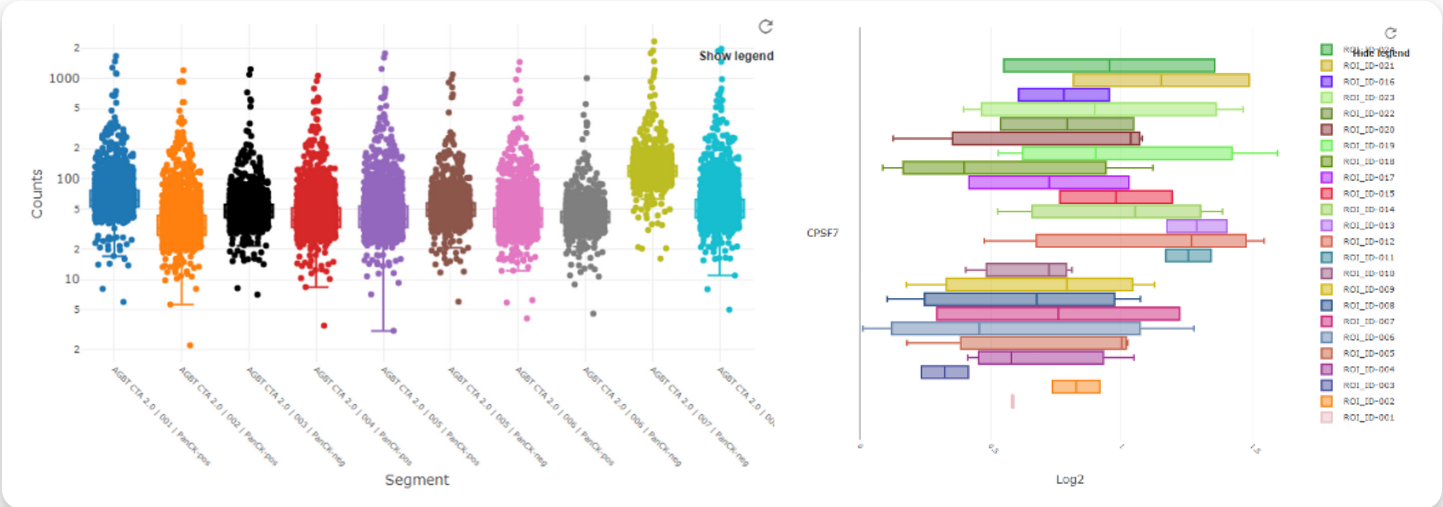
Streamlined interactive analysis moves raw count data from QC and normalization to statistical analysis, and visualization in minutes. The DSP Data Analysis Suite includes a study dashboard to quickly navigate various data transformations and visualizations, while maintaining connectivity to the Regions of Interest selected on the image. Using statistical and cluster analysis, significant patterns of expression across tissue morphology and potential gene signatures or biomarkers of disease can be identified.



Volcano plots and three-dimensional, interactive Principle Components Analysis (PCA) enable deeper insight to identify outliers, genes of interest, and gene signatures of interest:



Additional visualizations options in the DSP Data Analysis Suite include box plots and forest plots:



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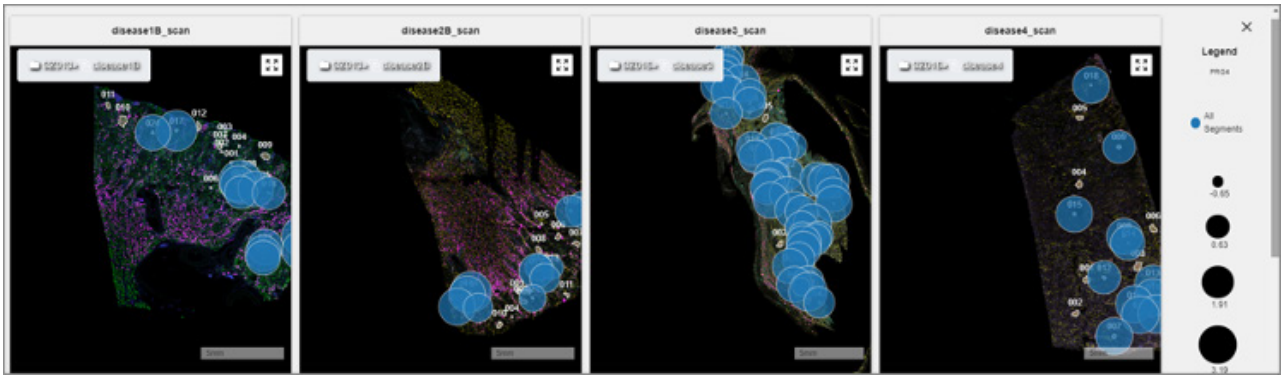
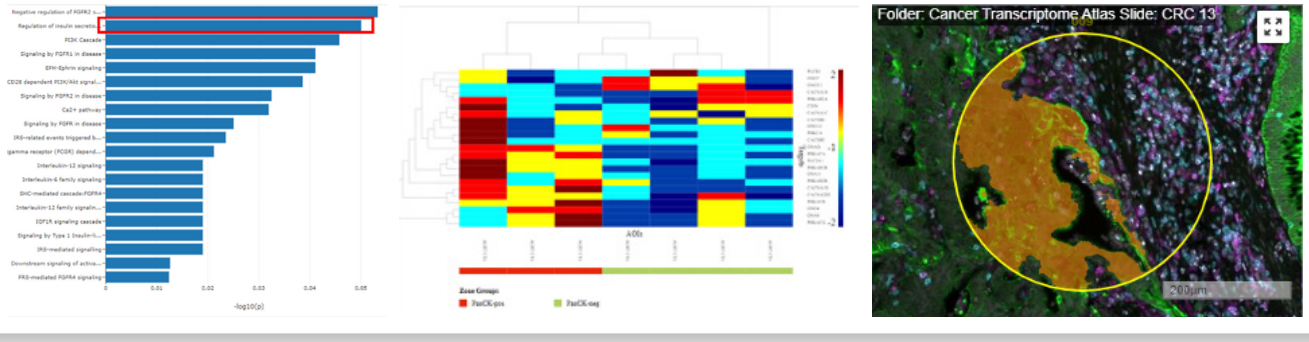
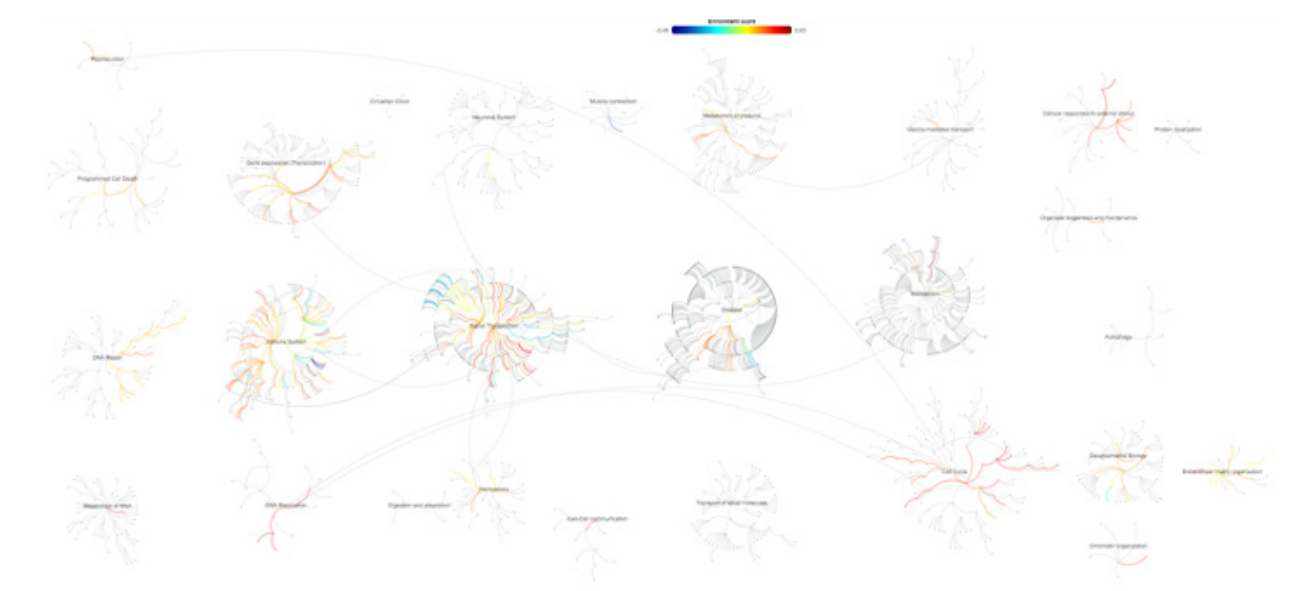
Explore gene expression in context to the tissue with the Expression Overlay feature. Color by tag or factor:



Continue to find biological meaning by determining the significantly dysregulated pathways using gene set enrichment analysis (GSEA) and drill down into the specific genes in the pathway to understand the differentially expressed genes potentially responsible for the observed phenotype or morphological changes:



Pathway enrichment scores can be visualized across multiple pathways to look at trends and identify significantly impacted biological areas.



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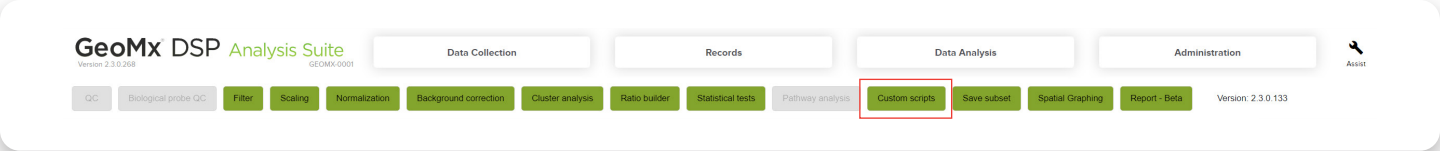
Custom Scripts

The task bar highlights the next step in the analysis process including the ability to use externally developed capabilities through the R-script Manager:

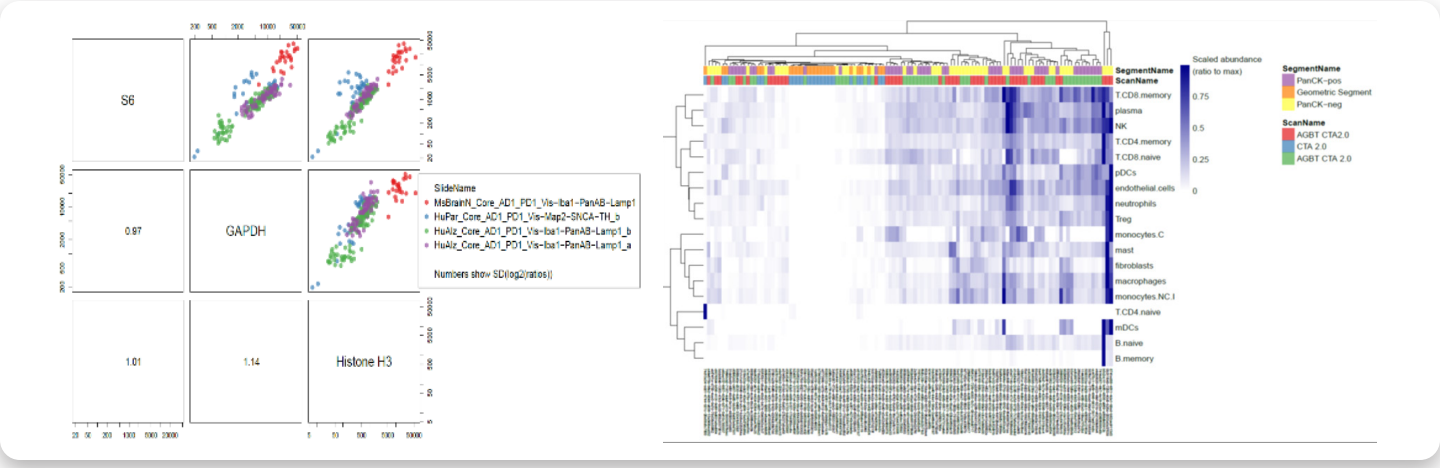


Access custom R-scripts from the **GeoScript Hub** to augment or create new plots and visualizations for DSP Data Analysis Suite. Scripts available include:

- QC/Normalization for RNA and Protein
- SpatialDecon for determining cell abundance
- Dimension Reduction: PCA, tSNE, and UMAP plots

Sharing GeoMx DSP Data

Once an analysis has been completed easily share with collaborators and colleagues by generating the GeoMx Data Analysis Summary report which is customized for each study.



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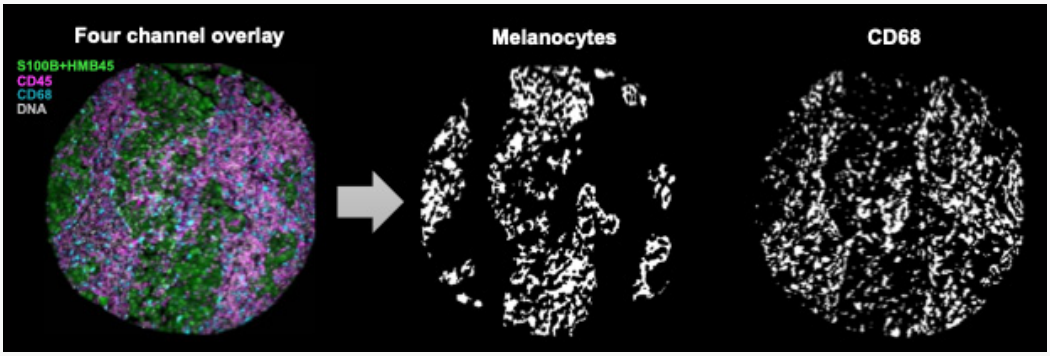
Case Study 1: Identification of Predictive Biomarkers in Melanoma

Background: Either single-agent or combinations of Immune checkpoint inhibitors are now regularly used in the clinic for the treatment of melanoma. Given the significant number of patients that are non-responsive to ICI, the inflammatory toxicities and the lack of ability to satisfactorily predict either, there is a necessity to discover more predictive biomarkers.

Experimental Question: Using tissue microarrays from pre-treatment biopsies, can DSP reveal novel predictive biomarkers either singularly or in combination across multiple biological compartments of the tumor and its immune microenvironment?

Experimental Design: The expression of 44 proteins was measured in the macrophage, T-cell and melanocyte compartments in the melanoma biopsies of 59 patients that underwent ICI therapy.

ROI Selection Strategy: Serial masks for each TMA core: CD68 (macrophages), CD45 (all lymphocytes except macrophages), S100B+PMEL17 (melanocytes), and DNA (non-tumor/non-lymphocyte cells) – remaining material from patient.



Conclusions

- **GeoMx DSP Enables Compartment Specific Biomarkers to be Discovered**
- **With limited patient samples, DSP has enabled meaningful investigation of these samples**
  - o Compartment masking leads to identification of mechanistically rational association with patient response
  - o Multiplex marker analysis allows for testing of precious material without wasting tissue
- **Expression of key markers is associated with patient outcome in this patient cohort**
  - o PD1, PDL1, and B2M in stromal compartments
  - o CD20 and IDO1 trend towards significance in tumor compartment
  - o Akt in the tumor is associated with patients with PD after a year, but not patients without PD
  - o PDL1, B2M, and IDO1 show increasing expression with durable response in several compartments
- **Tissue compartmentalization adds power to identify biomarkers of patient outcome**
  - o Whole slide analysis would bias results towards tumor heavy expression, washing out signal from stromal compartments

To read the full publication, visit https://clincancerres.aacrjournals.org/content/25/18/5503

Case Study 2: Effects of TREM2 R47H variant on neuropathological hallmarks of Alzheimer's Disease

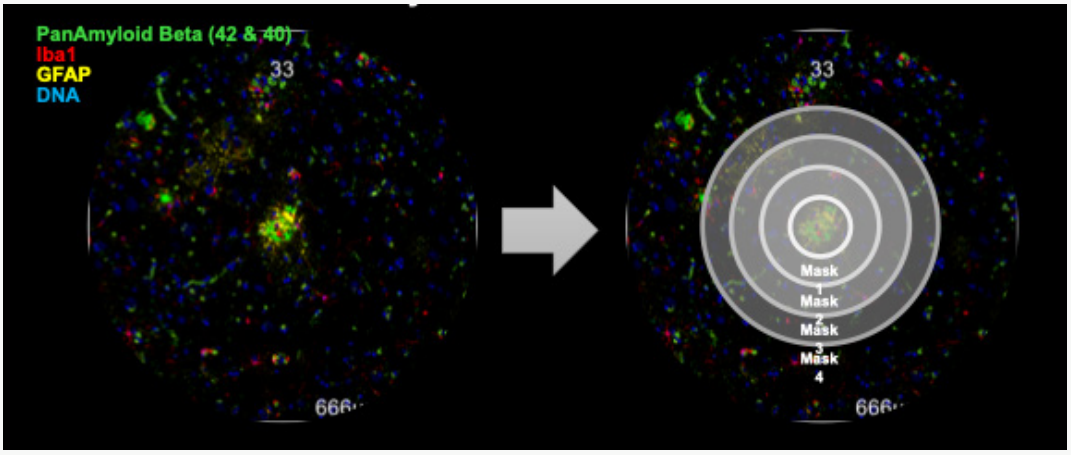
Background: Triggering receptor expressed on myeloid cells 2 (TREM2) is a microglia receptor that mediates phagocytosis and confers anti-inflammatory properties in microglia. Functional variants of TREM2 are associated with late-onset Alzheimer's disease (AD) and frontotemporal dementia (FTD).

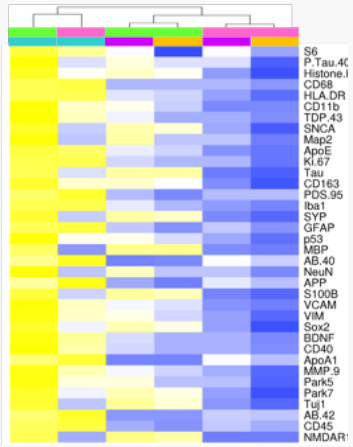
Experimental Questions:

- Can DSP serve to identify distinguishing features in microglia from TREM2 R47H carriers that will provide insight into the risk conferred by the TREM2 variant?
- Can DSP reveal differences in the nature of neuropathology in TREM2 variant carriers?

Experimental Design: Using post-mortem brain tissue from AD patients with and without TREM2 mutations, the expression of >40 proteins was measured in the hippocampus of 6 AD patients without TREM2 mutations, 6 TREM2 R47H carriers and non-AD controls at various distances from amyloid plaques.

ROI Selection Strategy: Concentric circles were selected to assess the expression profile of Amyloid-beta plaques and their microenvironment. Each ring was illuminated independently.





Conclusions:

- GeoMx DSP analysis provided an advantage over conventional IHC neuropathological assessment revealing variability in post-mortem brain tissue from patients with and without Trem2 risk variants, despite no apparent neuropathological differences.
- GeoMx DSP revealed profound differences in the plaque microenvironment between high AD and Trem2 cases.
- Multiplexed target analysis enabled a comprehensive evaluation of pathology from a single FFPE tissue section.
- GeoMx DSP data confirmed biochemical and immunohistochemical findings and revealed new biological insights.

To read the full publication, visit https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6939638/

Case Study 3: Spatial Profiling with RNA and Protein in Prostate Cancer

Background: Phenotypic subtypes of metastatic castrate resistant prostate cancer (CRPC) has emerged from adaption and resistance of cancer cells to therapeutics. Alternative late-stage therapeutic strategies are needed for metastasis, and better understanding of metastatic heterogeneity and immune composition of these phenotypic subtypes can inform therapeutic choices.

Experimental Questions and Design:

- High-throughput, multi-analyte profiling on TMAs, comprised of 178 samples from multiple metastases from 28 CRPC patients
- Assessed multiple regions-of-interests per patient to reveal inter- and intra-patient heterogeneity, with higher resolution than bulk RNA profiling
- Mapped and characterized the cellular and molecular immune composition of prostate cancer subtypes

Results:

- DSP revealed a higher degree of intra-tumor heterogeneity than bulk profiling, including detection and variation of the AR-V7 splice isoform, a biomarker for therapeutic resistance.
- High-throughput immune profiling revealed immune compositions associated with phenotypic subtypes of CRPC, including a new candidate immune checkpoint blockade target, B7-H3.

  To read the full publication, visit https://www.nature.com/articles/s41467-021-21615-4

Case Study 4: Whole Transcriptome Spatial Profiling

Background:

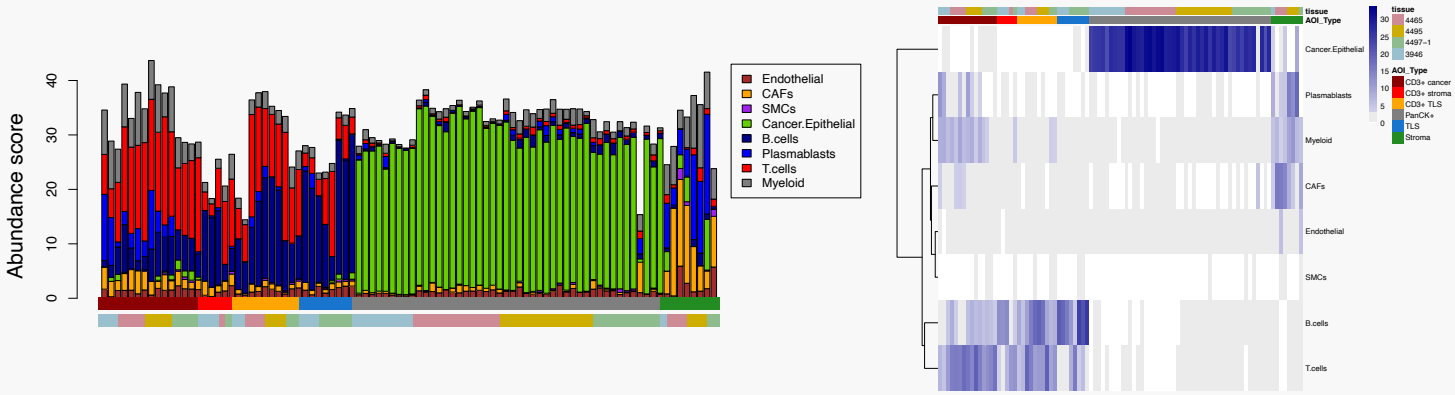
- Intratumoral heterogeneity within breast cancers may be involved with patient outcome and imply distinct mechanisms of tumor and TME evolution
- Primary, untreated, triple negative breast cancer samples were profiled using the whole transcriptome analysis and compared back to a biobank of single-cell RNA sequencing data from TNBC

Experimental Questions and Design:

- Segmentation was performed based on visual markers to characterize immune and stromal cells in the invasive edge, tumor core, and distant stromal regions.

Results:

- Identified unifying characteristics of specific cell types across tumors while detecting tumor specific expression patterns in each sample profiled
- Identified specific cell types associated with TLS structures within TNBC using cell type deconvolution analysis



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