986

A Biopharma Best Practices Framework to Enable Successful Execution of Spatial Biology Cohort Studies Using GeoMx® Digital Spatial Profiler

Esperanza Anguiano[1], Edward Bonnevie[2], Benjamin Chen[3], Sarah Church[1], Premi Haynes[3], Kelly Hunter[4], Anil Kesarwani[2], David Krull[2], Yan Liang[1], Maxine McClain[1], Corinne Ramos[5], Deniliz Rodriguez[3], Jessica Runyon[6], Julien Tessier[7], Joseph Beechem[1]

On Behalf of GeoMx DSP Biopharma and CRO Best Practices Consortium

nanoString

NanoString Technologies
530 Fairview Avenue North, Seattle, WA
98109

ABSTRACT

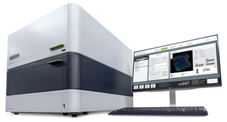
The interest and utility of high-plex spatial profiling of RNA and protein biomarkers has increased over the last few years. The implementation of high-plex analyte spatial platforms, such as GeoMx® Digital Spatial Profiler (DSP), is increasing within discovery and development of biomarkers associated with clinical outcome. The surge in spatial platforms comes with an increased adoption of digital pathology in translational and clinical research studies. The integration of these two workflows has the potential to benefit diagnostic and therapeutic development. This study aims to facilitate the implementation of DSP in tissue analysis workflows helping researchers involved in drug discovery and development efforts to (1) assess platform feasibility for their research, (2) design effective DSP experiments, and (3) enable generation of high-quality, analyzable spatial data from large cohort studies.

The GeoMx DSP Biopharma and CRO Consortium has developed consensus-based best practices incorporating expertise of members from biopharma and contract research organizations (CROs). Best practices guidelines for spatial profiling of tissue biopsies in drug discovery and development using GeoMx stands to advance current standard practices in tissue analysis. These recommendations encompass every step in the implementation of DSP for standard tissue analysis workflows, emphasizing the importance of multidisciplinary stakeholder involvement, defining experimental conditions and testing these prior to execution of large-scale studies, and considerations in assessing assay performance. This document offers a practical reference for optimal implementation of GeoMx DSP in exploratory sample analysis for use in research supporting drug discovery and development. Here we present a practical reference for the optimal implementation of GeoMx DSP in exploratory analysis for drug discovery and development studies. Best practices insights for the application of this technology to breast cancer research have been previously published (1) and should also be taken in consideration when designing relevant spatial studies.

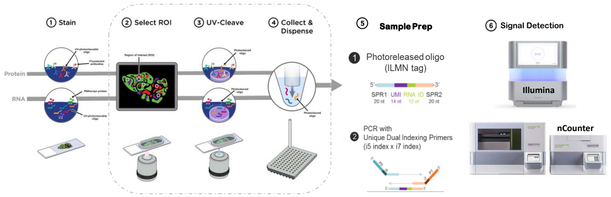
GeoMx® Digital Spatial Profiler Platform

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.

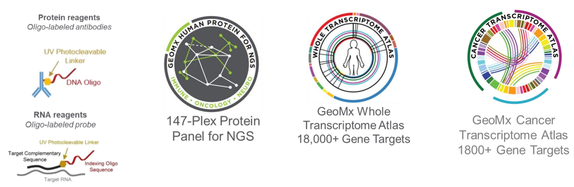
GeoMx® Digital Spatial Profiler



Sample Analysis Workflow



DSP Assays



Designing Spatial Biology Studies

Understanding the Need

Inclusion of key subject matter experts early in the DSP study planning is critical to achieving the best experimental strategy. Enable robust data analysis and interpretation by aligning perspectives, reviewing technical feasibility, and outlining project scope. Key considerations:

- Assemble Study Team: Pathologist, Research scientist, GeoMx Tech Lead, Bioinformatics, Histotechnologist
- What is the spatial scientific question and hypothesis?
- Study factors: species, disease area, tissue of origin, fixation method,
- How many subjects, conditions, time points?

Defining GeoMx DSP Experimental Plan

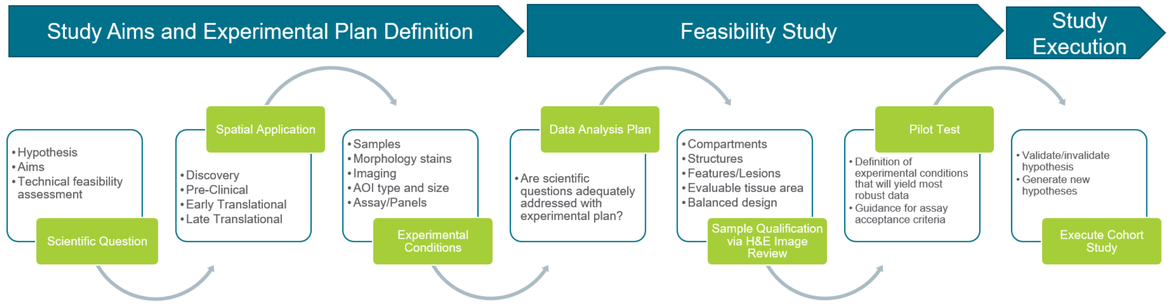
Avoid unnecessary complexity. Key questions to ask in the planning stage of study:

- Which tissue compartments/structures will be analyzed?
- What needs to be measured within compartments/structures?
- Which types of controls are available for use?
- What is being compared?
- What is the expected tissue heterogeneity across conditions?
- What is the expected intra- and inter-sample heterogeneity in study samples?
- Are H&E images available for study samples?
- How many morphology markers? Which markers/ Which channels?
- How many regions of interest for each marker? Each compartment?
- Which ROI selection strategy? Molecular segmentation? Contour? Geometric?
- What sensitivity level is required? What size ROI? What size AOI?

Consortium Members' Affiliation

[1] NanoString Technologies Inc., Seattle, WA
[2] GlaxoSmithKline, Collegeville, PA
[3] Bristol-Myers Squibb, Lawrenceville, NJ, Seattle, WA, Cambridge MA
[4] ProPath-UK Limited, Hereford, UK
[5] ImaBiotech, Loos, France
[6] Canopy Biosciences, Hayward, CA
[7] Sanofi, Cambridge, MA

Biopharma Best Practices Framework

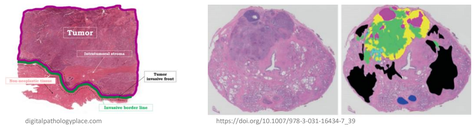


Best Practices Framework for Biopharma Spatial Research Studies defines interdependencies in the experimental workflow, giving researches awareness to support consistent design and execution of spatial biology experiments. Application of this framework will assist in yielding the most meaningful biological insight consistently. **(A).** The steps under "Study Design and Experimental Plan" help define scientific question(s) and application which are used to define experimental conditions and assay requirements .for the DSP experiment. **(B)**. Evaluation of experimental design is evaluated by developing a data analysis plan which is used to identify experimental variables and conditions that should be tested and qualified and carried through DSP cohort study. **(C)** Implementation of verified experimental conditions from pilot study help generate expectation of assay performance in cohort study.

Technical and Quality Control Considerations for DSP Experiments

Sample Selection and Qualification

Pathology review, manual or AI guided, of adjacent H&E stains is extremely valuable in determining sample adequacy for DSP and to streamline ROI selection workflow. This pre-assessment ensure tissue compartments of interest are represented in samples to be used in experiment and enables annotation of tissue architecture and features relevant to the scientific questions being addressed. This annotation can be highly useful in the execution of ROI selection at the bench during the DSP experiment.



digitalpathologyplace.com

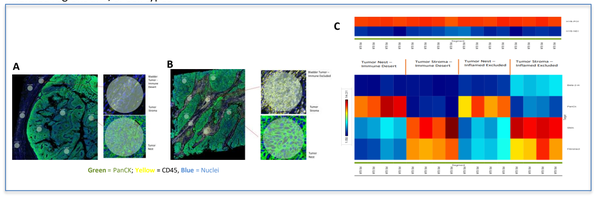
https://doi.org/10.1007/978-3-031-16434-7_39

Tissue Staining Optimization and Performance Verification

The GeoMx DSP enables users to select biologically relevant regions of interest (ROI) based on the appropriate selection and optimal staining of morphology markers for the tissue being examined. Staining patters are key in guiding ROI selection and in enabling molecular segmentation to isolate the expression patters of specific tissue compartments and cell types. As such, verification of the performance of morphology markers selected is critical to the successful design and execution of spatial biology experiments.

Fluorescent labeled antibodies or RNA *in situ* probes used to reveal tissue structures and morphology should be tested and optimized for the DSP's optical system and tissue type(s) of interest. Directly conjugated primary antibodies offer the most straightforward options for fluorescent staining. If conjugated antibodies are unavailable for a desired target, the "Morphology Marker Guidelines" (2) can be referenced for alternative, compatible methods including RNAScope™, IHC on serial section, fluorescent secondary antibodies, self-conjugated antibodies, and TSA.

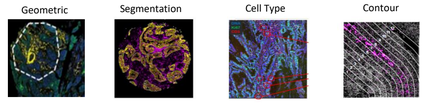
The most straight forward option for the Verification of morphology marker performance is orthogonal methods. Expression data generated from selected ROI can also be evaluated to confirm the successful staining and selection of intended regions and/or cell types.



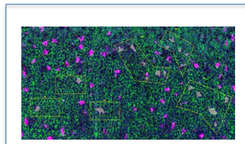
Verification of optimized staining protocol for Pan-cytokeratin, CD45, and Cyto13 markers. (**A and B**) Two bladder cancer samples of known immune phenotype (immune excluded and immune dessert) were used as controls. 4 ROIs from two differentiated tissue compartments (tumor stroma and tumor nest) were selected from each sample type and subjected to hybridization using the IO Protein Assay. (**C**) Bottom heatmap shows accurate clustering by ROI type (columns) driven by selected biological control markers (rows) and Top heatmap shows positive and negative hybridization probes, – mean expression of HK (n=3) and IgG (n=3) control probes, respectively.

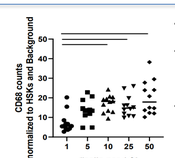
Defining ROI Size and ROI Selection Strategy

The ROI size and selection strategy is defined by the hypothesis being tested and it is possible that more than one ROI selection strategy can be applicable. The GeoMx Software offers the following options for selecting regions of interest.



Different applications and scientific questions will likely require different levels of sensitivity for detecting proteins and RNAs of interest in target tissues. NanoString has reported AOI size dependent effects on the limit of detection for the WTA assay (3). Given the expected intra- and inter tissue variability of cell density, this effect is expected, and it is recommended that the optimal ROI/AOI size is determined empirically for a given set of conditions (e.g., tissue type, morphology marker, and AOI selection strategy). This could be accomplished by sampling from different size AOIs and then evaluating assay performance by measuring signal-to-noise ratio as illustrated in the following schematic.





- Background levels have high variability in low area AOIs
- Background correction reveals AOI size-dependent background accounts for significant CD68 signal
- Based on background and housekeeper counts, an empirical threshold of 25 cells for a minimum AOI collection in the protein assay

It is important to keep ROI/AOI sizes similar across the entire study. Image analysis of ROIs using 3rd party software (e.g., Visiopharm) could help determine cell counts within ROIs. Cell count data collected over time could eventually inform optimal ROI/AOI size for similar experimental conditions. Information on Table below can be used as starting point to design lab-specific verification experiments and to help with suitability of application based on specific needs of the study.

	Small AOIs	Medium AOIs	Large AOIs
Area (circle diameter equivalent)	50µm	100-200µm	250µm
Number of cells	15	100	250
Detect medium- and high-expressing genes	√	√	√
Detect enriched pathways	√	√	√
Robust cell type deconvolution		√	√
Robust differential expression analysis		√	√
Detect low expressing genes			√

Considerations for DSP Library Pooling

NanoString recommends multiple options for library pooling. These should be carefully considered particularly when it's challenging to maintain ROI/AOI size similar across the entire experiment. Different library pooling strategies have been reported to affect end results in different tissue types (4) . It is recommended that existing best practices for NGS workflows are also implemented in this workflow.

Comprehensive Technical and Quality Parameters for DSP Assays

	Study Design	Samples	Morphology Markers	DSP Assay	ROI Selection	AOI Sampling	Signal Detection Workflow	Data Output
Technical Considerations	Hypothesis, Aims, Objectives, Variables, Study size	Biopsy technique, Sample preparation, Fixation method, Section thickness, Storage, adjacent H&E pathology review	Target marker, Marker type (RNA/ Protein/other), Antibody type, Staining technique, Detection system, Channel assignment	RNA, Protein, Proteogenomic Panel, Detection system (NGS, nCounter)	ROI selection strategy (geometric, segmentation, contour), Spatial regions, Compartments, Number of replicates	AOI size, assay specific workable input range, assay sensitivity requirement	**NGS**: Library pooling strategy, library normalization, sequencing depth **nCounter**: Sample preparation, pooling, normalization, scan settings	Immunofluorescence image, Gene expression; Protein expression
Output Quality Considerations	Platform feasibility assessment, Assay performance qualification requirements	Tissue integrity, Verification of tissue disease type, Total tissue area, Evaluable tissue area, analyte integrity	Signal-to-background noise, Sensitivity, Specificity, Orthogonal method verification	Performance of internal assay controls, Performance of assay positive (HK) and negative control probes, Orthogonal method verification, biological positive and negative controls	Marker specificity, Presence of regions across samples in study, Verify total size of evaluable tissue area supports experimental plan	Marker specificity, Image quality, Performance of internal assay controls, Signal-to-background ratio, dynamic range	**NGS**: Library quality, library pool concentration, sequencer run quality **nCounter**: Sample quality, concentration, nCounter probe binding efficiency	Marker specificity, Image quality, Performance of internal assay controls, Signal-to-background ratio, dynamic range, technical bias assessment, confirmation of "known" expression characteristics (biological controls)

Feasibility Assessment

The feasibility assessment should aim to test the set of assay parameters that have been defined by the multi-disciplinary team put together for the spatial biology study. Here it is assumed that staining and imaging conditions have been optimized. Optimized staining conditions are used to define empirically identify the ROI size that will best support the research study as well as the ROI selection strategy that would be most effective for the study. It is recommended that issue controls (e.g., tonsil) and representative samples (tissue and disease type) for the planned spatial study are used as part of feasibility experiments.

The outcome of the feasibility study is the definition of assay parameters that need to carry through execution of the intended study and/or similar type studies.

Data Analysis

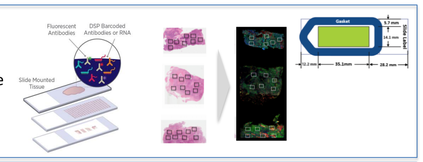
In collaboration with bioinformatician and/or statistician, defining a data analysis plan prior to execution of the DSP pilot experiment or actual spatial study can be advantageous and very valuable in informing decisions that might need to be made on the days experiment is being conducted. The analysis plan should clearly define the specific experimental variables (ROIs, AOIs, compartments, etc.) that will be compared within and across samples.

- Identify critical technical variables that need to be optimized and controlled for during execution of experiment
- In pilot study, confirm experimental plan will adequately address scientific questions
- Minimize batch effects by randomly assigning samples/variables across slides, DSP runs, instruments, sequencing, etc.
- From pilot study data, set assay performance expectations

Data quality control consists of evaluating signal-to-noise ratio based on internal assay positive (housekeeping targets) and negative control probes. The annotation of technical batch effect can be assessed in this processed if technical variables were evaluated. Identification of variables introducing bias in data can be very valuable for enabling the application of batch correction techniques. This process has been previously described for both protein (5) and RNA (6) data sets.

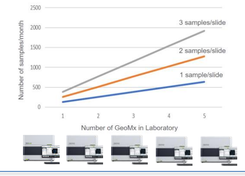
Considerations in the Analysis of Large Cohort Spatial Studies

- Number of samples/slide
- Number of ROI/sample
- Pre-annotated H&Es



- Automated workflow
- AI guided ROI selection

- Lab sample throughput
- Turn-around time
- Cost



Conclusions

- This best practices guidelines aimed to facilitate the implementation of DSP for those requiring a higher level of reliability from their spatial data, and still operate in research-use-only setting
- Best practices guidelines for spatial profiling of tissue biopsies in drug discovery and development using GeoMx stand to advance current standard practices in tissue analysis
- Emphasis of the importance of multidisciplinary team involved in defining, testing, analyzing and interpreting data from feasibility studies and during the execution of larger studies is highly recommended
- Manuscript with detailed recommendations to follow

Acknowledgments

Leslie Abad for helping form and manage Consortium meetings and agendas; and many other NanoString Team members who have indirectly contributed to this work

References

1. GeoMx Breast Cancer Consortium. Best Practices for Spatial Profiling for Breast Cancer Research with the GeoMx ® Digital Spatial Profiler. Cancers(Basel). 2021 Sep4;13(17):4456
2. Appelbe, O.K., Rhodes, M., and Furhman, K. (2021) *Morphology Marker Guidelines* {White paper}. NanoString Technologies.
3. Fropt, R., Griswold, M., Zimmerman, S, Nguyen, K, Reeves, J., Fuhrman K., and Rhodes M. (2021) *The GeoMx® Human Whole Transcritpome Atlas for the Digital Spatial Profiler: Design, Performance, and Experimental Guidelines* {White paper}. NanoString Technologies
4. Rodriguez, D., Sriganesh, J., Grinell-Vasquez, S., Haynes, P., and Stern M. Size selection optimization strategy of regions of interest and library pooling to improve sequencing performance and RNA quantification utilizing NanoString's GeoMx Digital Spatial Profiling (DSP) platform. Poster presented at: Spatial Biology for Immuno-Oncology Summit; January 2023, San Diego, CA
5. 2020. *Introduction to GeoMx® Normalization: Protein* {White paper}. NanoString Technologies
6. 2020. Introduction to GeoMx® Cancer Transcriptome Atlas: Normalization {White paper}. NanoString Technologies