GeoMx™ DSP Experimental Design Guideline

GeoMx DSP Introduction

Digital Spatial Profiling (DSP) is a new technology which enables spatial analysis of RNA and protein in tissue specimens on slides. Samples are stained with large panels of pre-mixed biological probes (each incorporating a unique, UV-cleavable DNA barcode) and fluorescently-labeled morphology reagents (used to visually or computationally elucidate the morphology of the tissue). Using the fluorescent morphology reagents for guidance, defined regions of interest (ROIs) are illuminated with UV light and the cleaved barcodes from these ROIs are then quantified. This allows for high-plex RNA or protein quantitation from spatially-resolved regions within the tissue. The resulting counts constitute an expression profile of key targets across ROIs and elucidate the biology specific to that region.

Experimental Design

For general GeoMx DSP operation instructions, see the **GeoMx DSP Online User Manual**, accessible from the help icon in the GeoMx DSP Control Center and at https://www.nanostring.com/geomx-online-user-manual. This Experimental Design document outlines considerations for custom experimental designs and important parameters to consider when setting up an experiment.

The **Scan Checklist**, available under **Support Documents** at www.nanostring.com/GeoMxDSP, is a helpful reminder of important tasks to check off before proceeding from one stage of testing to the next. *Ensuring you have selected the appropriate configuration files for your run is particularly important.*

Selecting morphology markers

Morphology markers are used to guide region of interest selection based on visual staining patterns. NanoString provides kits to profile common tissue morphology of interest. Each kit contains a nuclear stain and two fluorescently labelled antibodies against specific biological targets. This leaves an open fluorescent channel on the instrument for adding a third antibody for tissue visualization. This channel can be used for specific cell subtypes, unique architectural proteins, or other identifiers specific to your project. Alternatively, you can use just the nuclear stain from NanoString and customize the remaining three targets if the off the shelf kits do not work for your research question. Table 1 lists the GeoMx DSP channels available.

Table 1: GeoMx DSP channels

Channel	Excitation (peak/bandwidth)	Emission (peak/bandwidth)
FITC / 525 nm	480 / 28 nm	516 / 23 nm
Cy3 / 568 nm	538 / 19 nm	564 / 15 nm
Texas Red / 615 nm	588 / 19 nm	623 / 30 nm
Cy5 / 666 nm	645 / 19 nm	683 / 30 nm

Considerations for the customization of morphology markers include:

- When working with tissues known to be auto-fluorescent, consider choosing fluorophores in longer wavelengths.

- To replace the Cy5 / 666 nm channel for a full custom design, consider using DyLight™ 650 as the replacement dye. If using Dylight 650 and Alexa Fluor® 594, be aware of bleed-over.

Specific segmentation requirements

Segments are areas within an ROI which are defined by combinations of the presence and absence of specific morphology markers. For example, a *Tumor* segment can be generated by finding the areas within an ROI that are positive for a PanCK marker and a complementary *Immune Tumor Micro-Environment* segment can be defined as the area that is negative for PanCK AND positive for CD45 AND Positive for the DNA marker. Considerations when creating segment masks include:

- Segments may not overlap or extend outside their ROIs.

- Segments should be as contiguous as possible, with clean margins separating each segment from off-target tissue.

- Segments composed of numerous small particles will have reduced fidelity and higher background from adjacent tissue.

- Consider establishing the collection order of the different segments within an ROI starting with the smaller segments (based on area or number of cells) and finishing with the largest segment within that ROI.

- Refer to **GeoMx DSP Segmentation Guidelines**, available under **Support Documents** at www.nanostring.com/GeoMxDSP, for more detailed information on Advanced Parameters.

Additional considerations for performance

There are a number of different factors to consider when optimizing the GeoMx run. Considerations include:

- Consider a scanning only run (without ROI definition, UV illumination, or ROI collection) to test the performance of custom morphology markers. Strong staining is required for segmentation based on fluorescent markers. Poor fluorescent staining or low signal to noise can lead to poor or incorrect segmentation and thus to incorrect sampling.

- When choosing the exposure time, consider that the acceptable exposure time range for the GeoMx DSP instrument is 5ms - 9000ms (diminishing returns have been observed as exposure time is increased above 500ms). The exposure time selected will be applied to each field of view. Increasing the exposure time, therefore, may extend the run time, depending on the size of the tissue area defined for high-magnification scanning.

ROI selection strategy

ROI selection has the greatest impact on the final results and should be driven by the research questions, experimental design, and tissue type and quality. Tissue artifacts such as wrinkles and tears can give erroneous results. As with all IHC methods, edges of the tissue sometimes show excessive staining or

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labeling. Be aware of this type of artifact while selecting ROIs. Selecting ROIs based on features of interest can lead to a biased sampling of the tissue. Take the following considerations into account:

- Run appropriate controls (tissues or regions as positive/negative controls).

- When comparing between samples or sample groups, take representative tissue regions between the samples or sample groups.

- If using segmentation within ROIs, try to segment as simply as possible. Too many segmentation rules can reduce the effectiveness of automated segmentation. Always have the research questions in mind when making segmentation decisions.

ROI selection size constraints and considerations

ROI size determines total counts collected. Very small ROIs will have fewer counts, making rare markers harder to detect. The minimum ROI size tested at NanoString is a 32µm diameter circle with 93% of the Human IO and 81% of the Mouse IO panels being detected above background. If selecting ROIs smaller than a 32µm circle, consider that a reduction in sensitivity in some antibodies is possible. To reach limit of detection in Protein analysis, NanoString, in general, recommends capturing at least 20 cells in an ROI or segment, and for RNA analysis it's recommended to capture at least 200 cells. Larger ROI selections are encouraged for RNA analysis, since RNA targets are not as abundant as protein targets and there is no risk of saturating the cartridge.

For protein analysis, large ROIs can cover more area, but risk saturating the cartridge (see hybridization saturation guidance in the **GeoMx nCounter Readout User Manual**; Table 2 is an excerpt from this manual). Data normalization can attempt to correct for large differences in ROI size, however larger normalization factors can amplify noise resulting in skewed data and artificial expression differences. The same issue arises when creating ROIs of similar sizes, but with automatic segmentation resulting in highly variable segment sizes within the ROIs (e.g., when doing rare cell profiling or when looking at invasive tumor margins and subsequent segmenting for tumor and tumor microenvironment). Studies and samples built with ROIs (and segments) of a common size will yield the highest data quality.

For protein analysis only, the ideal size for geometric ROI circle diameter is 200 µm (the overall recommended range is 50 – 400 µm). Since automatic segmentation divides ROIs into multiple segments, ideal auto-segmentation ROI circle diameter is 300 µm (the overall recommended range is 100 – 500 µm). Once the plate is finalized in the DSP instrument, total segment area for entire columns will be listed in downloadable lab worksheet. Users will need to consult Table 2 to determine the appropriate amount of hyb product to pool for each column. This value may be different for each column.

Table 2: Hyb Saturation Table

Total segment area for entire column (µm²)	MAX/FLEX volume to pool	SPRINT volume to pool
≤47,000	15.0 µL	15.0 µL†
≤63,000	13.5 µL	9.0 µL†
≤140,000	6.8 µL	4.5 µL†
≤280,000	3.0 µL	2.0 µL
≤420,000	2.0 µL	1.3 µL*
≤560,000	1.5 µL	1.0 µL*
≤770,000	1.2 µL*	0.8 µL*
≤1,540,000	0.6 µL*	0.4 µL*
≤2,310,000	0.4 µL*	0.3 µL*
≤3,100,000	0.3 µL*	0.2 µL*

* Consider using serial dilutions in TE-Tween (10 mM Tris pH 8, 1 mM EDTA, 0.1% Tween-20) or similar for smaller volumes.

† Since the maximum loading volume for a SPRINT cartridge well is 30 µL, users may need to adjust any pools greater than 30 µLs.

Note, however, that certain tissue types may have extremely high levels of particular proteins (e.g., SMA (smooth muscle actin) in muscle tissue or HER2 in HER2+ breast cancer tissue), which may lead to saturation even after following the guideline table. If your intended tissue experiment falls into this category, please contact geomxsupport@nanostring.com to discuss attenuation strategies.

Two factors contribute to determining amount of hyb product to pool: 1. ROI sizes and 2. Number of rows to collapse in a plate. The impact of these factors is visualized in Figure 1 below. A third factor to consider (not depicted in Figure 1) is the percentage of the ROI area represented by each segment.
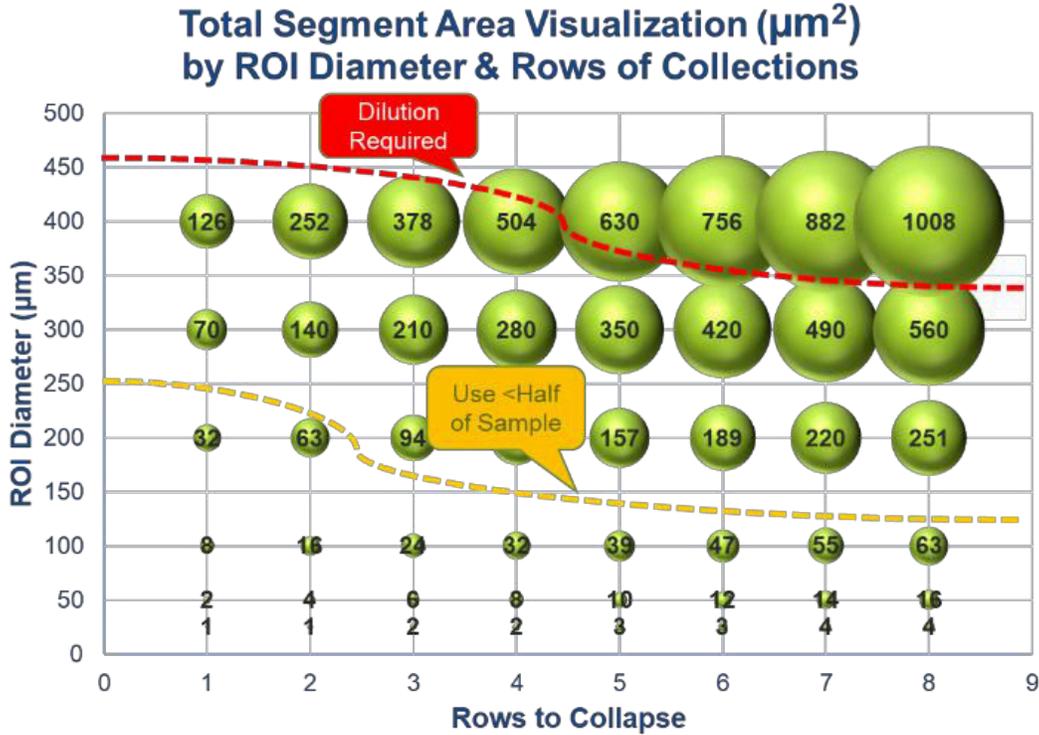
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Figure 1: Total segment area per column visualization. The samples which required dilution above red line were indicated with an (*) in the protein Hyb volume pooling guidance table.

Users can consult the ROI menu in the GeoMx Scan Workspace to check the sizes of each of the ROIs they have established.

As a validation of ROI size selection strategy, it's good practice to qualify the normalization factors (e.g. nuclei or area normalization factors) in the summary tab of the data analysis visualization panel. If there are outliers, there is a risk of noise amplification and skewed data resulting in incorrect results. The Data Analysis Suite allows you to tag outliers to further examine or to exclude from analysis.

Analysis considerations

- Consider running enough replicates within each sample or slide (on the ROI and segment level) and running biological replicates within each sample group (i.e. more patients of the same experimental condition). For clinical samples, NanoString has good experience with running at least 6-12 ROIs (or segments) per sample with at least 10 samples per experimental group (for clinical patient samples, more replicates might be necessary to get enough statistical power). Performing a statistical power analysis is recommended.

- Consider how consistent your ROI sizes are when normalizing. Compare ROIs and/or segments that are alike (see above).