



WHITEPAPER

# Morphology Marker Guidelines

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# Morphology Marker Guidelines

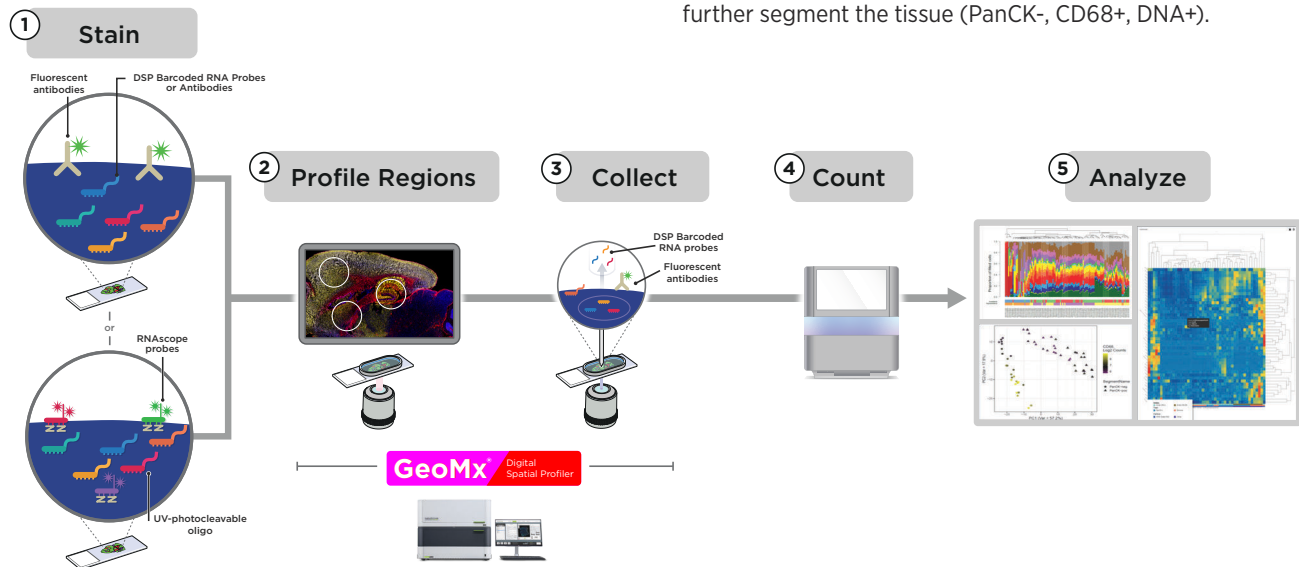
## Morphology Marker Guidelines for Spatial Profiling

The GeoMx DSP builds upon the importance of histology by allowing users to select biologically relevant regions of interest (ROI) based on the appropriate morphology markers for the tissue. The staining patterns not only guide ROI selection but also enable segmentation to isolate the expression patterns of specific compartments and cell types within a tissue (Figure 1).

GeoMx DSP provides the flexibility to use fluorescently labeled antibodies or RNA *in situ* probes to reveal tissue structure and morphology relevant to the research. The selected regions are then profiled for proteins or transcripts with DSP barcoded reagent. Here, we introduce a variety of methods to choose morphological markers and a detailed description of how to test and optimize the performance of those markers for the DSP's optical system.

## GeoMx® Digital Spatial Profiler (DSP)

The GeoMx DSP workflow integrates with current histology methods to quickly deliver robust and reproducible spatial omics data. Starting with FPPE or fresh frozen tissue, morphology markers are used to stain for protein or RNA targets to visualize tissue compartments or cell types. This allows the DSP to precisely select regions to profile based on the biology. The biological questions ultimately help to define the best profiling selection strategy. For example, a common question for which spatial profiling on GeoMx DSP is deployed is the identification of changes in the tumor and tumor microenvironment in relation to therapeutic response. To answer this question, tumor regions (PanCk) that are either enriched or depleted for immune cells (CD45) are selected and then segmented into tumor (PanCk+) and tumor microenvironment (PanCk-, DNA+) for molecular profiling across different clinical response groups. Where there are further hypotheses about specific cell types that may influence response, other markers, such as CD68 for macrophages, can be added to further segment the tissue (PanCk-, CD68+, DNA+).



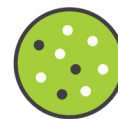
Profiling strategies aid in answering those questions:



**Geometric Profiling:** Assess tissue heterogeneity profiling standardized geometric shapes across distinct tissue regions



**Gridded Profiling:** Perform deep spatial mapping using a tunable gridding pattern that samples a large area



**Cell-type Profiling:** Reveal the function of distinct cell populations with cell type specific morphology markers guiding region selection and compartmentalization



**Segment Profiling:** Enrich for anatomical features using morphology markers to identify and profile distinct biological compartments within a region

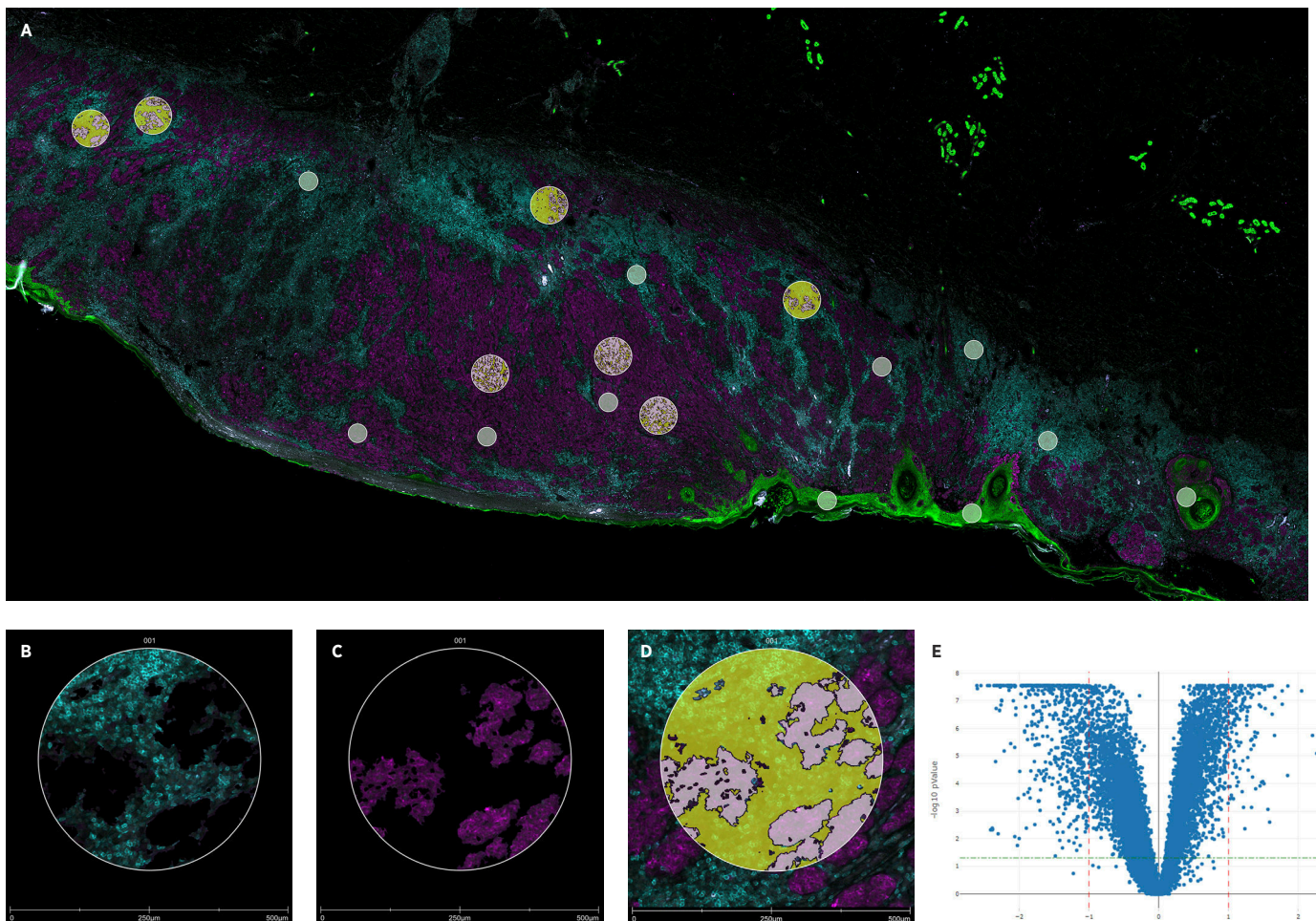


**Contour Profiling:** Evaluate how proximity affects biological response and the local microenvironment around a central structure using radiating regions

## Choosing morphology markers for GeoMx DSP

By understanding tissue morphology and key histological structures, powerful transcriptome information can be discovered with spatial biology. The underlying genetic code drives complex transcriptional and proteomic pathways that create organized tissue structure. In contrast, disease pathology often leads to a breakdown in these organized structures. By identifying regions of interest with morphology markers, the user is able to separate regions and compartments into meaningful biological groupings and analyze protein and RNA profiles.

There are multiple avenues to successful visualization of tissue morphology in a DSP experiment. Each tissue and GeoMx assay have different considerations when validating morphology markers for use in an experiment. Directly conjugated primary antibodies offer the most straightforward options for fluorescent staining. If conjugated antibodies are unavailable for a desired target, this document presents several alternative methods compatible with GeoMx staining. Note, the protocol for RNA profiling includes a Proteinase K treatment which can reduce the presence of specific epitopes for some antibody clones. NanoString recommends testing any non-GeoMx validated morphology markers on a test section and optimizing as needed.



**FIGURE 1:** DSP autosegmentation analysis of melanoma tissue based on MelanA (cyan) and CD45 (magenta) morphology marker staining. PanCk+ epithelial (green) A. Whole slide scan of melanoma tissue; B. Melanoma compartment identified as MelanA cells (cyan); C. Immune cells identified as MelanA- & CD45+ cells (magenta); D. Zoomed in ROI showing auto-segmented areas from B and C; E. Volcano plot showing differential expression of the Human Whole Transcriptome Atlas between melanoma and immune regions of interest.

There are multiple avenues to successful visualization of tissue morphology in a DSP experiment. Each tissue and GeoMx assay have different considerations when validating morphology markers for use in an experiment. While directly conjugated antibodies are the most straightforward, we have listed various options below.

Primary antibody options	Advantages	Considerations
<b>NanoString Morphology Markers</b> - These NanoString-validated morphology markers are immunofluorescent-conjugated antibodies bundled with SYTO13 nucleic acid stain, and will generally work with most tissues.	Validated for a diverse number of tissues and GeoMx assays	Morphology marker kits focused on general lineage markers such as CD45 for immune cells or PanCK for epithelial tissue
<b>NanoString Morphology Marker Reference List</b> - NanoString's Technology Access Program (TAP) has compiled a list of markers that they have had success with under specific conditions ( <a href="#">TAP list</a> ).	Markers used for pilot projects and observed to have worked on specific tissues	Have been used successfully before in the specific GeoMx assay and tissue
<b>Community Verified Morphology Markers</b> - Our customers have shared some of their custom morphology marker success stories with us to aid the greater NanoString user community.	Pre-screened markers observed to have worked on specific tissues	Have been used successfully before in the specific GeoMx assay and tissue
<b>Third Party Morphology Marker(s)</b> - If none of the options above work for your particular experiment, you have the option to choose your own morphology markers. See the "Selecting the Appropriate Antibody" section below.	Options only limited by what antibodies are available	May require screening multiple antibody clones to find one that works best for your tissue and assay of interest. Fluorescent conjugation may be required.

Alternative Options	Advantages	Considerations
<b>RNAscope</b> - NanoString has partnered with Advanced Cell Diagnostics to integrate up to three RNAscope probes into GeoMx RNA assays as morphology markers along with the nuclear dye SYTO13	Wide selection of RNA targets already available or the ability to build custom probes toward unique targets such key gene transcripts, TCR receptors or specific transgenes.	RNA staining often results in a punctate staining pattern which typically does not allow for auto-segmentation within the DSP software but does allow geometric profiling. Not compatible with GeoMx Protein assays.
<b>Staining of serial sections</b> - The image overlay tool in the DSP control center software allows for the use of serial section images in placing ROIs.	Can utilize any colorimetric slide stain to aid in ROI placement as well as image on a different microscope.	Used to select regions of interest (geometric profiling) within the tissue but not for auto-segmentation
<b>Fluorescent secondary antibodies</b> - The use of fluorescently labeled secondary to amplify the signal in your tissue of interest.	Useful for low expressing targets or antigens sensitive to proteinase K	Important to titrate antibodies to avoid high background signal and wash extensively to remove unbound antibodies.
<b>Self-Conjugated Antibodies</b> - Numerous kits are available to add a fluorochrome to the desired antibody	This approach utilizes antibody that may already be characterized in another application but lacks a commercial version with the needed fluorescent dye	Conjugation chemistries may inadvertently inactivate the epitope binding sites. It's important to verify the same staining pattern before and after conjugation.
<b>Tyramide Signal Amplification</b> - TSA can be used to amplify the signal of low abundance antibodies.	Increased signal-to-noise ratio can rescue staining of low abundance targets	Decrease in RNA detection in GeoMx RNA assays

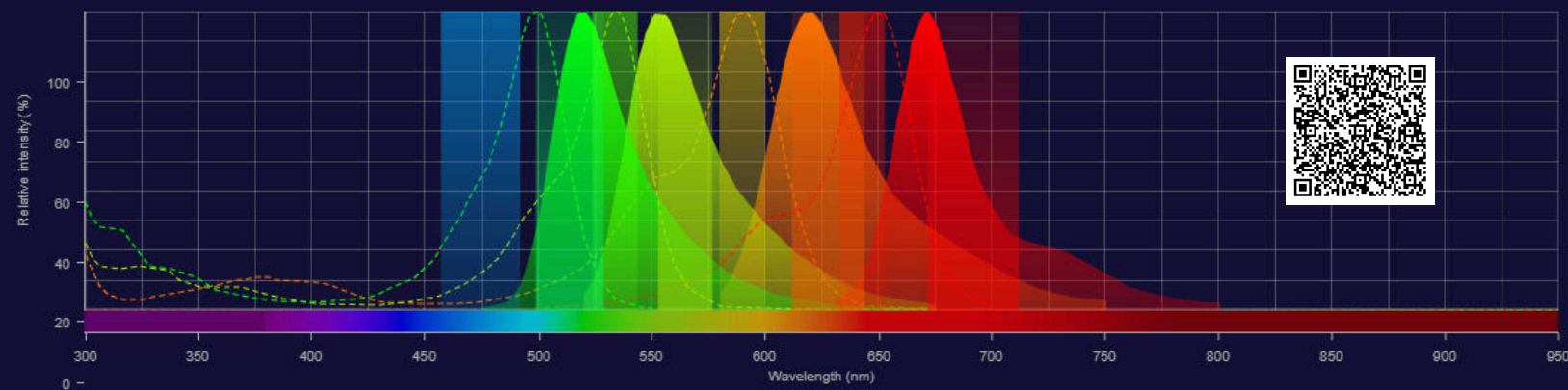
## DSP Optical System

GeoMx DSP's fluorescent imaging system utilizes LEDs with filter sets centered on standard FITC, Cy3, Texas Red, and Cy5 channels. These are optimal for using the fluorophores Alexa Fluor 488, 532, 594 and 647, respectively. The use of these fluorophores allows four channel fluorescent imaging with minimal overlap or crosstalk between channels. Alternative fluorophores can be used; however, compatibility of excitation and emission spectra should be confirmed before use in a GeoMx study.

NanoString Morphology Kits provide the DNA dye (SYTO 13) which is detected with the FITC/525 nm channel. An alternate SYTO DNA dye can be utilized to open that channel for other markers. Because excitation with UV cleaves the DSP barcode from their probe, DAPI staining is not compatible with DSP. For critical, low abundance target we recommend using the Cy5/666 nm channel for the best immunofluorescent signal-to-noise.

DSP channel / Em max	Excitation range	Emission range	Emission range
FITC / 525 nm	480 +/- 28 nm	516 +/- 23 nm	AF488*, FITC, DyLight488, Fluorescein, 5-FAM, Rhodamine Green
Cy3 / 568 nm	538 +/-19 nm	564 +/-15 nm	AF532*, Cy3, PE, TRITC
Texas Red / 615 nm	588 +/- 19 nm	623 +/- 30 nm	AF594*, Texas Red, DyLight594
Cy5 / 666 nm	645 +/- 19 nm	683 +/- 30 nm	AF647*, DyLight650, Cy5, AF660w

\* recommended first choice



**FIGURE 2:** Fluorescence SpectraViewer (ThermoFisher) displaying DSP's excitation and emission filters as well as excitation (dotted lines) and emission (solid, filled in graphs) for the four recommended fluorophores. AF488 = green, AF532 = yellow, AF594 = orange, AF647 = red.

*Note:* Taking advantage of a spectra viewer such as those offered by [ThermoFisher](#) (Figure 2) or [Novus](#) will help prevent unwanted overlap between imaging channels when determining which fluorophores are best for your experimental setup.

## Selecting the Appropriate Antibody

For ROI strategies built around segmentation, abundantly expressed targets attain a strong signal that is straightforward to segment thereby allowing multiple biological compartments to be profiled. For target staining resulting with a low fluorescent signal-to-noise, considering geometric regions such as circles, squares, rectangles, or polygons will better suit the needs of the study. If segmentation on weakly-staining targets is required, first optimizing the staining and auto-segmentation settings in the absence of GeoMx profiling reagents is encouraged. Enhancing the staining using amplification methods, such as secondary antibody or TSA, can be evaluated to amplify the signal of low expressing targets.

When choosing an antibody for your study, it is recommended to begin with those already validated for IF/IHC assays by the antibody provider. Once selected, it is important that they are tested using the specific staining protocols used for GeoMx. If the antibody of choice is not available already labeled with the preferred fluorophore, there are kits available for fluorescently labeling antibodies through direct conjugation or Zenon™ immunolabeling technology. Finally, monoclonal or polyclonal antibodies are both compatible with GeoMx assays. While NanoString typically recommends monoclonal antibodies for morphology markers, polyclonals have been shown to work.

	Monoclonal	Polyclonal
Pros	Designed against a single epitope and less likely to cross-react.	Often stronger signal by binding to multiple epitopes and more tolerant to tissue processing.
Cons	Tissue processing may lead to epitope loss.	More variation between antibody lots.

## Alternatives to directly conjugated antibodies

While directly conjugated primary antibodies are the most straightforward to integrate into GeoMx assays, other options exist that exhibit strong and specific signal to the target of interest.

The **image overlay tool** in the DSP control center software enables for the use of serial sections prepared using immunofluorescence, colorimetric IHC or ISH techniques (chromogenic DAB, digoxigenin, H&E, etc.) to be used for placement of geometric ROIs (polygons, circles, rectangles). There will undoubtedly be subtle differences between serial sections, so mapping ROI from one section to another will need a modest amount of adjustment.

**Fluorescent secondary antibodies** can be used for indirect immunofluorescence when a directly conjugated antibody is unavailable or signal amplification is needed. Considerations for this approach are below:

- Do not use anti-mouse secondary antibodies on mouse tissues
- Tissues with endogenous immunoglobulins can exhibit high background
- Include “secondary antibody only” control slide during fluorescence optimization to test for non-specific background signal from secondary antibody in your tissue of interest

**Tyramide signal amplification (TSA)** can be used to amplify the signal of low abundance antibodies (refer to the GeoMx DSP-NGS Slide Preparation user manual, Appendix VI for detailed workflow instructions). TSA typically delivers a higher signal-to-noise ratio than a directly conjugated antibody. Importantly, this TSA protocol moves proteinase K digestion to after immunolabeling, allowing for the use of antibodies that recognize proteinase K susceptible epitopes. Therefore, immunofluorescence with tyramide signal amplification may be preferable when the epitope is protease sensitive or when the use of directly conjugated fluorescent antibody results in a low signal. Considerations with this approach include:

- The addition of 4 hours to the GeoMx DSP workflow on day 1, but decreased time on day 2 prior to loading of the slides on the DSP instrument

- The TSA signal in the Cy3 channel bleeds into Texas Red channel; NanoString recommends using the Cy3 and Cy5 channels (not Texas Red) with this approach. If only one channel is needed, Cy5 is recommended. The Texas Red channel may still be utilized with a non-TSA amplified, directly conjugated antibody
- The optimization of amplification to prevent overexposure and artifacts

**RNAscope *in situ*** hybridization protocol has been validated to work with the GeoMx DSP RNA workflow (see GeoMx DSP slide prep manual, Appendix IV for detailed workflow instructions). One major advantage of using RNAscope probes is that a wide selection of available RNA targets can be used as visual markers (refer to the ACD catalog). Considerations with this approach include:

- For FFPE tissues, three RNAscope probes plus DNA stain can be used together on a tissue section.
- For frozen tissues, we recommend omitting a probe from the Cy3 channel as we have observed bleed over in this channel when attempting to use three RNAscope probes plus SYTO13 as the DNA stain.
- RNAscope signal will be visible as punctate dots, so built-in segmentation tools will only work for very highly expressed RNA targets. Use of polygon and geometric ROIs to select or avoid certain tissue areas is recommended.
- RNAscope uses TSA fluorophores that may need to be optimized to find the appropriate working dilutions to balance signal amplification and background noise.
- RNAscope can be combined with antibody based morphology markers utilizing the Dual ISH IHC protocol. Antibody epitopes are still sensitive to the proteinase K digestion that is part of the GeoMx DSP RNA workflow.

## Optimizing morphology marker in tissue

### Before you begin

Understanding the structure of the tissue at hand is a critical step in designing a successful spatial transcriptomics experiment. The expected staining pattern of your tissue of interest can offer insight into planning the appropriate ROI strategy. Consult the Human Protein Atlas, Allen Brain Atlases for both human and mouse, or other online databases or publications for comparison. Since diseases often alter the morphology of tissue, H&E or IF stains of a serial section can guide experimental design.

### Reagents needed in addition to GeoMx DSP Slide Preparation reagents (consult the GeoMx Slide Preparation user manual):

- Antibody to test. It can be useful to test multiple clones at the same time.
- Slides from your tissue of interest, at least one tissue section for each concentration to test.
- Slides from tissue(s) known to be negative for the antigen. At least one tissue section should be used to test for non-specific background using the optimized antibody concentration.

### Experimental Plan

NanoString recommends trying a range of dilutions around the supplier's recommendation on the control slides.(e.g. 0.5x, 1x and 2x the recommend dilution). If there is no recommendation from the supplier, typical working concentrations for immunofluorescent antibodies range from 0.5 - 20 µg/mL. Therefore, testing 1 µg/mL, 5 µg/mL, and 10 µg/mL will work as an initial screen. Assess the signal-to-noise by comparing regions of positive signal to regions in the tissue with negative signal. Avoid using glass as a measure of the background. Examine lower concentrations if you observe high background/non-specific staining or higher concentrations if you do not see strong, specific signal where you expect it in your tissue. Compare the observed staining pattern to previous work or online references.

At the optimized concentration that gives strong, specific signal in the tissue, try this same concentration on a known negative control tissue to check for non-specific staining. While this last step is optional, it will considerably increase the confidence of the staining pattern.

### Protocol Modifications for Antibody Optimization

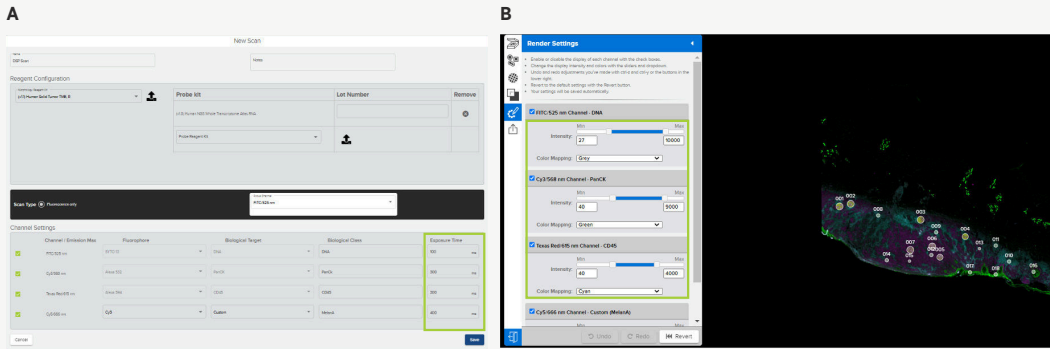
#### Dry Run Slide Preparation

**GeoMx Protein** - Follow the GeoMx Slide Preparation user manual for the protein assay with one notable exception. When preparing the overnight primary antibody incubation, instead of the core and module antibodies, add the same volume of buffer W.

**RNA** - Follow wthe GeoMx Slide Preparation user manual with one notable exception. When preparing the overnight *in situ* hybridization, instead of the RNA probe mix, add the same volume of 1X TE, pH 8.0.

#### Slide imaging using the GeoMx DSP Instrument

Either the DSP or another fluorescence microscope can be used to assess the staining pattern for the various slides. When assessing the bleed over, NanoString recommends imaging the slide directly



**FIGURE 3:** Set values in the DSP workflow to adjust image quality. A) adjust exposure time in the DSP Scan Setup to optimize single-to-noise B) Adjust render settings in the DSP Scan workspace for visual assessment of the tissue.

on the DSP. When evaluating the morphology marker staining, follow the same GeoMx instrument run process as in the user manual up to and including the slide scan. When setting the scan parameters (Figure 3A), rather than selecting a Morphology Reagent Kit, manually select the channels that you wish to evaluate and set the exposure time to 400 ms. If in the resulting scan images there is non-specific tissue background visible but there is no specific signal observed, increasing the exposure >400 ms will likely not help. If there is specific signal and some non-specific background visible, lowering the exposure time in 50 second intervals will help narrow in on the optimal exposure time.

Following completion of the scan, adjustments can be made to the render settings (Figure 3B). Adjusting the minimum threshold for a channel will decrease possible background by increasing the threshold or increase visible fluorescence by decreasing the threshold whereas adjusting the maximum threshold for a channel will decrease (greater value) or increase (lesser value) the overall fluorescence intensity that is visible in your scan.

Importantly, when comparing fluorescence across multiple scan images, care should be taken to maintain the same render settings in your channel of interest.

As stated, a fluorescence microscope can be used to evaluate the morphology marker staining. To avoid unintentional bias, evaluate all slides under as many of the same conditions (magnification, illumination intensity, exposure time, etc.). A final check should be done on the DSP.

### Troubleshooting

If increasing the antibody concentration up to 20 µg /mL does not

result in the expected specific signal, a different antibody clone may yield better results.

Various steps in the DSP protocol can be altered to optimize morphology marker staining. Changes far outside of the ranges described below or in the manual can lead to damaged tissue or decreased expression counts. Antigen retrieval and proteinase K digestion must be balanced against tissue integrity for the RNA assay. Reducing proteinase K concentration to 0.1 ug/mL can leave more of the epitope available for morphology marker staining. Alternatively, the incubation time can be decreased from 15 minutes to 5 or 10 minutes. If altering proteinase K digestion doesn't work then changes to the intensity of antigen retrieval by altering the incubation time may resolve this issue.

### Conclusion

Understanding tissue morphology is the first step in a well-designed spatial experiment. Much of the current understanding of disease derives from H&E and immunofluorescent stains. Morphology markers reveal the structural architecture of tissue and identify regions for further profiling. There are numerous morphology markers available from NanoString or third-party vendors. Many of these can be integrated into the GeoMx workflow and be utilized for tissue profiling. If you have any concerns about a particular marker or workflow, [GeoMxSupport@nanosttring.com](mailto:GeoMxSupport@nanosttring.com) can offer guidance based on the needs of your research. Developing the profiling strategy in tandem with the relevant morphology markers will maximize the learnings from the study.

For more information, please visit [nanosttring.com](https://nanosttring.com)

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