



WHITEPAPER

# Morphology Marker Guidelines

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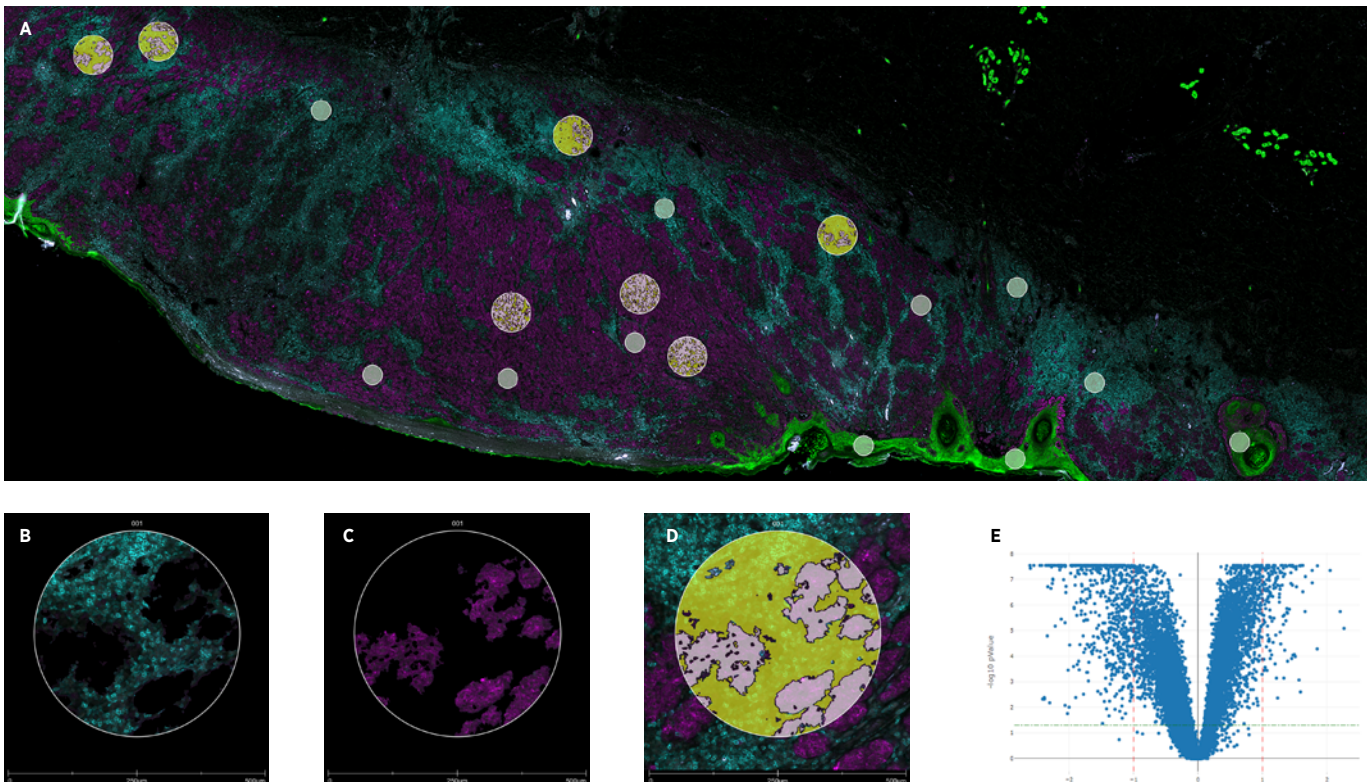
# Morphology Marker Guidelines for Spatial Profiling

GeoMx® Digital Spatial Profiling (DSP) enables researchers to answer questions about gene and protein expression in specific cell types and biological compartments within a tissue. The power of the technology lies in its targeted profiling: first, cell types and compartments are identified with fluorescently labeled morphology markers. Then, profiling reagents query the distinct populations to determine their expression patterns. Therefore, morphology markers are key to separating samples into meaningful biological groupings to analyze protein and RNA profiles (Figure 1). Here, we introduce methods to choose morphology markers and a detailed description of how to test and optimize the performance of those markers for your research.

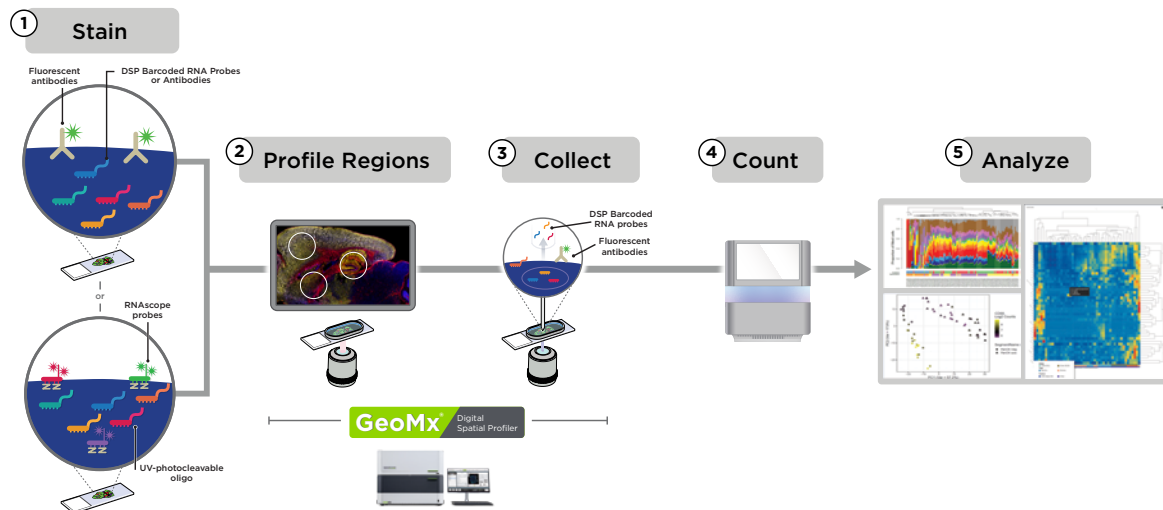
The GeoMx DSP workflow integrates with standard histology methods to quickly deliver robust and reproducible spatial omics data (Figure 2). Starting with FFPE, fresh frozen, or fixed frozen tissue, researchers use morphology markers to reveal tissue structure relevant to the research questions.

The tissue morphology guides region of interest (ROI) selection and, if desired, ROI segmentation into cell-type compartments.

Many profiling strategies are possible, with the optimal strategy determined by the research questions at hand (Figure 3). For example, a common question for which GeoMx DSP is deployed is to ascertain 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+ or 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+). Each of these individual segments are then profiled with detection probes separately.



**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.



**FIGURE 2:** GeoMx DSP Workflow



**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

**FIGURE 3:** Profiling Strategies for GeoMx DSP

**DSP Optical System**

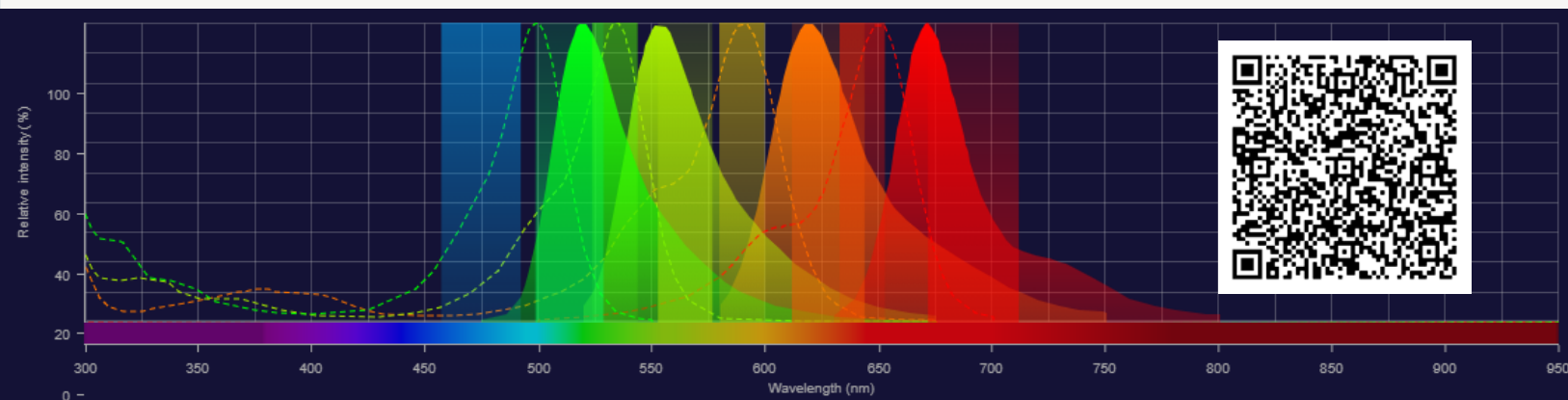
GeoMx DSP's fluorescence imaging system utilizes LEDs with filter sets centered on standard FITC, Cy3, Texas Red, and Cy5 channels (Table 1). These are optimal for the fluorophores Alexa Fluor 488, 532, 594 and 647, respectively. The use of these fluorophores allows four-channel fluorescence imaging with minimal overlap or bleed-through between channels. Alternative fluorophores can be used; however, compatibility of excitation and emission spectra should be confirmed by the user before use in a GeoMx study.

NanoString Morphology Marker Kits provide a DNA dye (SYTO 13) that is detected in the FITC/525 nm channel. An alternate SYTO DNA dye can be used to open that channel for other markers if needed. UV excitation cleaves the DSP barcodes from their probes, therefore, DAPI staining is not compatible with DSP. For critical, low abundance targets we recommend using the Cy5/666 nm channel for the best immunofluorescent signal-to-noise ratio.

**TABLE 1:** GeoMx DSP imaging channels

DSP channel / Em max	Excitation range	Emission range	Recommended fluorophores
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 4:** 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.

## Choosing Morphology Markers for GeoMx DSP

There are many avenues to successful visualization of tissue morphology in a GeoMx DSP experiment. Fluorescently conjugated primary antibodies offer the most straightforward option for staining (Table 2). NanoString has screened numerous commercially available, fluorescently conjugated antibodies for popular targets (list available [here](#)). If conjugated antibodies are unavailable for a desired target, users can turn to several alternative methods compatible with GeoMx staining (Table 3). Each tissue and assay have different considerations for morphology markers – for example, RNA sample preparation includes a Proteinase K treatment that can reduce the presence of specific epitopes for some antibody clones.

NanoString has performed extensive testing to ensure that our validated morphology markers work for a variety of tissues right out-of-the-box. Nevertheless, some testing and optimization may be needed for your specific tissue or sample type. For any non-GeoMx validated morphology markers, test the markers and optimize the tissue staining protocol as needed. GeoMx DSP software facilitates morphology marker testing with “Scan Only mode”, allowing scanning and ROI selection to be separated from sample collection.

**TABLE 2:** Directly-conjugated, primary antibody options as morphology markers.

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 R&D and Technology Access Program (TAP) teams have compiled a list of markers that they have had success with under specific conditions (available <a href="#">here</a> ).	Markers used for pilot projects and observed to have worked on specific tissues.	Demonstrated success in the specific GeoMx assay(s) and tissue(s) referenced.
<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.	Demonstrated success in the specific GeoMx assay(s) and tissue(s) referenced.
<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.

**TABLE 3:** Alternatives to directly-conjugated primary antibodies as morphology markers.

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 as 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.

## Selecting the Appropriate Antibody

For ROI strategies built around segmentation, abundantly expressed targets provide a strong signal that is straightforward to segment, thereby allowing multiple biological compartments to be profiled. For target staining resulting in a low fluorescent signal-to-noise, 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, users are encouraged to first optimize the staining and auto-segmentation settings in the absence of GeoMx profiling reagents (a “dry run”). GeoMx DSP software facilitates this testing with “Scan Only mode” for evaluation of morphology marker staining without collection from GeoMx DSP samples. Enhancing the staining using amplification methods, such as a secondary antibody or tyramide signal amplification (TSA) can 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. Next, test the antibody using the specific staining protocols provided for the GeoMx workflow of interest. If the antibody of choice is not available pre-labeled with the preferred fluorophore, another option is to use a kit for fluorescently labeling antibodies through direct conjugation or Zenon™ immunolabeling technology. Finally, both monoclonal or polyclonal antibodies are compatible with GeoMx assays. While NanoString typically recommends monoclonal antibodies for morphology markers, polyclonal antibodies have been demonstrated to work. See Table 4 for considerations.

**TABLE 4:** Monoclonal vs. Polyclonal antibodies as morphology markers.

	Monoclonal	Polyclonal
<b>Pros</b>	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.
<b>Cons</b>	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 also exhibit strong and specific signal to the target of interest. Refer to the Slide Preparation User Manuals’ appendices for more information and protocols for the techniques described here.

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 include:

- 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.
- This is only an option for the GeoMx RNA workflow.

Tyramide signal amplification (TSA) can be used to amplify the signal of low abundance antibodies. TSA typically delivers a higher signal-to-noise ratio than a directly-conjugated antibody. To be compatible with the GeoMx workflow, proteinase K digestion takes place after immunolabeling, allowing labeling antibodies that recognize proteinase K susceptible epitopes. Therefore, immunofluorescence with TSA may be preferable when the epitope is protease sensitive or when the use of directly-conjugated fluorescent antibody results in a low signal. Considerations for this approach include:

- The addition of 4 hours to the GeoMx DSP workflow on day 1, with decreased time on day 2 prior to loading of the slides on the DSP instrument.
- NanoString recommends using the Cy3 and Cy5 channels for TSA. 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 is often required.

The RNAscope in situ hybridization protocol has been validated to work with the GeoMx DSP RNA workflow. 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 for 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 through into this channel when attempting to use three RNAscope probes plus SYTO 13 as the DNA stain.
- RNAscope signal appears as punctate dots, so built-in GeoMx DSP 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.

If it is not feasible to stain the same tissue section that is subjected to GeoMx DSP probes, serial sections can be stained and used in combination with the image overlay tool in the GeoMx DSP software. A tissue section labeled using immunofluorescence, colorimetric IHC or ISH techniques (chromogenic DAB, digoxigenin, H&E, etc.) can be scanned, image saved, and subsequently overlaid on a tissue section slated for DSP collection. While the overlaid image will help guide ROI selection, the DSP will not recognize the staining so it cannot be used in an on-instrument segmentation. There will naturally be subtle differences between serial sections, so selecting ROI for one section based on another will require a modest amount of adjustment.

## Optimizing Morphology Marker in Tissue

### Before you begin

Understanding the structure of the tissue at hand is key to designing a successful spatial biology experiment. Consult the Human Protein Atlas, Allen Brain Atlases for human and mouse, or other online databases or publications to research appropriate morphology markers.

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

- Antibody to test. It may be efficient to test multiple clones at the same time.
- Sections from your tissue of interest: at least one tissue section for each antibody concentration to test.
- Sections from tissue(s) known to be negative for the antigen.
- Once the optimized antibody concentration is determined, dedicate at least one tissue section to test for non-specific background.

### Experimental Plan

If you have an antibody that works well for other staining protocols with your samples, begin your GeoMx DSP optimization using your usual antibody concentration for the protein assay, and double the concentration for the RNA assay. Otherwise, NanoString recommends testing a range of antibody concentrations centered around the supplier's recommendation (e.g. 0.5x, 1x and 2x the recommend dilution). If there is no recommendation from the supplier, try typical working concentrations for immunofluorescent antibodies (0.5 – 20 µg/mL; so for example 1 µg/mL, 5 µg/mL, and 10 µg/mL in an initial screen). Assess the signal-to-noise ratio 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.

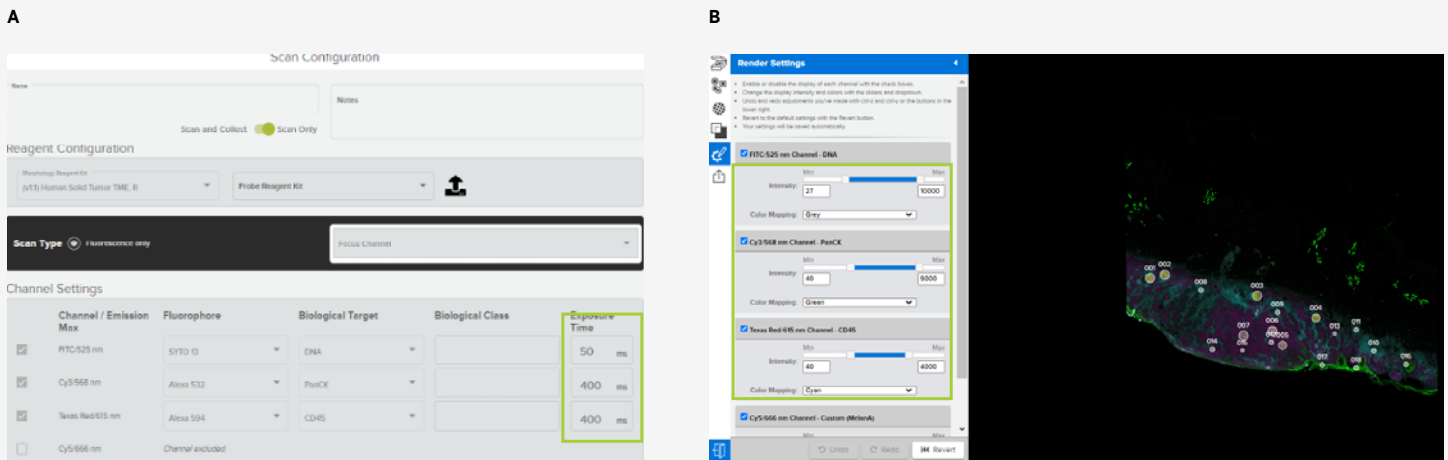
At the optimized concentration that gives strong, specific signal in the tissue, try this same concentration on a known negative control tissue to check for non-specific staining. While this last step is optional, it will considerably increase confidence in the staining results.

### Protocol Modifications for Antibody Optimization

#### “Dry Run” Slide Preparation

**GeoMx Protein** - Follow the GeoMx Slide Preparation user manual for the protein assay with one exception: when preparing the overnight primary antibody incubation, instead of the core and module antibodies, use an equal volume of Buffer W.

**RNA** - Follow the GeoMx Slide Preparation user manual with one exception: when preparing the overnight in situ hybridization, instead of the RNA probe mix, use an equal volume of 1X TE, pH 8.0.



**FIGURE 5:** 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.

### Slide imaging using the GeoMx DSP Instrument

Either the DSP or another fluorescence microscope can be used to assess the staining pattern on the test sections. Scanning on the GeoMx DSP instrument can be more time consuming and requires instrument buffers, but allows you to refine your segmentation strategy at the same time. (When assessing bleed-through, NanoString recommends imaging the slide on the DSP.)

To evaluate morphology marker staining on the DSP without collecting from the slide, take advantage of the GeoMx DSP's "Scan Only mode" (in software v3.0 and later). When setting up the scan parameters (Figure 5a), toggle the button to Scan Only mode. For the initial evaluation, we recommend setting each exposure time to 400 ms. If the resulting scan shows only non-specific signal or background signal, increasing the exposure above 400 ms will likely not help – a different concentration, or different incubation conditions, should be tested. (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.) If there is specific signal and some non-specific background visible, lowering the exposure time in 50 ms intervals will help narrow in on the optimal exposure time.

Following completion of the scan, adjust the appearance of the scan through the Render Settings (Figure 5B). Increase the minimum threshold (left slider) for a channel to decrease background; decrease the minimum threshold to increase visible fluorescence. Increase the maximum threshold (right slider) to decrease the overall fluorescence intensity; decrease the maximum threshold to increase the overall fluorescence intensity visible in the 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 above, a fluorescence microscope can also be used to evaluate morphology marker staining. For best practices, evaluate all slides under as many of the same conditions (magnification, illumination intensity, exposure time, etc.) as possible. A final check should be done on the DSP prior to running your biological samples of interest.



## ROI/AOI placement practice

Depending on tissue architecture and the scientific question being asked, ROI/AOI placement and segmentation fine-tuning can be a complex process. A dry run is thus an ideal opportunity for establishing and practicing a segmentation strategy. This can save a lot of time during the actual experimental run and lead to a smoother workflow.

## Troubleshooting

Various steps in the DSP protocol can be modified to help optimize morphology marker staining – for example, in RNA slide preparation, proteinase K digestion can be modified by reducing proteinase K concentration to 0.1 µg/mL or decreasing the incubation time from 15 minutes to 5-10 minutes (to leave more of the epitope available for morphology marker binding). Changes to Proteinase K digestion or heat-induced antigen retrieval can affect counts, so the balance between the two must be considered (and potentially tested empirically).

For more in-depth troubleshooting, please refer to “[GeoMx DSP IHC Workflow and Custom Morphology Markers: Troubleshooting, Tips, and Tricks](#)” (PMR-10172).

## Conclusion

Understanding tissue morphology is the first step in a well-designed spatial experiment. Morphology markers reveal the structural architecture of tissue and identify regions to profile. There are many morphology marker options, available from NanoString and other suppliers, that integrate into the GeoMx workflow and can be used for tissue profiling specific to your research question. Developing the profiling strategy in tandem with the appropriate morphology markers will maximize the learnings from your spatial biology study.

## Additional Resources

For more details on morphology markers and slide preparation, refer to the [GeoMx DSP Manual Slide Preparation User Manual](#) (MAN-10150) and [GeoMx DSP Automated Slide Preparation User Manual](#) (MAN-10151).

## Recommended NanoString University courses:

- [How to Use Morphology Markers with the GeoMx DSP](#)
- [Introduction to Region of Interest Selection](#)
- [Best Practices for Region of Interest Segmentation](#)



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

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