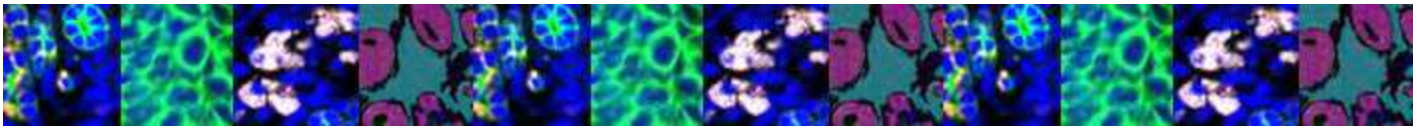
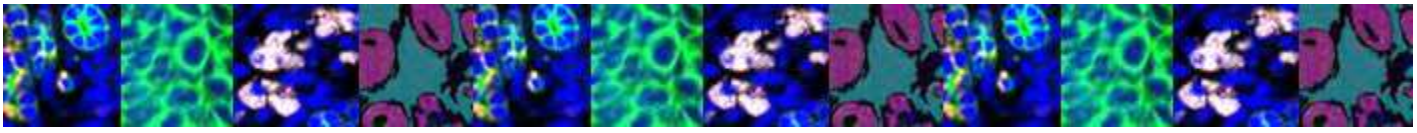


# A Guide to Designing and Preparing Digital Spatial Profiling (DSP) Projects

TRIP Lab



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# Introduction

The GeoMx Digital Spatial Profiler (DSP) developed by NanoString, is a technology that enables High-Plex Spatial and Molecular Analysis of RNA and Protein in tissue specimens on slides. Tissue slides are stained with large panels (>18,000 genes) of pre-mixed biological probes. Each panel contains unique, photo-cleavable DNA barcoded and fluorescently labeled morphology markers to visualize the morphology of the tissue. Using the morphology markers for guidance, ROIs are selected and illuminated with a UV light, then cleaved barcodes may be collected into a 96-well plate.

The plate will be processed by one of two methods, producing expression counts or a protein profile of key targets across the ROI. This quantified data is used to map back to the segmented tissue location yielding a spatially resolved digital profile of key pathways and varied gene expression levels.

With the nCounter platform, ~800 human or mouse genes and ~35 different pathways can be analyzed.

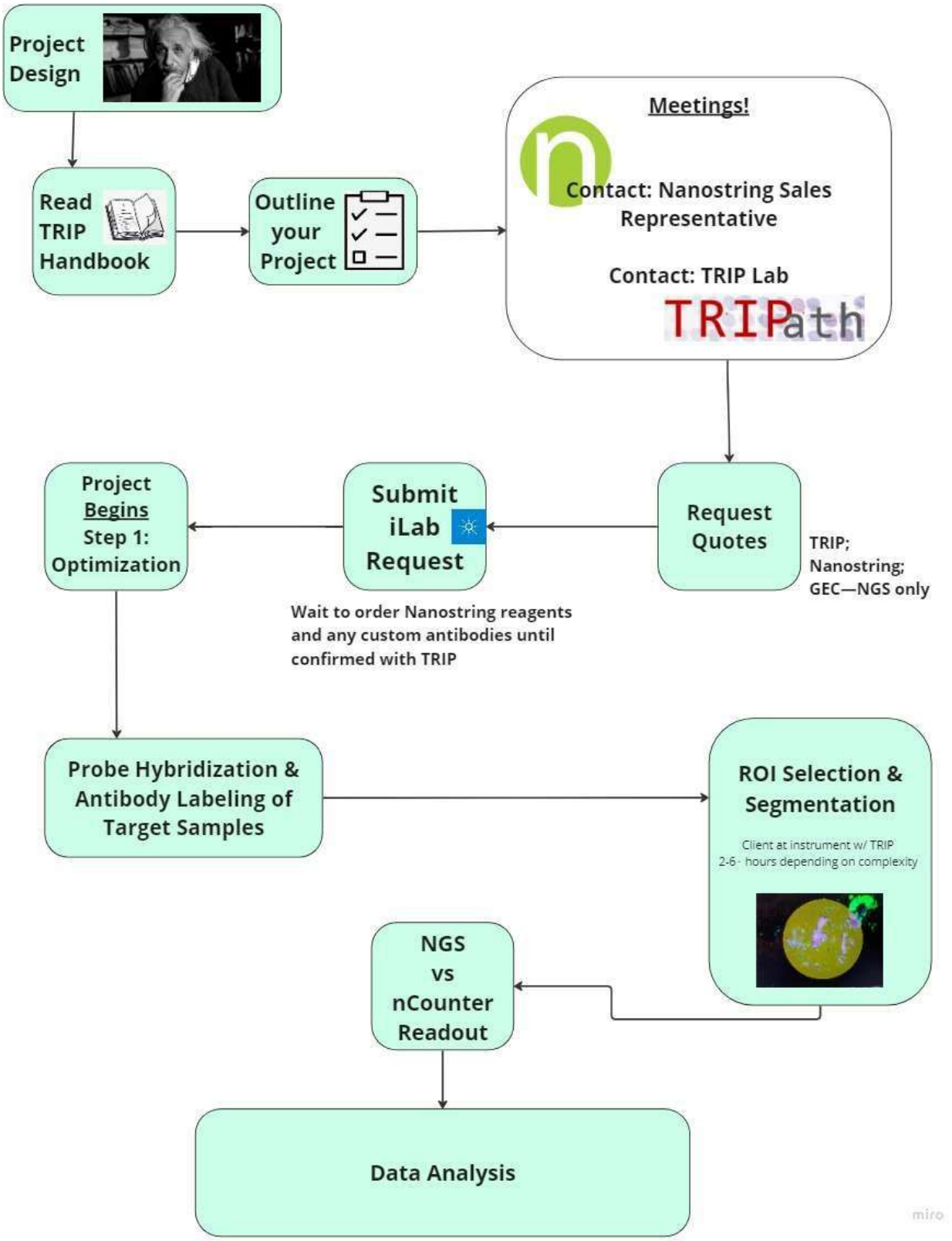
Alternatively, for High-Plex Spatial Profiling (>18,000 genes), Next Generation Sequencing (NGS) is performed in collaboration with the UWBC Gene Expression Center (GEC), DNA Sequencing Core, and the Bioinformatics Resource Center (BRC). These cores will work together by taking the raw sequencing reads from the Illumina, processing them through the GeoMx NGS pipeline, and converting them to DCC files to be uploaded to the workstation in TRIP, where analysis is performed.


Contacts

*TRIP Scientific Lab Manager*: Karla Esbona
kesbona@wisc.edu

*TRIP Spatial & Molecular Profiling Technician*: Rebecca Baus
rmbaus@wisc.edu

*TRIP website*: TRIP@pathology.wisc.edu




Design By: Maggie Baus
FlowChart Template by miro.com

# Project Design

When you reach out to TRIP Lab to discuss a new spatial project, it is important to be prepared with the information needed to communicate your needs for us to work together to accomplish your experimental goal. As seen below, we will highlight the main topics that you should familiarize yourself with and questions you should be able to answer prior to setting up a meeting with TRIP Lab.

## 1. Know how your tissue was collected, treated, and stored:

It is important to think about how the tissue was treated prior to coming into your possession. To avoid RNA degradation, transfer harvested tissue to formalin within 5 minutes, wear gloves, and spray work area with RNase Away (or equivalent). Cut tissue into small pieces to ensure formalin infiltrates the center of the tissue fully.

Know the length of time needed for formalin fixation based on your tissue type and size of the tissue. If you have questions, contact TRIP lab prior to harvesting. Over-fixation destroys antigens and creates difficulties in optimizing your morphology markers. After formalin fixation, transfer samples to 70% EtOH and bring to TRIP Lab. Storing in ethanol too long extends the fixation process, leading to over-fixation.

Avoid acidic decalcification reagents. Only EDTA-based decalcifiers may be used.

Many investigators will use archived FFPE blocks. Blocks <10 years old will yield the best results. TRIP will cut fresh slides from the FFPE blocks you provide. Pre-cut slides will not be accepted.

## 2. What is your biological question?

Be able to describe what you want to accomplish with Spatial GeoMx. Some examples include:

| | |
|---|---|
| Discover biomarkers predictive of therapeutic response | Understand disease mechanisms and progression |
| Profile the tumor microenvironment | Reveal molecular subtypes of diseases |
| Uncover drug mechanisms of action | Study Pre-Clinical Models |

## 3. What morphology markers will best answer your questions?

Selecting morphology markers that work for both GeoMx and your tissue type is one of the most challenging aspects of a GeoMx project. Not every antibody is able to withstand the harsh treatment that it will endure with this protocol. While you may want to use an antibody you are familiar working with, it is often faster and cheaper to use one of Nanostring's off-the-shelf Morphology Marker kits. These kits are validated for a diverse number of tissues and will require minimal optimization to confirm the staining pattern.

https://nanostring.com/products/geomx-digital-spatial-profiler/geomx-dsp-configuration-files/

If none of the markers within Nanostring's off-the-shelf Morphology kits meet your needs, Nanostring's Validated Morphology Marker Reference list should be utilized for selections as an alternative option. Contact your Nanostring Representative for the most up-to-date copy.

While these antibodies are validated by Nanostring, they are not validated for every tissue type. Nanostring's validated list provides details of which species and tissue have been tested. Selecting off this list may still require testing several clones in order to find one that works, adding additional costs to your budget. If you are interested in custom antibodies for all three channels, this could result in testing multiple antibody clones (3+) for each channel before finding one that is suitable. Optimizing custom antibodies is a time-consuming process and can significantly add to your budget and the length of time your project may take.

If your project needs are not met through Nanostring's off-the-shelf Morphology kits or any of the antibodies off Nanostring's Validated Morphology Marker Reference list, you can begin to look elsewhere. Keep in mind, you want an antibody that is raised in a species different from the tissue you are working with and it must be validated for IHC-Paraffin.

Antibody Selection Options

| | | |
|---|---|---|
| 1. | Off the shelf Morphology kits | Example: Solid Tumor Kit (PCK, CD45) |
| 2. | Nanostring Validated list | Contact Sales Rep for up to date list |
| 3. | Custom Antibodies | Look for an antibody that has been validated for IHC-Paraffin. |

## 4. Selecting a Custom Morphology Marker

When selecting a custom antibody, follow these important guidelines:

a) The species the tissue you are working with and the one the antibody is raised in cannot be the same.
b) Monoclonal antibodies will produce a more specific stain.
c) Look for an antibody with recommended concentration >1:50
   (We will not work with a 1:10 recommended dilution)
d) Antibodies from Abcam, Cell Signaling, and Novus have been successful for GeoMx.
e) The antibody must be validated for IHC-Paraffin. ICC-IF (cells in culture) alone will not be accepted.
f) Confirm the antibody is expressed in the species you are working with.
g) The antibody must be conjugated to AF532, AF594, or AF647.

| | |
|---|---|
| SYTO13 (nuclear stain) | AF488 |
| Used in most Nanostring morphology kits | AF532 |
| Used in most Nanostring morphology kits | AF594 |
| ***Open channel*** | AF647 |

AF488: This is reserved for the nuclear stain, SYTO13.

AF532: This is generally reserved for an antibody in one of Nanostring's off-the-shelf Morphology Kits, Pan-Cytokeratin from the Solid Tumor kit for example; however, it may be replaced with a custom antibody.

AF594: This is generally reserved for an antibody in one of Nanostring's off-the-shelf Morphology Kits, CD45 from the Solid Tumor kit for example; however, it may be replaced with a custom antibody.

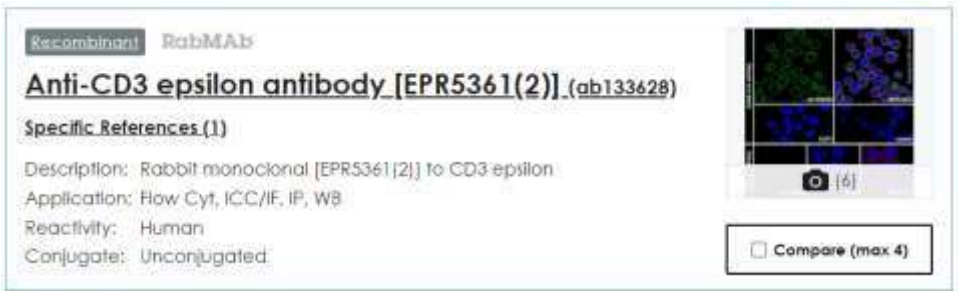
AF647: This is open for a custom antibody.

TRIP does not offer antibody conjugation as a service and we discourage labs from doing so themselves.

Prior to making antibody purchases, send all data sheets to TRIP for antibody approval.

## Practice comparing two antibodies to determine which is suitable for this project.

**Project:** Search for an antibody to use with Human tonsil tissue in conjunction with Nanostring's Solid Tumor Morphology Kit (PCK-AF532 and CD45-AF594) and nuclear marker, SYTO13.




*Abcam (Ab133628): recommended dilution ICC/P=assay dependent, IP=1:10*

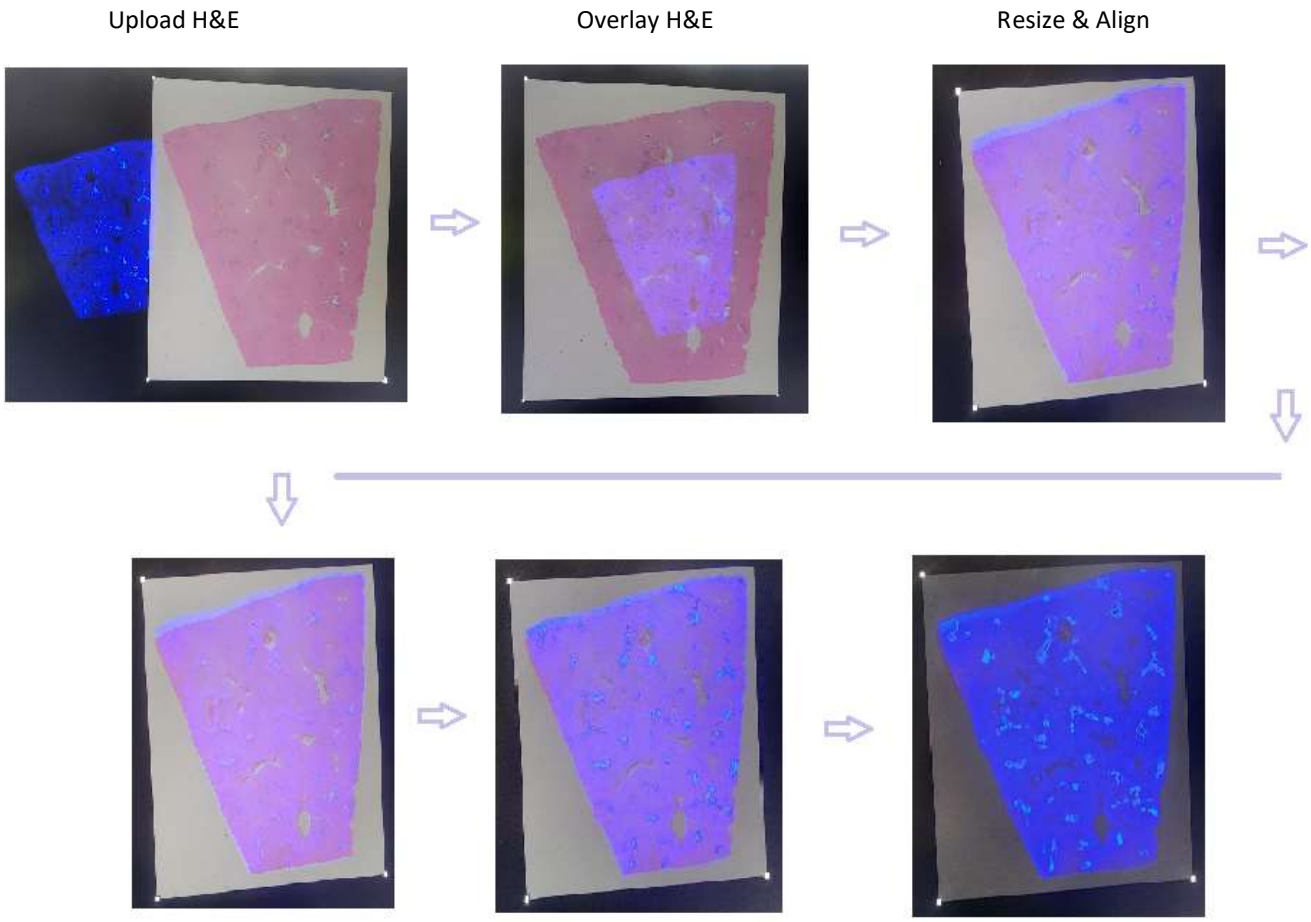

*Abcam (Ab225491): recommended dilution IHC-P = 1:1000*

| | CD3 | CD8 |
|---|---|---|
| Is the species the antibody is raised in different from the tissue you are working with? | **Yes** (Working with Hu, Raised in Rabbit) | **Yes** (Working with Hu, Raised in Rabbit |
| Is the antibody monoclonal? | **Yes** | **Yes** |
| Is the recommended concentration acceptable? | **No** (1:10) | **Yes** (1:1000) |
| Is it from a company TRIP has recommended? | **Yes** (Abcam) | **Yes** (Abcam) |
| Is it validated for IHC-Paraffin (IHC-P)? | **No** | **Yes** |
| Is the antibody expressed in the species you are working with (reacts in human)? | **Yes** | **Yes** (and mouse) |
| Is the antibody conjugated to the fluorophore in the open channel (AF647)? The nuclear stain is conjugated to AF488, PCK with AF532, and CD45 with AF594. | **No** (This antibody is unconjugated. TRIP does not conjugate antibodies and does not recommend labs do so either). | **Yes** |

In this scenario, **CD8** would be the correct antibody to select.

## 5. Using an H&E Slide to define Morphology

Using an H&E slide to define morphology and select ROIs is a method that requires minimal optimization. A single test slide allowing you to visualize your tissue with the nuclear stain and H&E overlay is all that is needed for you to approve the staining and move forward with your final tissue. This has shown to be an effective method when basing your ROI selection on morphology or known areas of expression.



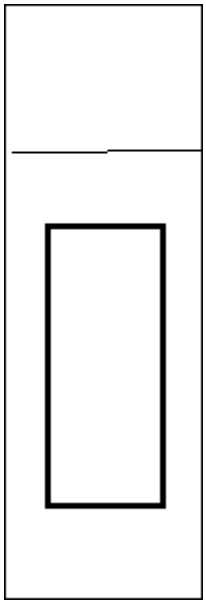

Fade out H&E to see tissue with nuclear stain, SYTO13, behind.

Test H&E overlay by Rebecca Baus for Wilmara Salgado-Pabon, Ariela Kaufman Burk 231012

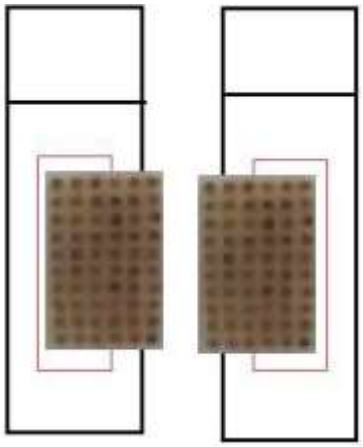
## 6. Tissue Size

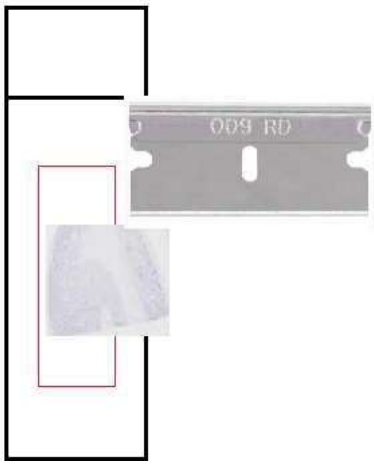
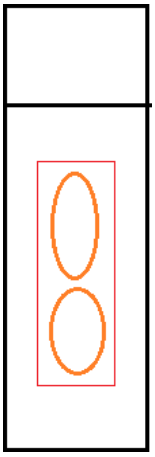
The FOV (field of view) for the GeoMx DSP instrument is ~33mm x 15mm (black rectangle). In order for the instrument to recognize a change from the edge of the gasket to the edge of the tissue, the size of your tissue should be no more than 30mm x 12mm (red rectangle).

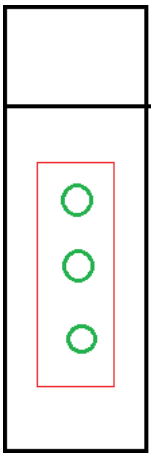
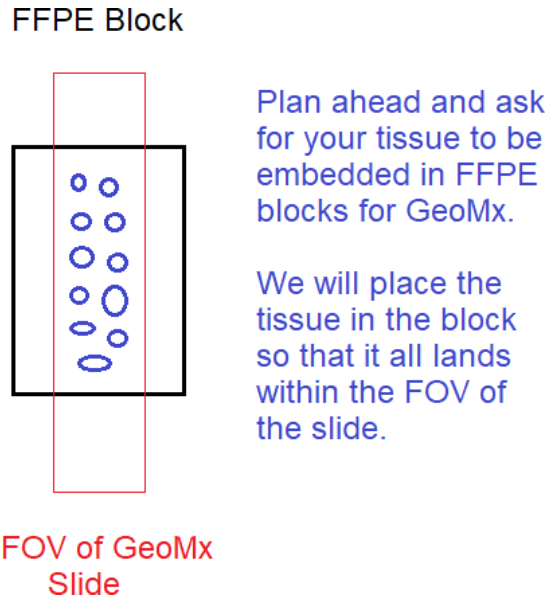
Image to Scale




We can accommodate a TMA by using two slides for full coverage of all cores. Two sections are cut where one slide maintains coverage of the left side of the TMA while the other maintains coverage of the right, generally with overlap.



| | |
|---|---|
|  |  |
| Large tissue requires a section of the target area to be placed within the FOV (red lines) while the remainder of the tissue is scraped off with a razor blade. | By selecting smaller target areas on a block, you may be able to place 2 samples per slide. |

| | |
|---|---|
|  |  |
| In some instances, where the samples are very small (ex. biopsies, eyes) we may be able to fit three samples per slide. | For very small tissue where you would like to fit >3 samples/slide, we will need to embed into a single block. If needed, we can melt and re-embed or create a TMA. |

# Contact Nanostring

Our Nanostring Sales Representative will be able to answer many of your initial questions. They have extensive knowledge of the Morphology Marker kits and the many different panels that Nanostring offers. They will provide the most up-to-date Morphology Marker Reference List (Validated Antibodies).

Request a reagent quote, but wait to place the order. Not all the GeoMx reagents in your order will come with the same expiration date. Prior to placing the order, discuss with Becky the details of your project, and wait for confirmation that the project meets the initial requirements before placing the order to avoid accidental expiring reagents. Do not ship orders to TRIP Lab. Label every reagent box with your name and Lab PI to avoid mix-ups with other lab's reagents.

# Contact TRIP Lab

Karla Esbona, TRIP's Scientific Lab Manager, meets virtually with clients for the initial GeoMx TRIP-Client meeting. At this meeting, you will have the opportunity to describe your project in detail and we can determine if this is something that is feasible for our lab.

At this point in the process you should already understand the technology, whether by reaching out to one of Nanostring's Field Application Specialists (FASs), by logging into Nanostring's free educational website, university.nanostring.com, to view the many training and introductory videos, attending a TRIP sponsored seminar, or by your own research.

# Request Sequencing Quote

The sequencing cost is based on the total area of all ROIs/AOIs across all plates, based on the DNA Sequencing facility's shared Illumina sequencing. In rare cases of very high total ROI area, it may be cheaper to buy your own lane of a NovaSeq S4 flow cell lane, which provides an average of 2250 million reads. An accurate estimate of these costs is provided after ROI/AOI selection is complete and the samples have been collected.

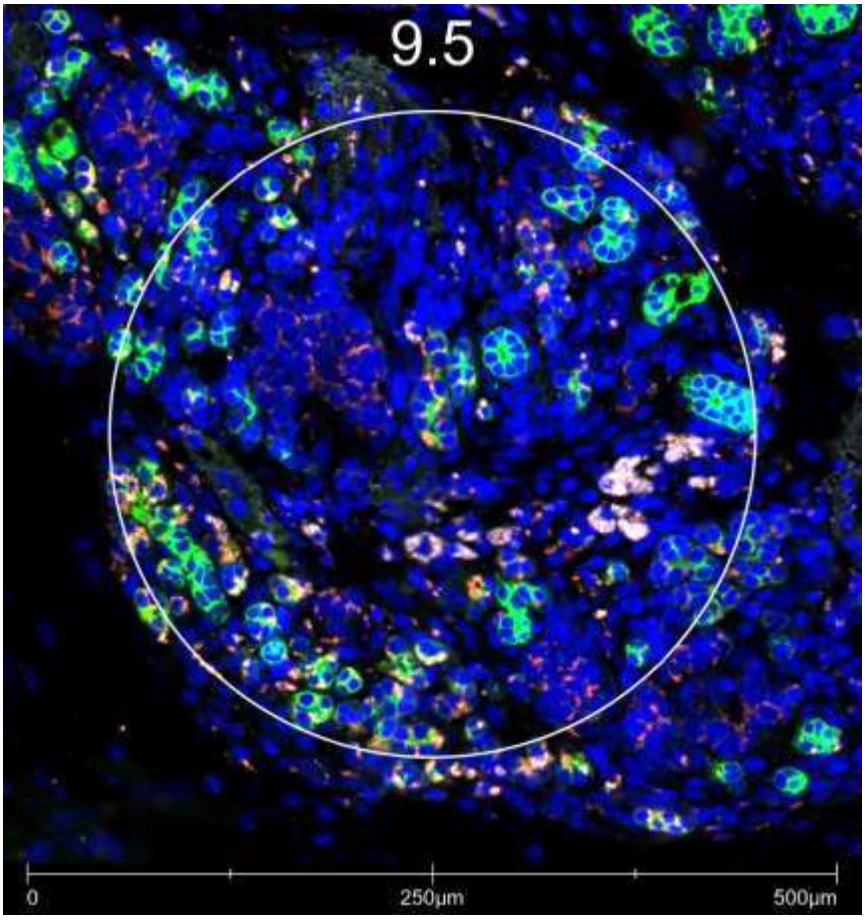
Provided by: Harrison Pantera, GEC Staff

# Submit iLab Request

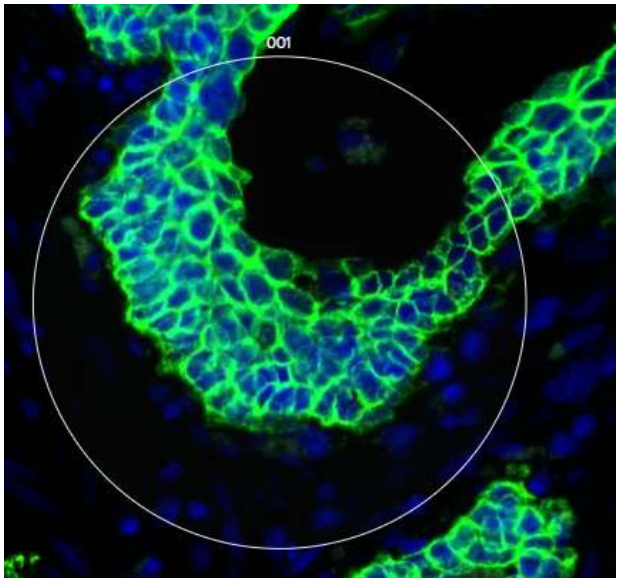
When you submit your iLab Request, select "Spatial & Molecular Profiling." From there you have two choices, NGS or nCounter readout. If you select NGS readout, then your samples are sent to GEC, ran on the Illumina and BRC will convert your files to .dcc format for analysis. If you select nCounter readout, then you are using nCounter kits with your GeoMx panel, your samples stay in TRIP lab, and you will be converting the files to .dcc format yourself for analysis.

# Understanding ROI Selection & Segmentation

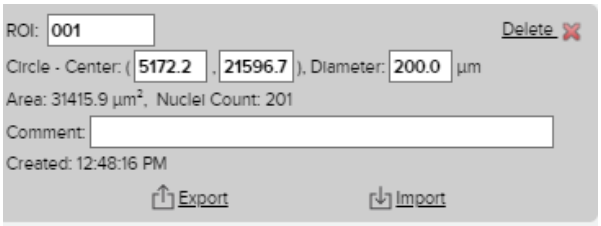
ROIs (regions of interest) are the regions on your sample that you are interested in collecting for analysis. For ROI selection, you will work with TRIP staff to draw circles, squares, or other geometric shapes to indicate the area you would like captured.



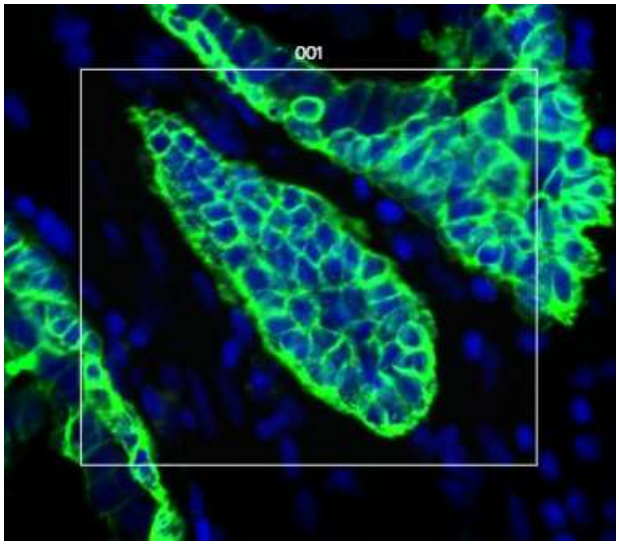

Dinh, Huy and Fitzpatrick, Megan 220216



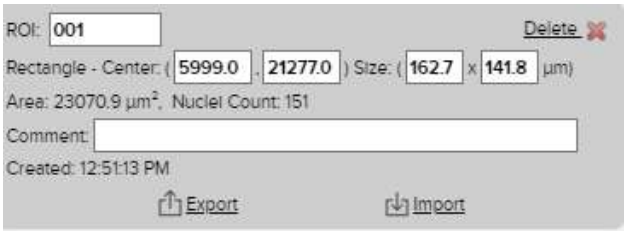

ROIs can be drawn in various shapes;
the most commonly used is the circle.



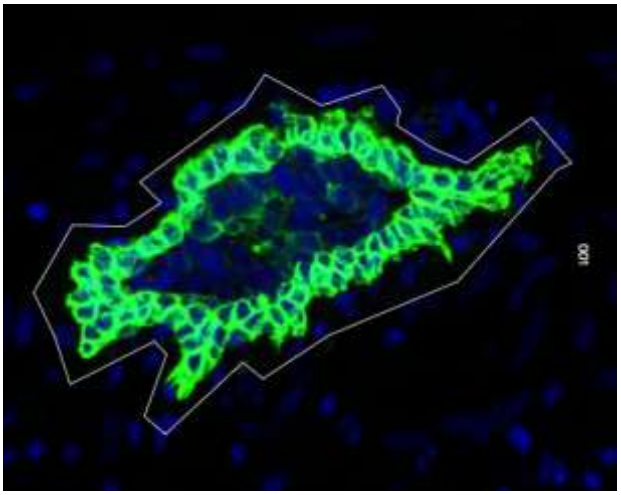

Test Image Rebecca Baus 2023



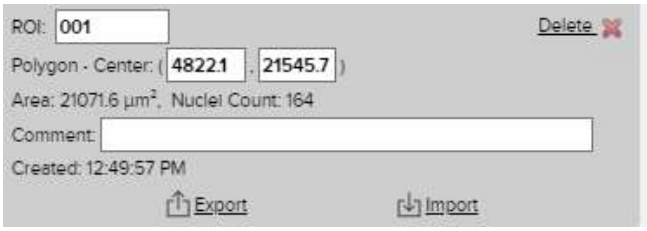

You can also draw a rectangle.



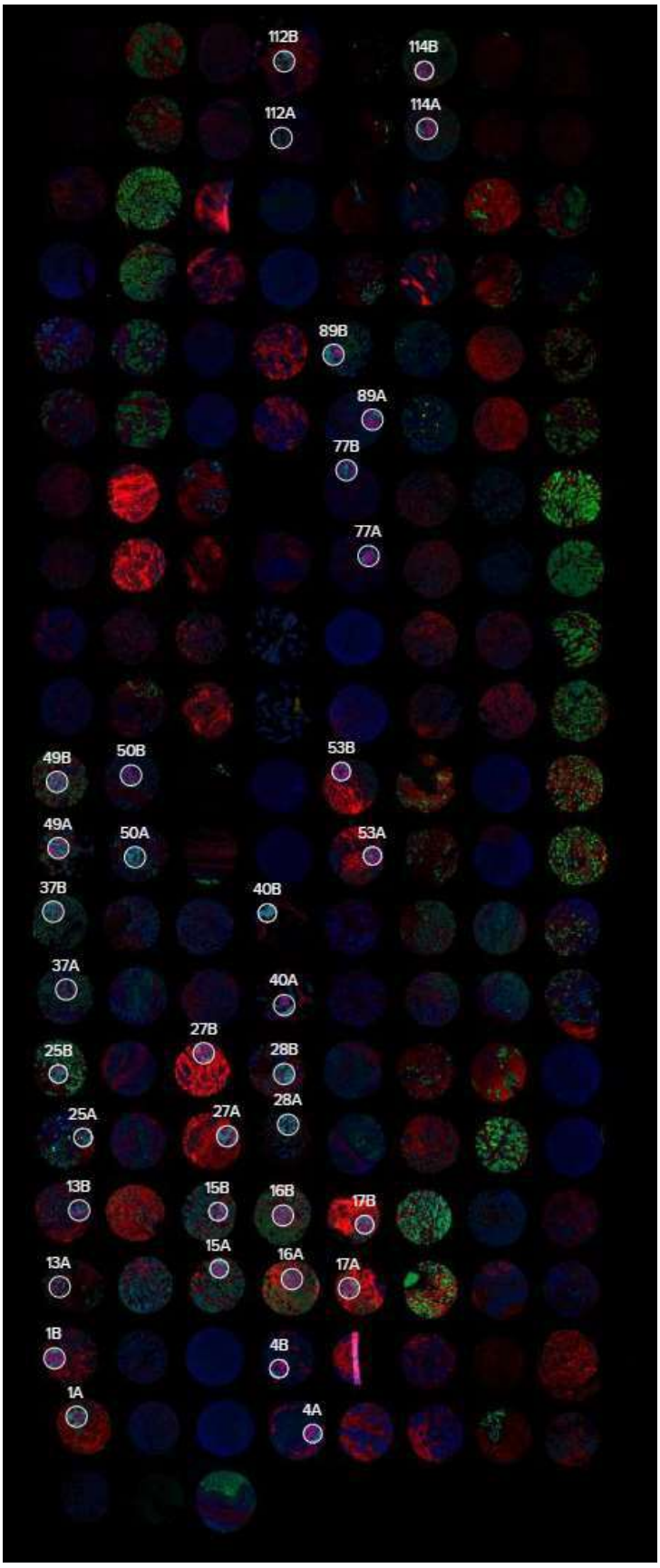

Test image Rebecca Baus 2023



Or a polygon.



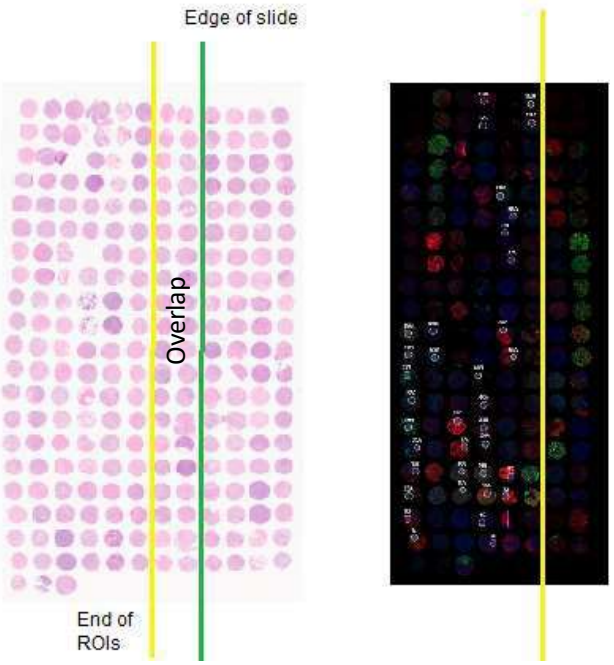

Test image Rebecca Baus 2023



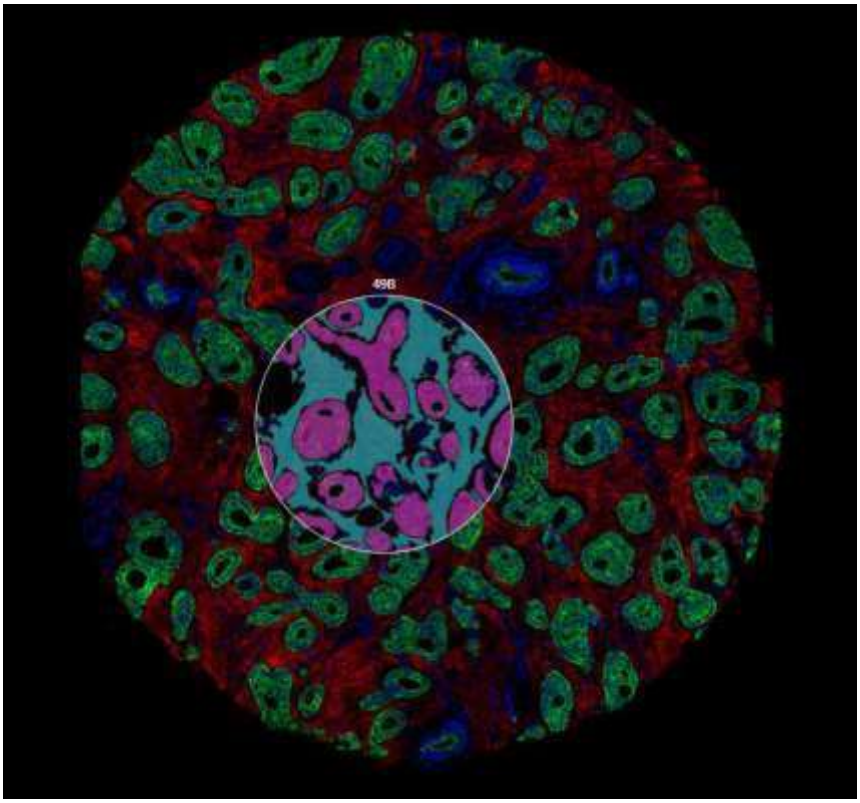

Many investigators will use archived FFPE blocks. Blocks <10 years old are preferred.

TMAs are beneficial when you want to visualize a large sample size. By splitting a TMA with 12 rows across 2 slides, allowing for some rows to overlap, you are able to get the full coverage of all cores in the block.

This is a scan of one slide. Half of the TMA block, including additional rows, ensures full coverage.




TMA for ROI selection used in GeoMx project.
Sheena Kerr, Suzanne Ponik & David Beebe 220614



To offer some perspective, this is an image of a single 1mm TMA core with a 400µm ROI

Sheena Kerr, Suzanne Ponik & David Beebe 220614

Once you have selected your ROIs, you have the option to collect them as a whole or further segment into defined regions. Segmentation allows researchers the opportunity to isolate expression patterns of specific compartments and cell types within a relevant region of the tissue and spatially analyze targets of interest.

Segmentation involves designating particular biological targets or combinations of biological targets as sub-regions. With the Solid Tumor Morphology kit, along with a nuclear stain, you would have the ability to segment and compare tumor vs stroma by setting your parameters as $PCK^+$ vs $PCK^-/CD45^+$. The image on the next page is an example of this comparison.

The areas of tumor ($PCK^+$) within the ROI are “masked” in purple and the stroma ($PCK^-/CD45^+$) in yellow.

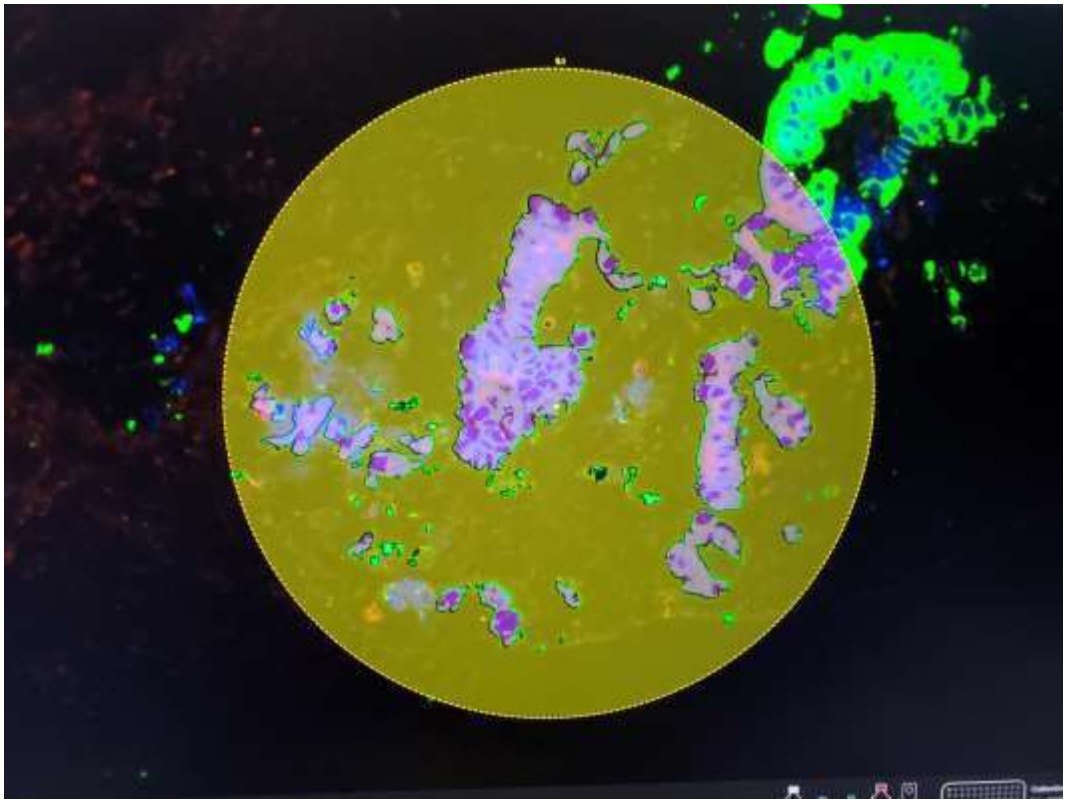


Image by Rebecca Baus 2023

Nanostring suggests segmenting as simply as possible. Too many segmentation rules can reduce the effectiveness of automated segmentation.

To determine how many 96-well plates you will need for a project, you must first know how many segments (or AOIs) you plan to have for each ROI. You may configure no more than 3 AOIs (Areas of Illumination) for collection per ROI.

In a 96-well plate, one well is reserved for the negative control. This leaves 95 wells for ROIs, divided by the number of AOIs. At least 8 ROIs per sample (or per treatment group) should be selected. For smaller tissue, such as biopsies, 3-4 is sufficient. For statistical power, there should be 4-6 biological replicates in each treatment group for comparison.

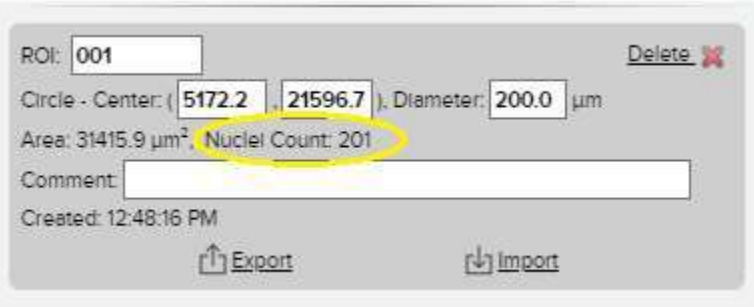
**Use this table to assist in determining your project needs.**

**ROI** = Region of Interest = the circle/rectangle/polygon you draw on your tissue

**AOI** = Area of Illumination = segments = the area divided within the ROI = depending on your scientific question, each segment may be a morphology marker expression

| How many: ROIs, AOIs/Segments, Plates ? | | | |
|---|---|---|---|
| | 1 tissue per slide | 2 tissues per slide | 3 tissues per slide |
| # of ROIs per tissue ( 0 segments ) | 23-24 ROIs (4 slides) = 1 plate | 11-12 ROIs (4 slides) = 1 plate | 7-8 ROIs (4 slides) = 1 plate |
| # of ROIs per tissue ( 2 segments ) | 11-12 ROIs (4 slides) = 1 plate | 5-6 ROIs (4 slides) = 1 plate<br>7-8 ROIs (3 slides) = 1 plate | 7-8 ROIs (4 slides) = 2 plates |
| # of ROIs per tissue ( 3 segments ) | 7-8 ROIs (4 slides) = 1 plate | 3-4 ROIs (4 slides) = 1 plate<br>5-6 ROIs (3 slides) = 1 plate<br>7-8 ROIs (2 slides) = 1 plate | 7 ROIs (3 slides) = 2 plates<br>10-11 ROIs (2 slides) = 2 plates<br>5 ROIs (2 slides) = 1 plate<br>10-11 ROIs (1 slide) = 1 plate |

Each segment should ideally have a minimum of 50 nuclei for RNA (20 for protein). When you are unable to meet the minimum standard, you may see low transcribing genes are omitted from your data. For some investigators, it may be more important to select a specific area of low nuclei count and sacrifice data from low transcribing genes than to not select the desired area at all.




The more ROIs you select, the more wells you will need to collect your samples.

# NGS vs nCounter Readout

**NGS Checklist**:

Set up an account with GEC and prepare a submission form.

Transport your 96-well plate to GEC for NGS.

Send Karla the numbers on both sides of your WISCARD so you have badge access to WIMR 2214A where the analysis workstation is located.

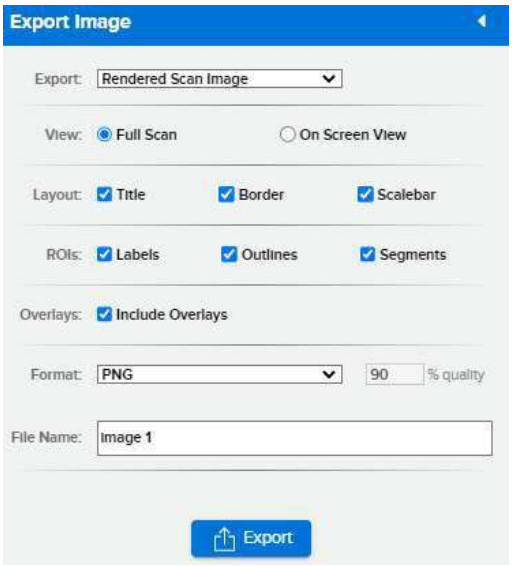
GEC staff will contact you to confirm the number of reads based off the lab worksheet file and approve the libraries created by GEC prior to submission to the DNA Sequencing Core.

When the sequencing data is available, you will receive an email from the DNA Sequencing Core with instructions on how to access your data. These are the FASTQ files. Be sure to save your data. They delete after 60 days. For internal/UW-Madison users, your data is also saved to your lab's Research Drive Account.

The Bioinformatics Resource Center (BRC) will convert your data to DCC files. You will receive an email from the BRC when the DCC files are ready. Copy these files to a 1-2TB external drive that you can take with you when you sign up on the Analysis Workstation at TRIP Lab.

Your username and password from Becky allows you to access your images and the GeoMx software on the Analysis Workstation. Here, you will upload your DCC for analysis.
Reserve your time on the workstation through iLab.
Log your time on the clipboard. Access is required for each slide to be visible in your account.

Save your images with the Export Image icon. Bring an external drive to store your images. All images saved on the desktop will be deleted.



**nCounter readout Checklist**:

☑ Send Karla the numbers on both sides of your WISCARD so you have badge access to WIMR 2214A where the analysis workstation is located.

☑ Your username and password from Becky allows you to access your images and the GeoMx software on the Analysis Workstation. Here you will upload your DCC for analysis. Reserve your time on the workstation through iLab. Log your time on the clipboard. Access is required for each slide to be visible in your account

# After Your Samples Leave TRIP

**NGS:**

NGS requires you to transport your samples to GEC for Library Prep and QC. Once it is complete (~2 days), you will receive an email to confirm the recommended number of sequencing reads and the expected cost.

Your samples are submitted for sequencing on the DNA Sequencing Core's shared Illumina sequencing system.

After the sequencing is finished (~2 weeks), you will get an email letting you know the data is available. Please note, this is not the final version of the data used at the Analysis Workstation. Simultaneously, the Bioinformatics Resource Center (BRC) will be converting the data files (<1 day) into an additional format, DCC file, using NanoString's NGS pipeline. You should get updates this is taking place. The DCC file format is then uploaded to the Analysis Workstation in TRIP Lab.

Provided by: Harrison Pantera, GEC Staff

**nCounter readout**:

With nCounter readout, your samples never leave TRIP Lab. TRIP staff will contact you when your FASTQ files are available for you to begin file conversion to DCC format.

# Data Analysis

## Data Analysis Resources:

https://university.nanostring.com/

GeoMx DSP Data Analysis Manual (v3.0 software), MAN-10154-03
GeoMx DSP Data Analysis Resource Guide, PMR-11294-02
Quick Guide: GeoMx DSP – NGS Data Analysis, MAN-10134-02
GeoMx nCounter Readout User Manual, MAN-10089-89
Quick Guide GeoMx DSP - nCounter Data Analysis, MAN-10105-03

Training Videos

GeoMx DSP: Data Analysis for NGS Readout – 60 min
https://university.nanostring.com/geomx-data-analysis-for-ngs-readout

GeoMx Academy: NGS Data Analysis for RNA – 98 min
https://university.nanostring.com/geomx-academy-ngs-data-analysis-for-rna

GeoMx Data Analysis for nCounter Readout – 91 min
https://university.nanostring.com/geomx-data-analysis-for-ncounter-readout

How to use the Spatial Decon Custom Script – 6 min
https://university.nanostring.com/how-to-use-the-spatial-decon-custom-script

Normalization of GeoMx DSP Protein Data using a Custom Script – 16 min
https://university.nanostring.com/normalization-of-geomx-dsp-protein-data-using-a-custom-script

How to run GeoMx DSP Data Analysis Workflows – 4 min
https://university.nanostring.com/how-to-run-geomx-dsp-data-analysis-workflows

How to create a GeoMx Data Analysis Report – 6 min
https://university.nanostring.com/how-to-create-a-geomx-data-analysis-report

Nanostring Data Analysis Training & Workshop Monthly Zoom Series

https://nanostring.zoom.us/meeting/register/tJAqdemtrT0rG9U2LLjnpaa_uU2O-gh_8j4M#/registration