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Department of Translational Molecular Pathology (TMP)

Immune Profiling Laboratory

Cancer Immune Monitoring and Analysis Center

The University of Texas MD Anderson Cancer Center

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THE UNIVERSITY OF TEXAS
MD Anderson
~~Cancer~~ Center

Making Cancer History®

SOP for Pathological Assessment and Region of Interest Selection using the nanoString GeoMx Digital Spatial Profiler

1. Technical platform

The GeoMx® Digital Spatial Profiling (DSP) technology is a novel platform developed by NanoString. This product relies upon antibodies or nucleic acid probes coupled to photocleavable oligonucleotide tags. After the incubation of antibodies to slide-mounted tissue sections, cell types of interest with fluorescent morphology markers to create fluorescent images for selecting region of interest (ROIs). The oligonucleotide tags are released from discrete regions of the tissue via UV exposure. Released tags are quantitated in an nCounter® and counts are mapped back to tissue location, yielding a spatially resolved digital profile of analyte abundance(1).

This SOP describes the **workflow and process for experimental design, pathological assessment and region of interest selection** using the GeoMx DSP(2) at the Translational Molecular Pathology Immunoprofiling Laboratory (TMP-IL).

2. Experimental design

Profiling tissue using the GeoMx Digital Spatial profiler requires several considerations to design and conduct DSP assay in order to reach scientific aims of a specific project. To tailor selection of visualization biomarkers, and selection of DSP panels and modules, the following information should be obtained during scientific discussion with collaborators. (See also **Annex 1.** TMP Experimental design form)

a. **Sample information:**
   i. Number of samples: Number of FFPE blocks
   ii. Number of slides: Specify if multiple samples can be placed in one slide or samples can be placed in TMA.
   iii. Tumor type: Histopathology diagnosis of samples.
   iv. Type of samples: Surgically resected, core biopsies, TMA, decalcified samples or others.

b. **Aims:**
   i. **Principal aim.**
   ii. **Specific Scientific aims.**

c. **GeoMx DSP biomarker/panel and ROI design:**
   i. **Morphology biomarkers:** Determine if the project will use commercially available GeoMx DSP morphology kits or if the laboratory will need to optimize a custom morphology biomarker

panel. A validation process will be performed for custom panels. The following morphology biomarkers are available and optimized at TMP-IL:

Markers	**Clone**	**Vendor**	**Catalog #**	**Labeling Fluorophore**
PanCK	AE1+AE3	Nanostring/Novus	NBP2-33200	AF532
CD45	2B11+PD7/26	Nanostring/CST	#13917	AF594
S100B	S100B/1706R	Nanostring / Novus	NBP2-54426	AF532
B3 Tubulin	EP1569Y	abcam	ab190575	AF647
CD20	IGEL/773	NovusBio	NBP2-47840	AF594
CD3e	UMAB54	Origene	AC211030	AF647
CD68	KP-1	Santa Cruz	SC-20060 AF647	AF647
CD68	KP-1	Santa Cruz	sc-20060 AF594	AF594
Claudin-4	3E2C1	LSBio	LS-C354893	AF594
NFH	EPR20020	abcam	ab223139	AF594
PanCK	AE1/AE3	NovusBio	NBP2-33200AF647	AF647
Synaptophysin	YE269	abcam	ab 196166	AF647

ii. **DSP profiling panel:** Protein or RNA.

iii. **DSP core panel and modules:** The following core panels and modules are currently commercially available for human samples (See also **Annex 2**).

Protein:

•*Immune Cell Profiling Core (Human protein core):* Includes key immuno-oncology targets and markers of immune cell types, including T cells, B cells, macrophages, NK cells, epithelia, and stroma. It also contains the controls needed to run any GeoMx DSP experiment.
Biomarkers: Beta-2-microglobulin; CD3; CD56; CTLA4; GZMB; PD-1; CD11c; CD4; CD68; Pan-cytokeratin; HLA-DR; PD-L1; CD20; CD45; CD8; Fibronectin; Ki-67; SMA; Ms IgG2a; Ms IgG1; Rb IgG; S6; Histone H3; GAPDH.

• *IO Drug Target Module:* Includes drug targets in development within the immuno-oncology space, including checkpoint molecules and metabolic mediators of immune function.
Biomarkers: 4-1BB; LAG3; ARG1; OX40L; B7-H3; STING; GITR; TIM-3; IDO1; VISTA

• *Immune Activation Status Module:* Includes additional checkpoint molecules that modulate T cell activation.
Biomarkers: CD127; CD25; CD27; CD40; CD44; CD80; ICOS; PD-L2

• *Immune Cell Typing Module:* Includes an expanded set of cell type markers to more deeply profile immune cell types covered in the Immune Cell Profiling Core and measure additional immune cell types, including T cell subsets.
Biomarkers: CD14; CD163; CD34; CD45RO; CD66b; FAPalpha; FOXP3

• *Pan-Tumor Module*: Includes an expanded set of markers for detecting specific tumor types, including ER+/HER2+ breast tumors, hematopoietic malignancies, and melanoma.

Biomarkers: BCL-2; EpCAM; ER alpha; HER2/ERBB2; MART1; NY-ESO-1; PR; PTEN; S100B

• *Cell Death Module:* Includes protein mediators of immunogenic and programmed cell death.
Biomarkers: BAD; BCL6; BCLXL; BIM; CD95/Fas; GZMA; NF1; p53; PARP; Cleaved Caspase 9

• *PI3K/AKT Signaling Module*: Includes key proteins involved in PI3K-AKT signal transduction, and phosphorylated protein products that measure pathway activation.
Biomarkers: Pan-AKT; MET; Phospho-AKT1(S473); Phospho-GSK3B (S9); Phospho-Tuberin (T1462); Phospho-GSK3A (S21)/Phospho-GSK3B (S9); INPP4B; PLCG1; Phospho-PRAS40 (T246)

• *MAPK Signaling Module:* Includes key proteins involved in MAPK signal transduction, and phosphorylated protein products that measure pathway activation
Biomarkers: EGFR; pan-RAS; BRAF; Phospho-c-RAF (S338); Phospho-JNK (T183/Y185); Phospho-MEK1 (S217/S221); Phospho-p38 MAPK (T180/Y182); Phospho-p44/42 MAPK ERK1/2 (T202/Y204); p44/42 MAPK E RK1/2; Phospho-p90 RSK (T359/S363)

RNA:

- GeoMx Immune pathways panel: 84-plex RNA panel + customs (96-plex)/nCounter
- Cancer Transcriptomic Atlas (CTA) / Next Generation Sequenceing: Covers +1,800 genes
- Whole Transcriptomic Atlas (WTA)/ Next Generation Sequenceing: Covers +18,000 genes

d. **Region of Interest (ROI) strategy**
   i. Number of ROI per sample.
   ii. Shape of ROI: rectangles, circles and/or polygons up to 660x785 µm .
   iii. Histological or phenotypic strategy for ROI placement.
   iv. Segmentation of ROI: The strategy should state which morphology biomarkers will be used to segment the area.

e. **Estimated time to complete the project.** For Protein and 84plex RNA panels the maximum capacity is 8 slides per week at 12 AOIs per sample (Total 96 AOIs per week)

3. Selection of cases and tissue qualification for DSP analysis

After a cohort of cases has been selected for DSP analysis, a pathological assessment of samples available is performed using freshly cut sections stained with H&E (preferable) or archived H&E slides, the latter is less preferable due to the risk of evaluation of a section that is not representative of tissue left in FFPE block. (See also Annex 3. check list for selection of cases and tissue qualification for DSP analysis)

a. **Pathology assessment of samples:** The laboratory requires that the H&E slides of potential tissue samples for DSP assay are evaluated by a pathologist prior to sending material for DSP assay. The H&E slides are digitally scanned using Aperio AT2 (Leica Biosystems). An excel file from collaborators containing relevant clinicopathological information of the samples should also be provided.

The following information is usually provided by collaborators but may vary among projects.

-MRN
-Tissue ID
-Time Point. If the patient has two TPs (TP0, TP1), check the experimental design for specific

instructions on sample selection.
-Diagnosis
-Organ site.
-Type of biopsy (surgical specimen, core biopsy, endoscopic biopsy, TMA) (A map of the TMA must be attached)
-Localization (primary organ vs metastasis in lymph nodes or other organs).
-Other

b. **Quality control:** A pathologist will perform a histological quality control of samples received using H&E slides (scanned and/or physical slide). The following information is usually evaluated for surgically resected tumor samples and can be tailored for specific projects. The following information is evaluated in surgical resected tumor samples
   - Presence of tumor cells (Yes/No): > than 100 viable tumor cells.
   - Presence of normal adjacent tissue.
   - Presence of Tumor associated Tertiary lymphoid aggregates (TLS).
   - Presence of Invasive margin (IM).
   - Percentage of tumor infiltrating lymphocytes (TILs)*.
   - Comments: Annotations of potential presence of artifacts that can interfere with the staining (background) including blood cells, fibrin, bone or necrosis.

*Note: TIL's assessment is performed in each sample to assess % of immune cells in tumor stroma using the guidelines of the International TILs Working Group 2014 (4).
In samples with heterogeneous tumor immune infiltration, we use a grid of 1500 um^2 to evaluate TILs% in each square to obtain an average of TILs %; this evaluation can be further used to select ROI for DSP analysis.

c. **Tissue qualification for DSP assay:** After histological evaluation a pathologist will perform the following:
   i. Select the best representative sample to run DSP assay (Considering experimental design strategies).
   ii. Annotate potential ROI areas (squares, circles or polygons of up to 660*785um^2) according to the experimental design strategy.

d. **Requisition of material to perform DSP assay:** After a pathologist have provided a list with the selected samples, the laboratory will request freshly-cut unstained sections processed according to nanoString GeoMx DSP protocol(5) to be sent to TMP-IL along with excel file containing information of slides.

e. **Storage of slides**: Once the laboratory receives unstained sections and a file with sample identification.

   i. Sections will be stored at 4ºC.
   ii. Slides must be labeled with sample identification that match with information of slides submitted in the excel file.
   iii. Slides labels should not contain protected health information (PHI).
   iv. Date of sectioning should be provided in the slide label, slide container or in an insert inside the container.

f. **Quality assurance of unstained sections:** Unstained sections are reviewed by a pathologist to asure that:
   - i. The sections are adequate with no excessive folding or dettachment
   - ii. Tissue fit the DSP scanning area
   - iii. No tissue overlap the gasket area or the tip calibration area.
   - iv. If multiple unstained slides are available to select for this assay, a pathologist will choose the unstained section with best tissue integrity.
   - v. Unstained sections should be similar in shape to H&E previously evaluated. If significative differences are found, H&E of a serial sections should be requested.

4. Selection of ROI in the scanned images using DSP:

Once the slides have being processed and the slides have been scanned in the DSP device, a pathologist will access the GeoMx DSP software through Google Chrome based app, via MD Anderson network, and will login using the username and password previously set up by the laboratory. Using the GeoMx DSP software tools, a pathologist will perform the following tasks (See also **Annex 4** Selection of Regions of interest and segmentation using GeoMx DSP software):

- i. Visualize the slide image which will allow to perform a verification of the immunofluorescence staining quality of morphology biomarkers, and
- ii. Select ROI and perform segmentation according to experimental design strategy.
- iii. Approve ROI and segment and send for collection.

5. References

1. Van TM, Blank CU. A user's perspective on GeoMxTM digital spatial profiling. Immuno-Oncology Technology. 2019;1:11-8.
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3. NanoString I. GeoMx® Protein Assays for Immuno-Oncology 2021 [cited 2022 09/05/2022]. Available from: chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://nanostring.com/wp-content/uploads/PB_MK3350_DSP_IO_Protein_PB_R28.pdf.
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5. NanoString I. GeoMx® DSP Automated Slide Preparation User Manual 2022 [updated April 2022; cited 2022 06/27/2022]. Available from: https://university.nanostring.com/geomx-dsp-automated-slide-preparation-user-manual/1209595.

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Signature (Luisa M. Solis Soto) **Date: 09/06/2022**

Annex 1. GeoMx DSP experimental design form.

GEOMX DSP EXPERIMENTAL DESIGN FORM
DSP project # (n= *number of slides*)

1) **Name of the project:**
2) **Project PI:**
3) **Cohort:**
4) **Sample information:**
    i) Number of samples: *Number of FFPE blocks*
    ii) Number of slides: *Specify if multiple samples can be placed in one slide or samples can be placed in TMA.*
    iii) Tumor type: *Histopathology diagnosis of samples.*
    iv) Type of samples: *Surgically resected, core biopsies, TMA, decalcified samples or others.*
  b) **Aims**
    i) **Principal aim:**
    ii) **Specific Scientific aims:**
  c) **GeoMx DSP biomarker/panel and ROI design:**
    i) **Morphology biomarkers:** *Determine if the project will use commercially available GeoMx DSP morphology kits or if the laboratory will need to optimize a custom morphology biomarker panel. A validation process will be performed for custom panels.*
    ii) **DSP profiling panel:** *Protein or RNA.*
    iii) **DSP core panel and modules:** *The following core panels and modules are currently commercially available: (See list in Annex 2)*

  d) **Region of Interest (ROI) strategy**
    i) Number of ROI per sample:
    ii) Shape of ROI: *rectangles, circles and/or polygons up to 660 x 785 µm.*
    iii) Histological or phenotypic strategy for ROI placement:
    iv) Segmentation of ROI: *The strategy should state which morphology biomarkers will be used to segment the area, order of collection and segment definitions*
      i. Segment 1:
        (a) Segment definition:
      ii. Segment 2:
        (a) Segment definition
      iii. Segment 3:
        (a) Segment definition
  e) **ROI strategy for tonsil control if included:**
    i) Number of ROI: 9 ROIs.
    ii) Shape of ROI: polygons up to 660 x 785 um
    iii) Histological or phenotypic strategy for ROI placement: 3 ROIs will be placed in germinal center; 3 ROI in interfollicular area; 3 ROI in reticulated epithelium area. No segmentation.
  f) **Plan to select ROIs per week:**
    i) 1st week: 8 cases
    ii) 2nd week: 7 cases + control-Tonsil
    iii) Estimated number of ROIs:
    iv) Estimated number of AOIs:

5) **TMP-IL Personnel**

a) GeoMx DSP Platform Operator:
b) GeoMx DSP Platform Lab member:
c) Sectioning:
d) GeoMx DSP Pathologists:
e) TMP-IL IHC /DSP Lab director:

6) **Estimated time to complete the project.** For Protein and 84plex RNA panels the maximum capacity is 8 slides per week at 12 AOI per sample (Total 96 AOI per week)

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Annex 2 GeoMX DSP panels commercially available for human specimens as for 09/06/2022

1. PROTEIN

Nanostring DSP Protein Immuno-oncology Panels

Immune Cell Profiling Core

PD-1
CD68
HLA-DR
Ki-67
Beta-2-microglobulin
CD11c
CD20
CD3
CD4
CD45
CD56
CD8
CTLA4
GZMB
PD-L1
PanCk
SMA
Fibronectin
Rb IgG
Ms IgG1
Ms IgG2a
Histone H3
S6
GAPDH

IO Drug Target Module

4-1BB
LAG3
OX40L
Tim-3
VISTA
ARG1
B7-H3
IDO1
STING
GITR

Immune Activation Status Module

CD127
CD25
CD80
ICOS
PD-L2
CD40
CD44
CD27

Immune Cell Typing Module

CD45RO
FOXP3
CD34
CD66b
FAP-alpha
CD14
CD163

Cell Death Module

BAD
BCL6
BCLXL
BIM
CD95/Fas
GZMA
NF1
p53
PARP
Cleaved Caspase 9

PI3K/AKT Signaling Module

Pan-AKT
MET
Phospho-AKT1(S473)
Phospho-GSK3B (S9)
Phospho-Tuberin (T1462)
Phospho-GSK3A (S21)/Phospho-GSK3B (S9)
INPP4B
PLCG1
Phospho-PRAS40 (T246)

MAPK Signaling Module

EGFR
pan-RAS
BRAF
Phospho-c-RAF (S338)
Phospho-JNK (T183/Y185)
Phospho-MEK1 (S217/S221)
Phospho-p38 MAPK (T180/Y182)
Phospho-p44/42 MAPK ERK1/2 (T202/Y204)
p44/42 MAPK E RK1/2
Phospho-p90 RSK (T359/S363)

2. RNA:

2.1. GeoMx Immune Pathway Panel Human RNA Core for nCounter (84-plex+custom, 96-plex)

Probe Name	Gene(s)
AKT1	AKT1
ARG1	ARG1
B2M	B2M
BATF3	BATF3
BCL2	BCL2
CCL5	CCL5
CCND1	CCND1

CD27	CD27
CD274	CD274
CD276	CD276
CD3E	CD3E
CD4	CD4
CD40	CD40
CD40LG	CD40LG
CD44	CD44
CD47	CD47
CD68	CD68
CD74	CD74
CD86	CD86
CD8A	CD8A
CMKLR1	CMKLR1
CSF1R	CSF1R
CTLA4	CTLA4
CTNNB1	CTNNB1
CXCL10	CXCL10
CXCL9	CXCL9
CXCR6	CXCR6
DKK2	DKK2
EPCAM	EPCAM
FAS	FAS
FOXP3	FOXP3
GZMB	GZMB
HAVCR2	HAVCR2
HIF1A	HIF1A
HLA-DQ	HLA-DQA1
HLA-DRB	HLA-DRB1,HLA-DRB3,HLA-DRB5,HLA-DRB4,HLA-DPB1
HLA-E	HLA-E
ICAM1	ICAM1
ICOSLG	ICOSLG,LOC102723996
IDO1	IDO1
IFNAR1	IFNAR1
IFNG	IFNG
IFNGR1	IFNGR1
IL12B	IL12B
IL15	IL15
IL6	IL6
ITGAM	ITGAM
ITGAV	ITGAV
ITGAX	ITGAX
ITGB2	ITGB2
ITGB8	ITGB8

KRT	KRT18,KRT10,KRT17,KRT19,KRT6A,KRT6B,KRT6C,KRT7,KRT14
LAG3	LAG3
LY6E	LY6E
MKI67	MKI67
MS4A1	MS4A1
NKG7	NKG7
pan-melanocyte	PMEL,S100B,SOX10
PDCD1	PDCD1
PDCD1LG2	PDCD1LG2
PECAM1	PECAM1
PSMB10	PSMB10
PTEN	PTENP1,PTEN
PTPRC	PTPRC
STAT1	STAT1
STAT2	STAT2
STAT3	STAT3
TBX21	TBX21
TIGIT	TIGIT
TNF	TNF
TNFRSF9	TNFRSF9
VEGFA	VEGFA
VSIR	VSIR
Internal Reference Genes	
OAZ1	OAZ1
POLR2A	POLR2A
RAB7A	RAB7A
SDHA	SDHA,SDHAP1,SDHAP2,LOC220729,SDHAP3
UBB	UBB,UBBP4
Negative Controls	
NegPrb1	
NegPrb2	
NegPrb3	
NegPrb4	
NegPrb5	
NegPrb6	

2.2 Cancer Transcriptomic Atlas (CTA)/Next Generation Sequencer: +1,800 genes

Signaling pathways covered by CTA		
Table 1. Adaptive Immunity	**Table 4. Cell Function**	**Table 7. Signaling Pathways**
Adaptive Immunity	Cell Function	Signaling Pathways
B cells	Apoptosis	AMPK Signaling
BCR Signaling	Autophagy	Androgen Signaling

Cancer Antigens
MHC Class I Antigen Presentation
MHC Class II Antigen Presentation
T-cell Checkpoints
T cells
TCR Signaling
TH1 Differentiation
TH17 Differentiation
TH2 Differentiation
TH9 Differentiation
Treg Differentiation

Table 2. Innate Immunity
Innate Immunity
Complement System
Dendritic Cells
DNA Sensing
Glycan Sensing
Host Defense Peptides
Inflammasomes
Myeloid Inflammation

Neutrophil Degranulation
NK Activity
NLR Signaling
RAGE Signaling
RNA Sensing
TLR Signaling

Table 3. Immune Response
Immune Response
Chemokine Signaling

Cytotoxicity
IL-1 Signaling
IL-17 Signaling

IL-2 Signaling
IL-6 Signaling
Immune Exhaustion
Interferon Response Genes
Lymphocyte Regulation
Lymphocyte Trafficking
NF-kB Signaling
Other Interleukin Signaling
Prostaglandin Inflammation
TNF Signaling
Type I Interferon Signaling
Type II Interferon Signaling

Cell Adhesion & Motility

Cell Cycle

Cilium Assembly
Differentiation
DNA Damage Repair
EMT
Endocytosis
Epigenetic Modification
Immortality & Stemness
Ion Transport
Lysosome
Oxidative Stress
Phagocytosis
Proteotoxic Stress
RNA Processing
Senescence

Table 5. Metabolism
Metabolism
Amino Acid Synthesis & Transport
Arginine Metabolism

Fatty Acid Oxidation
Fatty Acid Synthesis
Glutamine Metabolism
Glycolysis & Glucose Transport
Glycosylation
IDH1/2

Lipid Metabolism
Mitochondrial Metabolism / TCA
Nucleotide Synthesis
Pentose Phosphate
Tryptophan & Kynurenine Metabolism
Vitamin & Cofactor Metabolism

Table 6. Physiology & Disease
Physiology & Disease
Angiotensin System
Circadian Clock
Drug Resistance
Glioma
Leukemia
Matrix Remodeling and Metastasis
Melanoma
Neuroendocrine Function
Prostate Cancer

EGFR Signaling

ERBB2 Signaling

Estrogen Signaling
FGFR Signaling
FoxO Signaling
GPCR Signaling
Hedgehog Signaling
HIF1 Signaling
Insulin Signaling
JAK-STAT Signaling
MAPK Signaling
MET Signaling
mTOR Signaling
Myc
NO Signaling
Notch Signaling
p53 Signaling
PDGF Signaling
PI3K-Akt Signaling
PPAR Signaling
Purinergic Signaling
Retinoic Acid Signaling
TGF-beta Signaling
VEGF Signaling
Wnt Signaling

Table 8. Tissue Comparment
Tissue Compartment
Tumor
Immune

Stroma

Table 9. Biological Category
Tissue Compartment
Tumor Biology
Immune Response
Microenvironment

Type III Interferon Signaling

---END---

Annex 3. Checklist for selection of cases and tissue qualification for DSP assay

GeoMX DSP check list for selection of cases and tissue qualification

DSP project #

1. **Pathology assessment of samples:**

Check if the following steps have been completed:

__ H&E slides scanned in Aperio System.
-Aperio Project ID: _________________
__File with Clinicopathological information received
Check if the following information was received:
__MRN
__Tissue ID
__Time Point. If the patient has two TPs (TP0, TP1), check the experimental design for specific instructions on sample selection.
__Final Diagnosis. (Apollo provides with Primary and Reviewed diagnosis).
__Organ site.
__Type of biopsy (surgical specimen, core biopsy, endoscopic biopsy, TMA) (A map of the TMA must be requested)
__Localization (primary organ vs metastasis in lymph nodes or other organs).
__Other

2. **Quality control:**

Check if the following information was evaluated

__Presence of tumor cells (Yes/No): > than 100 viable tumor cells.
__Presence of normal adjacent tissue.
__Presence of Tumor associated Tertiary lymphoid aggregates (TLS).
__Presence of Invasive margin (IM).
__% of tumor infiltrating lymphocytes (TILs)*.
__Comments: Annotate potential presence of artifacts that can interfere with the staining (background) including blood cells, fibrin, bone or necrosis. Areas with artifacts, mucin or necrosis as well as samples with inadequate processing issues will be excluded for the further selection of regions of interest (ROIs).

3. **Tissue qualification for DSP assay:**

Check if the following steps have been completed:

__ Sample selected for DSP assay annotated.
__ROI plan annotated : estimated number of ROI according to research strategy and type of biopsy.
__AOI plan annotated: estimated number of AOI based in segmentation strategy and ROI.

4. **Requisition of material to perform DSP assay:**

Check if the following steps have been completed:

___Unstained sections requested (Date)

5. **Storage of slides**:

Check if the following steps have been completed:

___Unstained slides received.

Date:
Location:
Total number of sections:_____
-Number of sections per sample/case
Total number of sample/cases:

6. **Quality assurance of unstained sections:**

Check if the following steps have been completed:
Unstained sections are smilar to H&E previously reviewed?
-Yes.
-No.

Note: If unstained section varies significatively to the H&E slide reviewed, an H&E of a serial section should be requested for optimal ROI selection.

Number of slides with adequate tissue sections: ____
(Tissue sections do not have excessive folding or dettachment. Tissue section fit the DSP scanning area. There is not tissue in the tip calibration area)

Slide with issues:_____
__Tissue sections are suboptimal. DSP can be performed with limitations:
Slide ID:
__Tissue sections are not adequate. DSP cannot be performed:
Slide ID:
The following issues have being observed:
__Tissue sections have excessive folding
__Tissue sections have extensive dettachment.
__Tissue sections do not fit the DSP scanning area.
__There is tissue in the tip calibration area.
__Other ………..
___ unstained section selected for DSP assay.

--END--

Annex 4: Selection of ROI in the scanned images using GeoMx Digital image analysis software

Selection of ROI and segmentation using the GeoMx Digital image analysis software

1. Prepare pre-assessment files including excel file containing information of samples and H&E images with the potential placement of ROI. H&E image slides can be visualized using Aperio Image Scope (eSlide manager) or Halo (IndicaLab). If a grid with Tumor Infiltrating Lymphocytes (TILs) % was prepared, open file containing the information.

2. Log in into GeoMx DSP software through Google Chrome based app

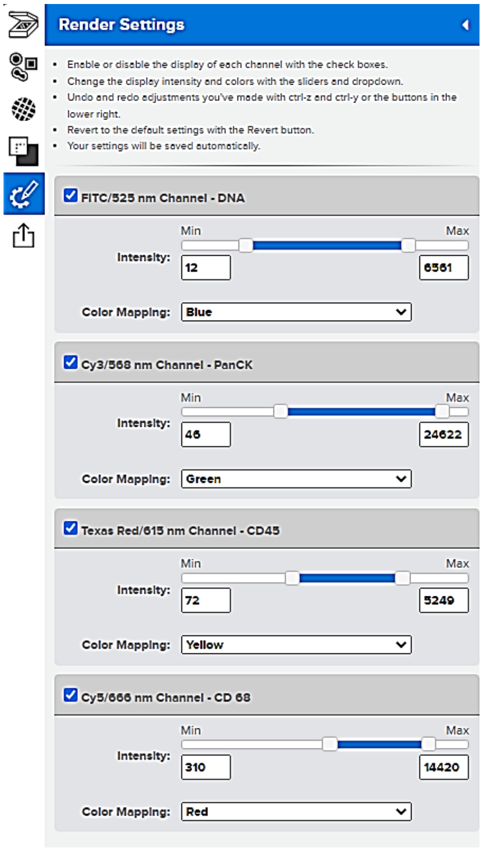
   https://10.104.100.32/login (DSP#1)
   https://10.104.100.33/login (DSP#2)

3. Search for the scanned slide at the bottom of the screen (from slot 1 to slot 4), and click on the selected slide. The image should consist of immune fluorescent slide with multiple channels.

4. Review that the DSP ID (located at the top left of the image) and scan number information (S1;, S2, etc.) in the image slide match the corresponding ID from file.

5. Adjust intensity of the IF staining by scrolling to desire channel and adjusting threshold. Pick desired color for the selected channer image. Compare the scanned tissue with the H&E slide for orientation (screenshot 1). The laboratory should have provided information on the morphology markers, fluorophores and DSP channel.

Screenshot 1



Render Settings

- Enable or disable the display of each channel with the check boxes.
- Change the display intensity and colors with the sliders and dropdown.
- Undo and redo adjustments you've made with ctrl-z and ctrl-y or the buttons in the lower right.
- Revert to the default settings with the Revert button.
- Your settings will be saved automatically.

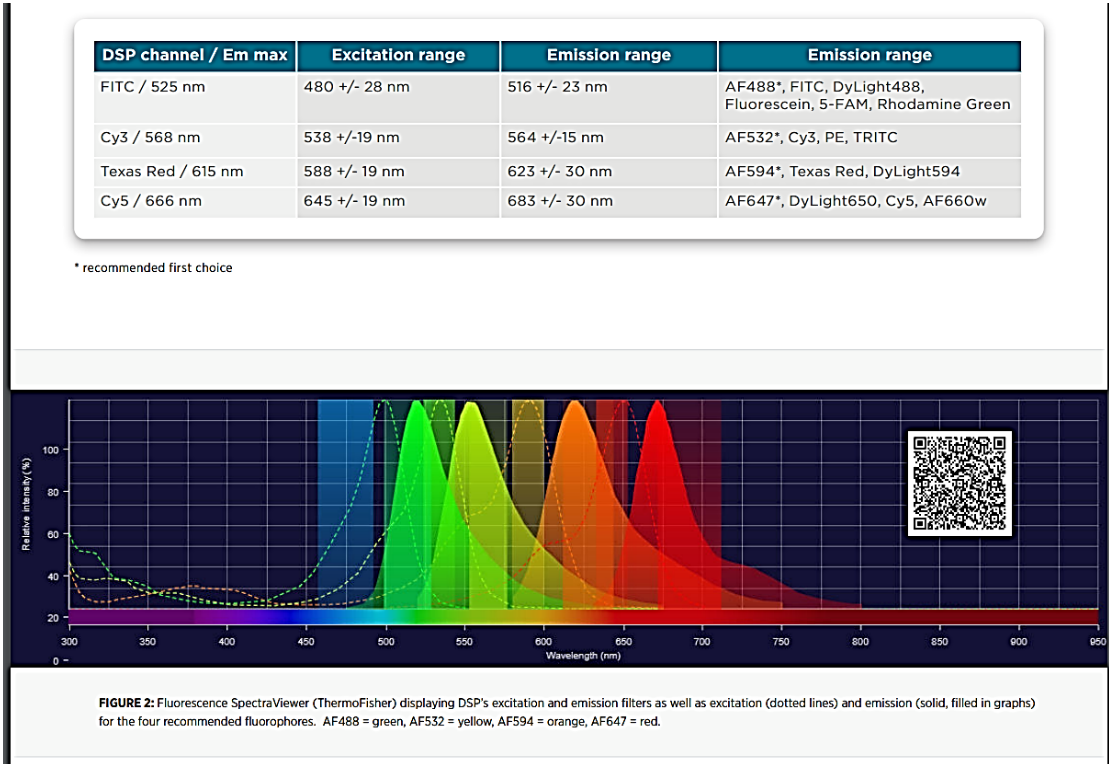
☑ FITC/525 nm Channel - DNA
Intensity: Min 12, Max 6561
Color Mapping: Blue

☑ Cy3/568 nm Channel - PanCK
Intensity: Min 46, Max 24622
Color Mapping: Green

☑ Texas Red/615 nm Channel - CD45
Intensity: Min 72, Max 5249
Color Mapping: Yellow

☑ Cy5/666 nm Channel - CD 68
Intensity: Min 310, Max 14420
Color Mapping: Red

6. A quick guide for DSP channels and fluorophores is provided below (screenshot 2).
   Screenshot 2:



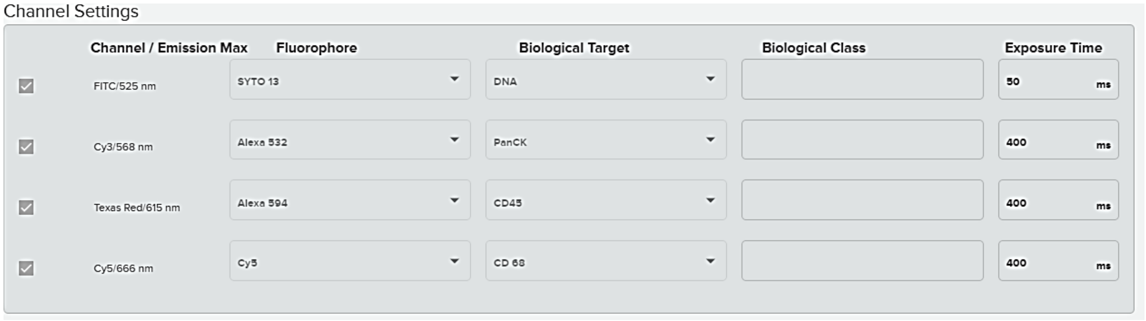
DSP channel / Em max	Excitation range	Emission range	Emission range
FITC / 525 nm	480 +/- 28 nm	516 +/- 23 nm	AF488*, FITC, DyLight488, Fluorescein, 5-FAM, Rhodamine Green
Cy3 / 568 nm	538 +/-19 nm	564 +/-15 nm	AF532*, Cy3, PE, TRITC
Texas Red / 615 nm	588 +/- 19 nm	623 +/- 30 nm	AF594*, Texas Red, DyLight594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

7. The information of any specific slide can be accessed by clicking in “scan parameters” (screenshot 3)

<u>Screenshot 3:</u>

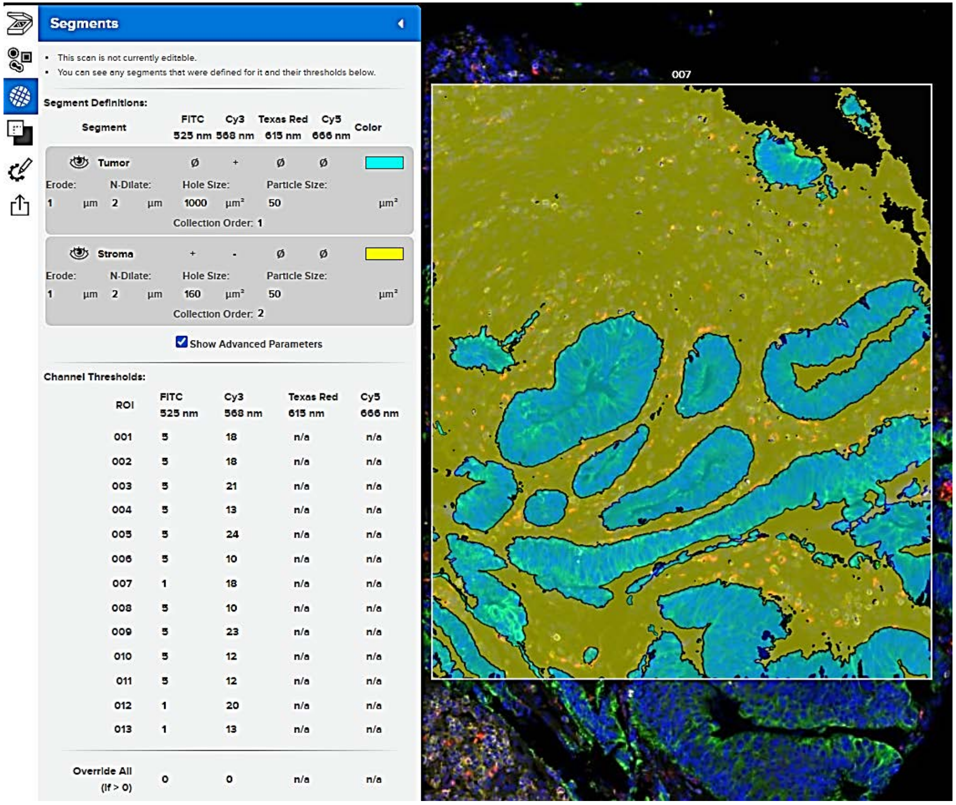


Channel Settings

	Channel / Emission Max	Fluorophore	Biological Target	Biological Class	Exposure Time
☑	FITC/525 nm	SYTO 13	DNA		50 ms
☑	Cy3/568 nm	Alexa 532	PanCK		400 ms
☑	Texas Red/615 nm	Alexa 594	CD45		400 ms
☑	Cy5/666 nm	Cy5	CD 68		400 ms

8. Select all ROIs in the sample following the experimental design strategy. Note that areas with artifacts should be excluded.
9. Generate segments (e.g. Tumor; Stroma; and Immune) according to the experimental design strategy and replicate the same strategy for segment generation including order and segment definitions in each sample from the same experiment to keep consistency.
    - Depending of the type of sample, additional segments can be generated to exclude background or empty spaces based on biomarker expression patterns (e.g. Glass; Elastic fibers; Blood).
    - Pick a desired color per segment; we recommend using different colors to segment mark-ups to have a better contrast with the visualization markers.
10. Click in advanced parameters for segment generation: Choose desired Hole size and Particle size settings (The initial settings recommended for tumor segment is Hole size:1000 and Particle size:50; and for Stroma or Immune segments Hole size: 160 Particle size: 10 (immune) or 50 (stroma); Erode um: 1.). Settings can be tailored to specific projects based in biomarker and architectural patterns of biological compartments).
11. Save the changes.
12. Adjust immunofluorescence intensities thresholds by each ROI to reach the best segmentation parameters. Turn on and off the mark-ups to make sure segmentation parameters are optimal.
13. Note that changing parameters for segmentation after this step will reset IF intensities and thresholds should be adjusted accordingly.
14. Example of Segment definitions and channel thresholds is shown in screenshot 4:

Screenshot 4:



- This scan is not currently editable.
- You can see any segments that were defined for it and their thresholds below.

Segment Definitions:

Segment	FITC 525 nm	Cy3 568 nm	Texas Red 615 nm	Cy5 666 nm	Color
Tumor	Ø	+	Ø	Ø	🟦
Stroma	+	-	Ø	Ø	🟨

Tumor: Erode: 1 µm N-Dilate: 2 µm Hole Size: 1000 µm² Particle Size: 50 µm²
Collection Order: 1

Stroma: Erode: 1 µm N-Dilate: 2 µm Hole Size: 160 µm² Particle Size: 50 µm²
Collection Order: 2

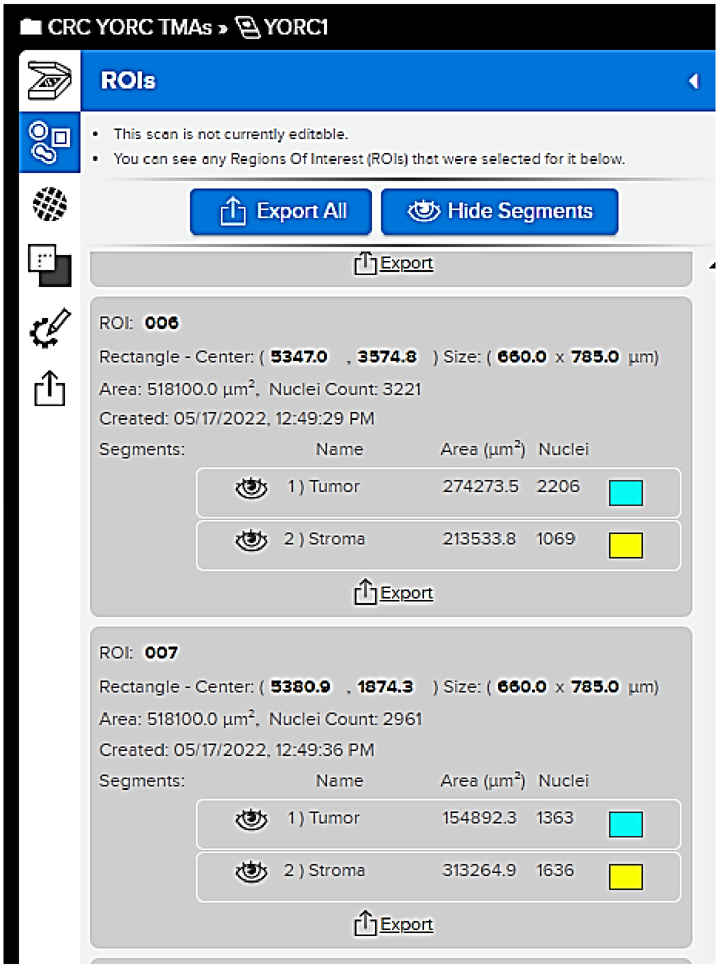
☑ Show Advanced Parameters

Channel Thresholds:

ROI	FITC 525 nm	Cy3 568 nm	Texas Red 615 nm	Cy5 666 nm
001	5	18	n/a	n/a
002	5	18	n/a	n/a
003	5	21	n/a	n/a
004	5	13	n/a	n/a
005	5	24	n/a	n/a
006	5	10	n/a	n/a
007	1	18	n/a	n/a
008	5	10	n/a	n/a
009	5	23	n/a	n/a
010	5	12	n/a	n/a
011	5	12	n/a	n/a
012	1	20	n/a	n/a
013	1	13	n/a	n/a
Override All (If > 0)	0	0	n/a	n/a

15. Exclude any segment generated that do not qualify for DSP analysis including segments with suboptimal number of cells and segments generated to avoid artifacts (e.g. glass, elastic fibers)
16. Review the count of nuclei by segment and exclude any segment with less than 20 nuclei for protein and less than 200 cells for RNA. DSP counts of nuclei may show an inaccurate nuclei count, then it is recommended to confirm number of nuclei by direct observation of image (screenshot 5).

Screenshot 5



- This scan is not currently editable.
- You can see any Regions Of Interest (ROIs) that were selected for it below.

ROI: **006**

Rectangle - Center: (**5347.0** , **3574.8**) Size: (**660.0** x **785.0** µm)

Area: 518100.0 µm², Nuclei Count: 3221

Created: 05/17/2022, 12:49:29 PM

Segments:	Name	Area (µm²)	Nuclei	
	1) Tumor	274273.5	2206	🟦
	2) Stroma	213533.8	1069	🟨

Export

ROI: **007**

Rectangle - Center: (**5380.9** , **1874.3**) Size: (**660.0** x **785.0** µm)

Area: 518100.0 µm², Nuclei Count: 2961

Created: 05/17/2022, 12:49:36 PM

Segments:	Name	Area (µm²)	Nuclei	
	1) Tumor	154892.3	1363	🟦
	2) Stroma	313264.9	1636	🟨

Export

17. Once this is finalized and segments have passed pathology QC, send AOI for collection.
18. Continue the selection of ROI and segmentation in the next slide.
19. Communicate to the lab when Selection of ROI have been completed, so the lab can proceed with hybridization and proper steps for mapping back the DSP counts to image slide.
20. After the DSP counts are mapped back to DSP device. An excel file should be ready in order to merge pathological annotation details to the GeoMx DSP analysis suite software. This file can be filled in while performing segmentation or after the DSP counts are mapped back in order to confirm ROI and segment information.
21. The excel file should contain the following information.
    a. Batch_HE: *Batch number of H&E slides received*
    b. Slide_ID_DSP: *This Slide ID must match the Slide ID used to label the slides scanned in the DSP device. Of note, a second scan or repeated cases of the same sample need to be coded with "t" or "s".*
        i. "t" *if new sections from the same block is stained and scanned.*
        ii. *"s" if new scan from the same stained slide is performed (e.g. PA03, PA03s2)*
    c. ROI (label): *This cell must match ROI (label) in the DSP annotated regions. It needs to be entered with apostrophe (e.g. '001) to match DSP template file*
    d. Segment (Name / Label): *This cell must match Segment label used when segmenting images in the DSP device.*
    e. Case_ID: *Unique identification of the sample included in the analysis.*
    f. TID: *Tissue ID provided by collaborators.*

g. Primary_Dx: *Information extracted from collaborators.*
h. Strategy: *Indicates the strategy used in each ROI (e.g. Tumor vs. Stroma; tumor vs. Immune, no segmentation).*
i. ROI_Type: e.g. *Intratumoral vs Invasive margin vs. TLS per experimental design strategy.*
j. Compartment: *Biological compartment after segmentation or the whole ROI if not segmented (e.g. tumor vs. Immune).*
k. QC_CK: *Quality of staining of tumor biomarker (e.g. Cytokeratin) (Ok, background; low).*
l. QC_CD45: *Quality of staining of CD45 in immune cells (Ok, background; low).*
m. QC_Number_nuclei_Immune: *Quality control of the number of nuclei obtained from the "Immune"segment by DSP platform (visual assesment; e.g. ok/hypersegmentd/hiposegmented).*
n. QC_Number_nuclei_Tumor: *Quality control of the number of nuclei obtained from the "Tumor" segment by DSP platform (visual assesment; e.g. ok/hypersegmented/hiposegmented).*
o. QC_Number_nuclei_Stroma: *Quality control of the number of nuclei obtained from the "Stroma" segment by DSP platform (visual assesment; e.g. ok/hypersegmented/hiposegmented).*

---END---