IN-DEPTH: Polaris-GeoMx protocol

This protocol outlines the required reagents and step-by-step procedures for performing Orion imaging followed by GeoMx spatial transcriptomics on the same tissue section. This was originally described in the publication "*Same-Slide Spatial Multi-Omics Integration Reveals Tumor Virus-Linked Spatial Reorganization of the Tumor Microenvironment"*, with modifications adapted from protocols provided by Polaris (Akoya Biosciences) and Bruker Corporation (NanoString, GeoMx). Please cite when using this protocol.

Reagents checklist:

Reagent	**Catalog #**	**Storage**
Dako pH9.0 Antigen Retrieval Buffer	Agilent (S236784-2)	4ºC
Nuclease-free water	Invitrogen (10977015)	RT
10X PBS (pH 7.4)	Gibco (70011069)	RT
Xylene	Sigma (534056)	RT
100% Ethanol	Fisher Scientific (07-678-007)	RT
Opal 7-color automation IHC kit (Opal PKI Blocking Buffer, Opal Fluorophore reagents, Spectral DAPI solution)	Akoya Biosciences (NEL821001KT)	4ºC
Bond Wash solution	Leica Biosciences (AR9590)	4ºC
Bond Epitope retrieval solution ER1 low pH	Leica Biosystems (AR9961)	4ºC
20X SSC Buffer	Sigma (SRE0068)	RT
HybriSlip hybridization covers (22 mm x 40 mm x 0.25 mm)	Grace Bio-Labs (714022)	RT
100% formamide	ThermoFisher (AM9342)	4ºC
GeoMx Instrument Buff Kit PCLN (Buffer S, Buffer R)	NanoString (100474)	RT for Buffer S, 4ºC for Buffer R
GeoMx Whole Transcriptome Atlas - Human RNA for NGS	NanoString (121401102)	-20ºC
GeoMx Nuclear Stain Morphology Kit (SYTO13)	NanoString (121300303)	-20ºC
DSP collection plate	NanoString (100473)	RT
96-well PCR plate	Applied Biosystems (4483354)	RT
Plate seal	Applied Biosystems (4306311)	RT
GeoMx seq Code Pack - Primer plates: AB/CD/EF/GH Seq Code Master Mix	NanoString (121400202/121400203/121400204)	-20ºC
AMPure beads	Beckman Coulter (A63881)	4ºC
DNA LoBind tubes (1.5 ml)	Eppendorf (022431021)	RT
Elution Buffer (0.05% Tween-20, 10mM Tris pH 8.0 in nuclease-free water)	/	RT
Hematoxylin	StatLab (HXMMHPT)	RT
Blueing Solution	StatLab (HXB00588E)	RT
Eosin Y Solution	StatLab (STE0243)	RT

DAY 1

Tissue preparation and antibody staining

1. **Deparaffinize:** *Time needed: 1 hour*
   a. Bake FFPE tissue sections in an oven at 70°C for 1 hr.

2. While the tissue sections are baking, prepare the PT module for use later:
   a. Prepare 1X Dako pH 9.0 Antigen Retrieval Buffer from the 10X stock using nuclease-free water in a slide holder.
   b. Cover the slide holder with a lid or tin foil to prevent contamination of buffer with surrounding PBS.
   c. Transfer the slide holder containing the buffer into the PT module.
   d. Set the following retrieval program:

Prewarm	75°C
Heat	97°C, 20 mins
Cool	65°C

   e. Ensure that the PT module is filled with enough 1X PBS.
   f. Start prewarming the PT module.

3. **Rehydration:** *Time needed: ~45 mins*
   a. Prepare 95%, 80% and 75% EtOH by diluting 100% EtOH with nuclease-free water.
   b. Examine the tissue sections to ensure all the FFPE wax has melted.
   c. Incubate the tissue sections twice with xylene for 5mins each.
   d. Dip the tissue sections for 10 times every 30 second for a total of 3 mins each following exactly the order listed below:
      i. Xylene (x3 rounds)
      ii. 100% EtOH (x2 rounds)
      iii. 95% EtOH (x2 rounds)
      iv. 80% EtOH
      v. 70% EtOH
      vi. ddH_2O (x3 rounds)

4. **Heat-Induced Epitope Retrieval:** *Time needed: ~1 hour*
   a. Transfer the tissue sections to the prewarmed slide holder containing 1X Dako pH 9.0 Antigen Retrieval Buffer in the PT module prepared in Step.2, and begin the retrieval program.
   b. After the PT module cools to 65°C, remove the slide holder together with the tissue sections from the PT module.
   c. Place the slide holder (with the tissue sections inside) onto a lab bench.
   d. Allow it to cool for 15 mins.

5. **Wash:** *Time needed: ~5 mins*
   a. Wash tissue sections with 1X PBS for 5 mins on a belly dancer.

The following steps followed the Vectra Polaris protocol from Akoya Biosciences and performed on the Leica BOND RX autostainer.

6. **Reagent preparation:** *Time needed: ~ 15 mins*
   a. Thaw Opal polymer HRP and keep on ice.
   b. Dilute antibody targets and corresponding Opal polymer HRP accordingly. ***Note:*** *All antibody targets should be titrated prior to the actual experiment. Concentration may varies depending on tissue type, refer to Akoya Biosciences for recommended working concentration.*

7. **Automated steps (Immunostaining, counterstaining, epitope retrieval):** *Time needed: ~ 12 hours*
   a. Load the tissue sections into the Leica BOND RX autostainer accordingly.
   b. Begin the program with the following steps (all steps are with 150µl of solution):
      i. Wash with Bond Wash Solution twice at RT.
      ii. Incubate with PKI Blocking Buffer once for 5 mins at RT.
      iii. Incubate with the first primary antibody target for 30 mins at RT.
      iv. Wash with Bond Wash Solution thrice at RT, with 1 min incubation during the 2nd wash.
      v. Incubate with Opal Polymer HRP corresponding to the first antibody target for 10 mins at RT.
      vi. Wash with Bond Wash Solution four times at RT, with 1 min incubation during the 2nd wash.
      vii. Incubate with Opal Fluorophore reagent for the first target for 10 mins at RT.
      viii. Wash with Bond Wash Solution four times at RT, with 1 min incubation during the 2nd wash.
      ix. Wash with Bond ER Solution 1 once at RT.

x. Wash with Bond ER Solution 1 once at 95°C.
xi. Incubate with Bond ER Solution 1 for 20 mins at 95°C.
xii. Wash with Bond ER Solution 1 once at RT.
xiii. Wash with Bond Wash Solution thrice at RT, with 1 min incubation during the 2nd wash.
xiv. Repeat Step ii – xiii for each of the antibody targets.
xv. Incubate with Spectra DAPI solution for 5 mins at RT.
xvi. Wash with Bond Wash Solution thrice at RT, with 1 min incubation during the 2nd wash.

Day 3

8. Polaris imaging: *Time needed: ~3 hours*

a. Remove the tissue section from the Leica BOND RX autostainer.
b. Transfer the tissue section to a coplin jar filled with 1X PBS. Wash tissue sections twice at RT for 5 mins each on a belly dancer at low speed.
c. Carefully wipe away excess solution from the slide using a KimWipe.
d. Mount the tissue slide with a glass coverslip with 1X PBS. Slightly seal the coverslip around with nail polish if necessary.
e. Begin imaging.

After Polaris imaging, image quality assessment will be performed. Re-imaging might be needed for markers not passing the quality check. Proceed to the next step ONLY when all markers have passed quality check. *Time needed: ~15 mins*

9. Coverslip removal after imaging:

a. Dip a Q-tip with xylene and gently remove the nail polish around the coverslip.
b. Transfer the tissue section in a coplin jar of 1X PBS.
c. Incubate for 5 mins at RT on a belly dancer at low speed.
d. Let the coverslip detach from the tissue slide by itself. Do not remove it by force as this may damage the tissue section.
e. When the coverslip has detached, transfer the tissue slide to a new coplin jar containing 1X PBS.

Note: *It is strongly recommended to perform GeoMx tissue preparation and probe hybridization immediately after Polaris imaging to preserve RNA quality.*

TISSUE PREPARATION (GeoMx)

Note: *There is no turning back once the tissue is processed for GeoMx, when ready, proceed to Step.10. All GeoMx steps are based entirely on the GeoMx protocol from Bruker Cooperation.* *Time needed: 16-24 hours*

10. Probe hybridization:

a. Turn on and pre-warm the HybEZ Oven to 37°C.
b. Prepare and thaw the following reagents:
   i. GeoMx RNA detection probes: Thaw and keep on ice. Do not vortex and re-freeze probes. Once thawed, they can be stored at 4°C for up to 3 months.
   ii. Record probe's lot number. It is needed for setting up GeoMx RNA capture in the latter steps.
   iii. Buffer R: Warm to RT. Do not vortex.
c. Prepare Hybridization solution as follows:

Hybridization solution	**2 slides + 10%**
Buffer R	60 µl
RNA detection probes	7.5 µl
Custom probes or nuclease-free water	7.5 µl *(**Note:** For 1 custom probe set, add 3.75 µl probes with 3.75 µl nuclease-free water; For 2 custom probe sets, add 3.75 µl each of the probe set.)*
Total	75 µl

d. Gently mix by pipetting without introducing any bubbles.
e. Arrange fresh Kimwipes on the bottom of the HybEZ Tray, then wet with nuclease-free water. ***Note:*** *Ensure no excess water is floating on the Kimwipes to avoid mixing with probes on the tissue section.*
f. Remove tissue section from 1X PBS, gently wipe away excess 1X PBS and place in the HybEZ Tray.
g. Slowly pipette 70 µl Hybridization solution to the tissue section without introducing any bubbles.
h. Peel off the plastic film from the Grace Bio-Labs HybriSlip and gently place the side covered by the plastic film over the tissue section without introducing any bubbles.
i. Close the hybridization chamber and incubate at 37°C overnight (16-24 hrs).

Day 4

Note: *From henceforth, minimize exposure of the tissue sections to light.*

All wash steps on a belly dancer below should be set at low speed.

Ensure that cell type annotation and the phenotype maps are ready at this point, and have a clear sense of which ROIs to select based on study design and tissue morphology.

11. Post-hybridization wash and nuclear staining: *Time needed: ~40 mins*

a. Prepare the following:
   i. 2x and 4x SSC Buffers by diluting the 20x stock in nuclease-free water.
   ii. Two coplin jars of Stringent Wash Buffer (40 ml each) by mixing 1:1 of 100% formamide with 4x SSC buffer.
   iii. Prewarm Stringent Wash Buffer in a 37°C water bath.
   iv. Remove SYTO13 from the -20°C freezer and thaw at RT. Make 100nM SYTO13 by diluting the stock in 2x SSC buffer.
   v. One coplin jar of 2x SSC buffer.
b. Remove the tissue sections from the HybEZ Oven.
c. Gently dip the tissue section in the coplin jar containing 2x SSC buffer. Allow the coverslip to fall off by itself. Proceed to the next step immediately once the coverslip has fallen off.
d. Transfer the tissue section to the pre-warmed Stringent Wash Buffer at 37°C.
e. Incubate for 5 mins.
f. Repeat incubation using the 2nd coplin jar containing the pre-warmed Stringent Wash Buffer.
g. Transfer the tissue sections to the coplin jar containing 2x SSC buffer, wash tissue sections for 5 mins on a belly dancer.
h. Stain tissue sections with the 100nM SYTO13 prepared in the previous step for 5 mins at RT in the dark.
i. Wash tissue sections twice in 2x SSC buffer for 5 mins each on a belly dancer.

12. GeoMx transcript collection: *Time needed: ~3 hours per plate*

a. Load and secure the tissue section into the slide holder accordingly. Slowly add 3ml Buffer S along the side of the holder to the tissue section. ***Note:*** *Be careful not to add directly on top of the tissue as this may cause damage.*
b. Gently clean the bottom of each tissue section with 70% EtOH using a KimWipe, then wipe off with another dry KimWipe.
c. Follow the instructions from the GeoMx DSP for scanning, image capture and drawing ROI. ***Note:*** *Exposure of 10ms for SYTO13 was used in our study.* Adjust the exposure accordingly.
d. After drawing the ROIs, export the omi.tiff files and record the x- and y-coordinates of each of the ROI drawn.
e. Generate the phenotype maps based on the ROI info and save each ROI per folder in a thumb drive. ***Note:*** *Refer to https://sizunjianglab.github.io/IN-DEPTH/ for more details.*
f. Import the phenotype maps of each of the ROI to the GeoMx DSP one by one accordingly. ***Note:*** *This is extremely important, importing all phenotype maps at once will freeze the GeoMx DSP.*
g. After importing the phenotype maps of each ROI, carefully inspect whether the alignment is correct.
h. Begin transcript collection as per GeoMx DSP instructions.
i. Replace the collection plate as soon as possible after a plate has been filled up. One full plate will take around 3 hours to complete collection.
j. Dry the plate by leaving on a clean bench top overnight or incubating in a thermocycler at 65°C for 1 hr.
k. Check that the DSP collection plate wells are completely dry and seal it with a plate seal.
l. Store the dried aspirates at -20°C until ready for library construction.

Day 5

13. <u>Library construction:</u>

Time needed: ~2 hours per plate

a. After GeoMx has finished collecting aspirates and all plates were dried, centrifuge the plates at 1000xg for 30 sec.
b. Carefully remove the plate seal and rehydrate each well with 10 µl nuclease-free water. ***Note:*** *Use different pipette tips for individual wells to avoid contamination. Well A01 of each plate is automatically assigned as NTC and it should be empty.*
c. Reseal the plate and centrifuge at 1000xg for 30 sec. Allow each aspirate to solubilized by incubating at RT for 10 mins. Centrifuge at 1000xg for 30 sec once incubation is done.
d. While incubating, thaw Primer Plates and 5X PCR Master Mix at RT. ***Note:*** *Each DSP collection plate must be assigned to a unique Primer Plate (A-H).*
e. Centrifuge Primer Plate and 5X PCR Master Mix at 1000xg for 30 sec.
f. Using a new 96-well PCR plate per collection plate, set up the PCR reaction for each well as follows:

Reaction	**Volume**
Primer	4 µl
Rehydrated aspirate	4 µl
5X PCR Master Mix	2 µl
Total	10 µl

g. Pipette 10 times to mix thoroughly and seal the PCR plates with a new plate seal.
h. Centrifuge PCR plates at 1000 xg for 30 sec.
i. Store PCR plates at 4°C until ready for reaction.
j. Begin PCR reaction in a thermal cycler with the following program:
   i. Set the lid temperature to 100 °C.
   ii. Program:

Step		**Temperature**	**Run time**
1	UDG incubation	37°C	30 mins
2	UDG deactivation	50°C	10 mins
3	Initial denaturation	95°C	3 mins
4	Denaturation	95°C	15 sec
5	Anneal	65°C	60 sec
6	Extend	68°C	30 sec
7	Return to Step 4 for a total of 18 times		
8	Final extension	68°C	5 mins
9	Hold	12°C	∞

   iii. Initiate the program.

14. Pooling and AMPure cleanup:

Time needed: ~ 90 mins

a. Once PCR is completed, centrifuge each plate at 1000xg for 30 sec.
b. For each PCR plate, pool the PCR products into two separate DNA LoBind tubes:
   i. Tube 1 (Sample): Transfer 4 µl from each sample and NTC well (A01)
   ii. Tube 2 (NTC): Transfer 6 µl from each NTC well (A01)
c. Prepare the following fresh every time:
   i. 5 ml 80% EtOH (v/v)
   ii. 1 ml Elution Buffer
d. Measure the exact volume in each LoBind tube using a pipette and record the volume. ***Note:*** *This step is critical, as it determines the volume of AMPure XP beads to be used, which directly influences the size selection of the DNA fragments during cleanup.*
e. Resuspend the AMPure XP beads by vortexing until being completely resuspended.
f. Add 1.2 times volume of AMPure XP beads into each LoBind tube containing the pooled samples accordingly.
g. Carefully pipette 10 times to mix thoroughly.
h. Pulse centrifuge the tube for 1 sec and incubate at RT for 5 mins.
i. Pellet the beads by placing the tube into a magnetic stand for 5 mins. Ensure that the solution is clear before proceeding to the next step.
j. Carefully open the tube and pipette away the solution from the tube without disturbing the beads.
k. The beads now contain the library.
l. Gently add 1 ml of 80% EtOH to each tube without disturbing the beads. Incubate for 30 sec and carefully pipette away the solution.
m. Repeat Step.18l for a total of two times. ***Note:*** *Remove any residual EtOH with a P20 pipette if needed.*
n. Air dry the beads for 5 mins in the magnetic stand. ***Note:*** *Do not dry it for over 5 mins as this will affect library recovery.*
o. Remove the tube from the magnetic stand and resuspend the beads with 54 µl of Elution Buffer. Pipette 20 times to mix thoroughly.
p. Pellet the beads by placing the tube into a magnetic stand for 5 mins. Ensure that the solution is clear before proceeding to the next step.
q. Transfer 50 µl of solution from each tube to a new LoBind tube without disturbing the beads.
r. The solution now contains the library.
s. Add 60 µl of AMPure XP beads to each tube. Carefully pipette 10 times to mix thoroughly.
t. Pulse centrifuge the tube for 1 sec and incubate at RT for 5 mins.
u. Pellet the beads by placing the tube into a magnetic stand for 5 mins. Ensure that the solution is clear before proceeding to the next step.
v. Carefully open the tube and pipette away the solution from the tube without disturbing the beads.
w. The beads now contain the library.
x. Gently add 1 ml of 80% EtOH to each tube without disturbing the beads. Incubate for 30 sec and carefully pipette away the solution.
y. Repeat Step.18l for a total of two times. ***Note:*** *Remove any residual EtOH with a P20 pipette if needed.*
z. Air dry the beads for 5 mins in the magnetic stand. ***Note:*** *Do not dry it for over 5 mins as this will affect library recovery.*
aa. Remove the tube from the magnetic stand and resuspend the according to the number of wells pooled in that tube:

Number of wells	Volume of Elution Buffer to add
96	48 µl
48	24 µl
24	16 µl
12	12 µl
NTC	5 µl

bb. Pipette 20 times to mix thoroughly.
cc. Pulse centrifuge the tube for 1 sec and incubate at RT for 5 mins.
dd. Pellet the beads by placing the tube into a magnetic stand for 5 mins. Ensure that the solution is clear before proceeding to the next step.
ee. Transfer as much solution from each tube to a new LoBind tube without disturbing the beads.
ff. The solution now contains the library.
gg. Analyze sample purity and concentration using Tapestation or Bioanalyzer. The sequencing library can be stored at -20C until it is ready for sequencing.

After sequencing sample QC

15. Perform H&E staining and imaging:

a. Incubate tissue section with Hematoxylin solution for 5 mins at RT.
b. Wash tissue section by gently dipping it in a jar filled with nuclease-free water for 15 times. Repeat this step with new jar of nuclease-free water until the solution is clear of Hematoxylin.
c. Remove tissue section from the jar and gently wipe away the excess water using a Kimwipe.
d. Incubate tissue section with Blueing solution for 1 min, RT.
e. Wash tissue section by gently dipping it in a jar filled with nuclease-free water for 15 times. Repeat this step for 2 more times using a new jar of nuclease-free water each time.
f. Remove tissue section from the jar and gently wipe away the excess water using a Kimwipe.
g. Submerge tissue section in a jar filed with 95% Ethanol and incubate for 1 min at RT.
h. Remove tissue section from the jar and gently wipe away the excess ethanol using a Kimwipe.
i. Incubate tissue section with Eosin solution for 1 min at RT.
j. Wash tissue section by gently dipping it in a jar filled with 95% Ethanol for 12 times. Repeat this step for 2 times using a new jar of 95% Ethanol each time.
k. Dehydrate tissue section by gently dipping it 12 times in 80% Ethanol followed by 95% Ethanol, 100% EtOH and finally with xylene.
l. Mount the tissue section with a glass coverslip using xylene-based mounting solution.
m. Allow the mount to adhere by laying the slide flat for at least 1 hr.
n. Image the tissue section using a slide scanner.