IN-DEPTH: Orion-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 RareCyte (Orion) and Bruker Corporation (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
AF Quench buffer (4.5% H2O2, 24mM NaOH in 1X PBS)	/	4ºC
Surfactant Wash Buffer (in wash bottle) (0.025% Triton X-100 in 1X PBS)	/	RT
Image-iT FX Signal Enhancer	Thermo Fisher (I36933)	RT
Orion ArgoFluor-conjugated Primary Antibodies (of choice)	RareCyte	4ºC
Nuclear staining cocktail (Hoescht 33342)	Invitrogen (H3570)	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.

Optional: Circle tissue area with Pappen.
***Note:** Do not draw too close to the tissue area as this might affect subsequent staining procedures.*

The following steps followed Orion protocol from RareCyte.

6. **AF quenching with White light and UV light:** *Time needed: ~ 2 hours*
   a. Place the Light Box Risers on each corner of the lower White Light Box.
   b. Place the microtiter plate lids on the lower White Light Box, each lid can support a maximum of 4 tissue sections.
   c. Pour 40ml of AF Quench buffer into each microtiter plate lid.
   d. Remove tissue sections from 1X PBS and carefully wipe away excess solution with KimWipe.
   e. Lay and submerge the tissue section flat (with tissue facing upward) in the microtiter plate lid with AF Quench buffer.
   f. Move on to the next tissue section if applicable.
   g. Ensure all tissue sections are completely submerged in the AF Quench buffer.
   h. Place an additional microtiter plate lid over the original to form a quenching chamber.
   i. Place upper White Light Box on Risers with light surface facing down towards to the quenching chamber.
   j. Begin quenching for 60 mins.
   k. After that, transfer the quenching chamber to the UV Transilluminator.
   l. Close the Transilluminator lid and quench for 30 mins at 365 nm HIGH setting.
   m. Prepare three coplin jars filled with 1X PBS.

n. After quenching is completed, remove tissue sections from the quenching chamber and carefully wipe away excess solution from the slide using a KimWipe.
o. Transfer the tissue section to the coplin jar filled with 1X PBS.
p. Top off 1X PBS to fully cover the top of the slides.
q. Incubate for 1 min on a belly dancer at low speed.
r. Repeat Step.6n-q for a total of two times with fresh 1X PBS each time.
s. Transfer the tissue section to a new jar of 1X PBS and proceed to the next section.

Note: *Ensure temperature is below 37°C throughout both quenching process. Stop the procedure and let the tissue to cool for 10 mins if the temperature goes beyond 37°C.*

AF Quench buffer should be completely removed; any residual buffer will degrade the fluorophores. Change gloves between washes to ensure no residual buffer is carried over to the next washes.

7. **Blocking:** *Time needed: ~20 mins*
   a. Remove slides from 1X PBS and carefully remove excess buffer using a KimWipe.
   b. Place the tissue section flat on a humid box with the tissue facing upwards.
   c. Gently add Surfactant Wash Buffer onto the tissue section. Decant the buffer by pouring the buffer out while securing the tissue section.
   d. Carefully wipe the back and side of the tissue slide with KimWipe.
   e. Place the tissue section flat on a humid box with the tissue facing upwards.
   f. Add 4-6 drops (~200-300µl) of Image-iT FX Signal Enhancer to cover the tissue section.
   g. Close the lid of the humid box and let incubate for 15 mins at RT.

8. **Labeled Primary Antibody Cocktail staining:** *Time needed: ~130 mins*
   a. Gently add Surfactant Wash Buffer onto the tissue section. Decant the buffer by pouring the buffer out while securing the tissue section. Repeat for a total of two washes.
   b. Carefully wipe the back and side of the tissue slide with KimWipe.
   c. Place the tissue section flat on a humid box with the tissue facing upwards.
   d. Pipette 300µl of Labeled Primary Antibody Staining Cocktail (including Orion ArgoFluor-conjugated Primary Antibodies and Hapten (DIG/Biotin)-conjugated primary antibodies) per tissue section.
   e. Close the lid of the humid box and let incubate for 120 mins at RT.

9. **Nuclear/Secondary Cocktail staining:** *Time needed: ~40 mins*
   a. Gently add Surfactant Wash Buffer onto the tissue section. Decant the buffer by pouring the buffer out while securing the tissue section. Repeat for a total of two washes.
   b. Carefully wipe the back and side of the tissue slide with KimWipe.
   c. Place the tissue section flat on a humid box with the tissue facing upwards.
   d. Pipette 300µl of Nuclear/Secondary Staining Cocktail (including Hoechst 33342 and ArgoFluor-conjugated anti-DIG or Streptavidin Secondaries) per tissue section.
   e. Close the lid of the humid box and let incubate for 30 mins at RT.
   f. Gently add Surfactant Wash Buffer onto the tissue section. Decant the buffer by pouring the buffer out while securing the tissue section.
   g. Remove the tissue sections from the humid box and transfer it to a coplin jar filled with 1X PBS.

10. **Orion imaging:** *Time needed: ~1-2 days*
   a. Carefully wipe away excess solution from the slide using a KimWipe.
   b. Mount the tissue slide with a glass coverslip with 1X PBS. Slightly seal the coverslip around with nail polish if necessary.
   c. Begin imaging.

Note: *This differs from the standard Orion protocol, where tissue is typically dehydrated and mounted using mounting media.*

Day 3

After Orion 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*

11. **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 Orion 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.12. All GeoMx steps are based entirely on the GeoMx protocol from Bruker Cooperation.*

Time needed: 16-24 hours

12. 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.*

Time needed: ~40 mins

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.

13. Post-hybridization wash and nuclear staining:

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.

14. 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. *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 65ºC 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 -20ºC until ready for library construction.

Day 5

15. Library construction:

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 4ºC until ready for reaction.
j. Begin PCR reaction in a thermal cycler with the following program:
   i. Set the lid temperature to 100 ºC.
   ii. Program:

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

   iii. Initiate the program.

Time needed: ~ 90 mins

16. Pooling and AMPure cleanup:

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:

bb. Number of wells	cc. Volume of Elution Buffer to add
dd. 96	ee. 48 µl
ff. 48	gg. 24 µl
hh. 24	ii. 16 µl
jj. 12	kk. 12 µl
ll. NTC	mm.5 µl

nn. Pipette 20 times to mix thoroughly.
oo. Pulse centrifuge the tube for 1 sec and incubate at RT for 5 mins.
pp. 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.
qq. Transfer as much solution from each tube to a new LoBind tube without disturbing the beads.
rr. The solution now contains the library.
ss. 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

17. 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.