IN-DEPTH: CODEX-CosMx protocol

This protocol outlines the required reagents and step-by-step procedures for performing CODEX imaging followed by CosMx 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 Akoya Biosciences (PhenoCycler Fusion) and Bruker Corporation (NanoString, CosMx). 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
S2 Buffer (2.5 mM EDTA, 0.5× DPBS, 0.25% BSA, 0.02% NaN3, 250 mM NaCl, 61 mM Na2HPO4, 39 mM NaH2PO4)	/	4ºC
10X PBS (pH 7.4)	Gibco (70011069)	RT
Xylene	Sigma (534056)	RT
100% Ethanol	Fisher Scientific (07-678-007)	RT
BBDG Buffer (5% normal donkey serum, 0.05% NaN3 in 1× TBS-T)	/	-20ºC
Mouse IgG (B1) (50 µg/mL mouse IgG, diluted from 1 mg/ml stock in S2 Buffer)	Sigma (I5381-10mg)	-20ºC
Rat IgG (B2) (50 µg/mL rat IgG, diluted from 1 mg/ml stock in S2 Buffer)	Sigma (I4141-10mg)	-20ºC
Sheared salmon sperm DNA (500 µg/ml)	ThermoFisher (AM9680)	-20ºC
Oligo Block Buffer (50nM, diluted from stock with 500 nM of each oligo in 1× TE pH 8.0)	/	4ºC
Antibody Diluent (5% Donkey Serum, 0.05% NaN3 in 1× TBS-T)	/	-20ºC
FFPE Block Buffer (0.05 mg/ml mouse IgG, 0.05 mg/ml rat IgG, 0.5 mg/ml ssDNA, 100nM of each oligo in S2 Buffer)	/	4ºC
Hoechst 3342 Solution	Invitrogen (H3570)	4ºC
Plate Buffer (500 µg/mL sheared salmon sperm DNA in 1× CODEX buffer)	/	4ºC
16% PFA	EMS Diasum (15740-04)	RT (4 ºC when opened)
100% MeOH (ice cold aliquot in 50ml Falcone tubes)	Sigma (179337)	-20ºC
BS3 Final fixation	ThermoFisher (21580)	-20ºC
1X CODEX Buffer (10mM Tris pH 7.5, 0.02% NaN3, 0.1% Triton X-100, 10 mM MgCl2-6H2O, 150mM NaCl)	/	RT
20X SSC Buffer	Sigma (SRE0068)	RT
10% NBF Fixation Buffer	EMS Diasum (15740-04)	RT
NBF Stop Buffer (0.1M Tris and 0.1M Glycine in nuclease-free water)	/	RT
CosMx RNA inst buffer kit	NanoString (100480)	RT
CosMx SMI Flow cell	NanoString (122000061)	RT
Cleaning Tray Kit	NanoString (122000152)	4ºC
CosMx Slide prep kit, FFPE RNA (Buffer R, CosMx RNA Blocking buffer, NHS acetate buffer, CosMx fiducials, Incubation frames, Proteinase K)	NanoString (121500006)	4ºC/-20ºC
NHS-Acetate	Thermo Scientific (26777)	-20ºC
CMx RNA 1000plx 2sl Imaging tray	NanoString (122000156)	4ºC
CMx Hs Univ Cell Panel RNA kit	NanoString (121500002)	-20ºC
CMx RNAse Inhibitor	NanoString (121500004)	-20ºC
CMx Hs Univ cell Segmentation Kit, RNA	NanoString (121500020)	-80ºC
CMx Hs CD68 Marker Kit, RNA	NanoString (121500022)	-80ºC
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.

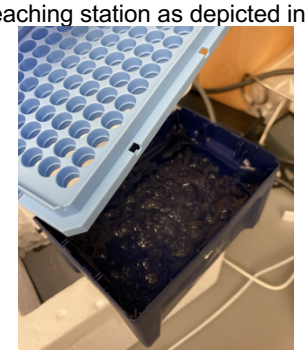
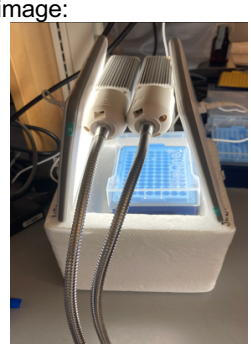
3. **Rehydration:** *Time needed: ~45 mins*
   a. Prepare 95%, 80% and 75% EtOH by diluting 100% EtOH with nuclease-free water.
   b. Examine the tissue sections to ensure all the FFPE wax has melted.
   c. Incubate the tissue sections twice with xylene for 5mins each.
   d. Dip the tissue sections for 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: ~30 mins*
   a. Wash tissue sections with S2 Buffer in a coplin jar for 20 mins on a belly dancer.
   b. Proceed to blocking buffer preparation during S2 Buffer wash.
   c. Transfer tissue sections to another coplin jar filled with 1X TBST and wash 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.*

6. **Blocking and Photobleaching:** *Time needed: ~1 hour*
   a. Remove BBDG, salmon DNA, B1 and B2 buffers from -20°C, thaw on ice. Vortex to mix.
   b. Remove Oligo block from 4 °C, keep on ice until use.
   c. Set up the blocking and photobleaching station as depicted in the image:

d. Depending on the size of the tissue, 200µl of blocking buffer is normally needed for each tissue section.
e. Prepare blocking buffer as follows:

Reagent	Stock conc.	Working conc.	Volume (1 tissue section)
BBDG	/	/	150 µl
Oligo block	500nM	50nM	20 µl
ssDNA	20X	1X	10 µl
Mouse IgG / B1	20X	1X	10 µl
Rat IgG / B2	20X	1X	10 µl

c. Add 100µl of blocking buffer to cover each tissue section.
d. Place tissue section in a humid box (with ice slurry) and photobleach it under happy light for 1 h.
e. Replenish blocking buffer to the tissue section after 30 mins or whenever necessary to prevent tissue section from drying.
f. Proceed to the next step while waiting for blocking and photobleaching to finish.

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

7. **Primary antibody preparation:** *Time needed: ~40 mins*
   a. Remove antibody diluent buffer from -20°C, thaw on ice. Vortex to mix.
   b. Remove all conjugated antibodies from 4°C and centrifuge at 12,500 x *g* for 8 mins.
   c. In an empty 1.5ml epitube, pipette and transfer 300µl of antibody diluent.
   d. Add the desired concentration of each conjugated antibody into the epitube. Mix well by pipetting gently.
   e. Pre-wet a 50-kDa centrifugal filter column with 400 µl S2 buffer and centrifuge at 12,500 x *g* for 2 mins.
   f. Carefully pipette the remaining liquid inside the filter, discard the column flow-through.
   g. Transfer the antibody mix to the pre-wetted 50-kDa centrifugal filter column and centrifuge at 12,500 x *g* for 8 mins.
   h. To collect the antibodies, invert the 50-kDa filter into a new collection tube and centrifuge at 12,500 x *g* for 1 min. ***Note:*** *Do not discard the filter at this stage.*
   i. Measure the volume of antibodies and calculate the amount of antibody diluent needed with the following formula:

      Total staining volume - volume of antibodies - ¼ total staining volume FFPE Block

   j. Add the amount of antibody diluent needed to the 50-kDa filter from Step.7h and pipette gently to collect any residual antibodies.
   k. Invert the 50-kDa filter into the collection tube containing the collected antibodies and centrifuge at 12,500 x *g* for 1 min.
   l. Add FFPE block to the collected antibodies. Mix well by pipetting gently.
   m. Pre-wet a 0.1 µm centrifugal filter column with 400 µl S2 buffer and centrifuge at 12,500 x *g* for 2 mins.
   n. Transfer the centrifugal filter into a new 1.5ml epitube. Discard the collection tube.
   o. Transfer the collected antibodies into the pre-wetted 0.1 µm centrifugal filter column.
   p. Centrifuge the 0.1 µm centrifugal filter column at 12,500 x *g* for 2 mins to remove antibody aggregates.
   q. The column flow-through now contains the antibody mix.

Note: *Antibodies should be treated with extra care. Avoid any bubbles during pipetting.*

8. **Primary antibody staining:** *Time needed: 16-24 hours*
   a. After blocking and photobleaching, remove excess blocking buffer by soaking gently with a Kimwipe.
   b. Place the tissue section in a humid box.
   c. Add the antibody mix prepared in Step.7 onto tissue.
   d. Seal the humid box with parafilm and incubate at 4°C for 16-24 h.

DAY 2-3

CODEX imaging

9. **CODEX reporter plate preparation:** *Time needed: ~30 mins*
   a. Calculate the amount of CODEX plate buffer that is needed (250 µl * total # cycles).
   b. Prepare CODEX Plate Buffer by diluting Hoechst 3342 in Plate Buffer at 1:300.
   c. Remove the reporter oligos needed from 4°C and centrifuge at 12,500 x g for 2 mins.
   d. Dilute reporter oligos at 1:10 for each cycle accordingly based on the following calculations:
      i. 250µl of Plate Buffer for blank cycles.
      ii. 247.5µl Plate Buffer for cycles with one reporter.
      iii. 245µl Plate Buffer for cycles with two reporters.
   e. Add reporter mix for each cycle accordingly to a black 96-well plate.

f. Seal the reporter plate with an Aluminum microplate seal.
g. Keep at 4°C until ready for CODEX imaging.

10. Post-stain washes and fixation: *Time needed: ~1 hour*

i. After antibody incubation has finished, remove the tissue section from the humid box.
ii. Gently wash the tissue section with S2 buffer in a coplin jar twice for 2 mins each on a belly dancer.
iii. Prepare 200µl of 1.6% PFA in 1X PBS from 16% PFA stock and 10X PBS.
iv. Remove the tissue section from the coplin jar, gently wipe off excess buffer with a Kimwipe.
v. Place the tissue section in a humid box and add 1.6% PFA, PBS onto the tissue.
vi. Cover the humid box with tin foil and incubate for 10 mins at room temperature.
vii. After incubation has finished, remove the tissue section from the humid box.
viii. Gently remove the excess 1.6% PFA, PBS by soaking with a Kimwipe.
ix. Gently rinse the tissue section with 1X PBS in a coplin jar twice and wash for 2 mins on a belly dancer.
x. Remove 100% MeOH aliquot from -20°C, fill a slider mailer and place it in ice.
xi. Transfer the tissue section from the coplin jar after wash to the slide mailer containing the ice-cold MeOH and incubate for 5 mins in ice.
xii. After incubation has finished, remove the tissue section from the MeOH and gently remove the excess MeOH by soaking with a Kimwipe.
xiii. Gently rinse the tissue section with 1X PBS in a coplin jar twice and wash for 2 mins on a belly dancer.
xiv. Prepare 150µl fresh final fixative per tissue section by diluting CODEX BS3 final fixative with 1X PBS at 1:50.
xv. Remove the tissue section from the coplin jar, gently wipe off excess buffer with a Kimwipe.
xvi. Place the tissue section on a humid box and add 75µl fresh final fixative to each tissue section.
xvii. Cover the humid box with tin foil and incubate at room temperature for 20 mins.
xviii. At the 10-min mark, add the remaining 75µl fresh final fixative to each tissue section, and continue the incubation.
xix. After incubation has finished, remove the tissue section from the humid box and gently remove the excess fixative by soaking with a Kimwipe.
xx. Gently wash the tissue section with 1X CODEX buffer in a coplin jar twice for 5 mins each.
xxi. Store the tissue section in 1X CODEX buffer at 4°C until ready for imaging.

Note: *1.6% PFA in 1X PBS must be prepared by diluting from 10X PBS.*

Final fixative BS3 must be prepared immediately before fixation.

11. Flow cell assembly: *Time needed: ~20 mins*

i. If Pappen was used to encircle the tissue area, gently scrape away the Pappen marks with the wooden end of a Q-tip. Be extremely careful not to disrupt or touch the tissue.
ii. Remove any sticker label from the tissue section. ***Note:*** *This is very important, slight elevation from the sticker will result in flow cell or sample slide breakage.*
iii. Remove flow cell from the case and dispose the plastic cover slide.
iv. Visually inspect to ensure there is no cracks on the flow cell. Use a dust blower to remove any dust on the flow cell.
v. Peel off the plastic frame that covers the adhesive on the flow cell.
vi. Place the flow cell on the stage of the Assembly Device with the adhesive side facing up.
vii. Remove the tissue section from the coplin jar and gently wipe away any excessive buffer from the slide. Be extremely careful not to disturb the tissue. ***Note:*** *Be careful not to dehydrate the tissue.*
viii. Align the tissue section with the flow cell with the sample facing down on top of the flow cell.
ix. Slide the stage all the way into the Assembly Device and lower the lever in one single action all the way down.
x. Wait for 30 secs to ensure proper seal.
xi. Raise the lever and transfer the flow cell-assembled tissue section to a coplin jar containing 1X CODEX buffer.
xii. Incubate for at least 10 mins to allow the flow cell to completely adhere to the tissue section.
xiii. The tissue section is now ready for CODEX imaging.

12. CODEX imaging *Time needed: 1-3 days*

i. Refer to PhenoCycler Fusion protocol from Akoya Biosciences for detailed procedure on setting up the software.

Note: *Exposure times for each antibody markers should be titrated prior to the actual experiment. For reference, please refer to the Supplementary Table 6_Antibody Panel from the original publication "Same-Slide Spatial Multi-Omics Integration Reveals Tumor Virus-Linked Spatial Reorganization of the Tumor Microenvironment" and previous studies from our lab (https://sizunjianglab.com/publications/).*

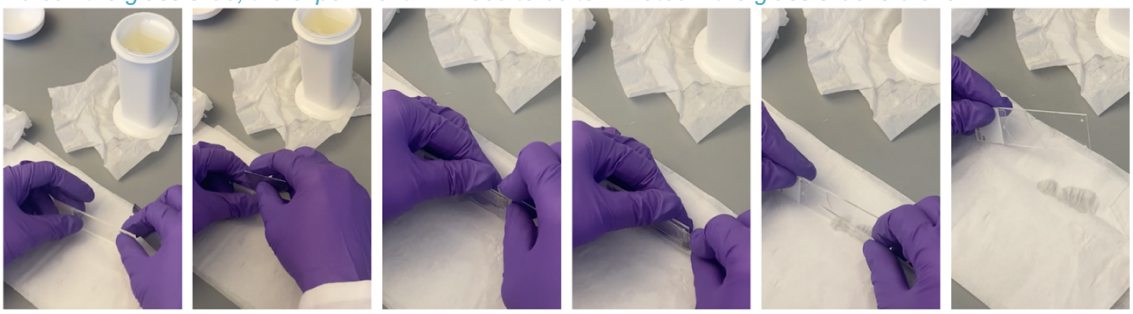
Day 4

After CODEX 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: ~30 mins*

13. Flow cell removal after fusion:

a. Prepare 2xSSC buffer by diluting 20X SSC buffer stock with nuclease-free water.
b. Remove the tissue section from the PhenoCycler Fusion slide carrier and transfer it into a coplin jar containing 2xSSC buffer.
c. Use a razor to remove the flow cell. Dip the tissue section in 2xSSC buffer as needed to ensure that the tissue section does not dry up. *Note: the flow cell will break during the process, it is completely normal.*
d. After the flow is removed from the tissue section, use a Q-tip soaked with methanol to scrap of the residual adhesive from the slide.
e. After the adhesive is removed, transfer the tissue sections into a new coplin jar containing 1X PBS.

Note: *Tutorial video on flow cell removal can be found in https://sizunjianglab.github.io/IN-DEPTH/ . Be careful not to break the glass slide, the experiment will need to be terminated if the glass slide is broken.*



It is important to remove as much residual adhesive from the tissue section as possible, remaining adhesive might affect subsequent CosMx processes.

It is strongly recommended to perform CosMx tissue preparation and probe hybridization immediately after CODEX imaging to preserve RNA quality.

TISSUE PREPARATION (CosMx)

Note: *There is no turning back once the tissue is processed for CosMx, when ready, proceed to Step.14. All CosMx steps are based entirely on the CosMx protocol from Bruker Cooperation.* *Time needed: ~1 hour*

14. Tissue digestion:

a. Prepare two coplin jars, one filled with nuclease-free water and the other with 100% EtOH.
b. Dip the tissue sections in nuclease-free water for 15 sec and in 100% EtOH for 3 mins.
c. Remove the tissue sections from 100% EtOH, carefully remove excessive EtOH with a KimWipe.
d. Lay the tissue section flat (with the tissue facing upward) on a clean KimWipe on a benchtop to allow it to air-dry for 30 mins.
e. During the drying, prepare 2µg/ml Proteinase K digestion solution by diluting the stock with 1X PBS. ***Note:*** *400µl is needed per slide, keep the dilution digestion solution in ice.*
f. Turn on and prewarm the HybEz oven to 40°C.
g. 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.*
h. After drying, carefully mount the incubation frame onto the tissue section. Ensure the frame is tightly adhere on the slide. Use a clean razor to remove excess plastic along the edges of the slide.
i. Load the tissue section into the HybEz tray.
j. Slowly pipette 400µl of Proteinase K digestion solution onto the tissue section. Ensure that the solution completely covers the tissue and is kept within the incubation frame.
k. Close the lid of the HybEz tray and place it in the HybEz oven for 15 mins.
l. While digesting, proceed to fiducials preparation in the next steps.

15. Fiducials preparation and incubation: *Time needed: ~15 mins*

a. Warm the following reagents to RT:
   i. Fiducials (protected from light).
   ii. NHS-Acetate
b. Prepare the fiducials as follows: ***Note:*** *It is important to just prepare it right before use to prevent clumping which will affect image registration.*
   i. Vortex fiducials for 1 min.

ii. Sonicate fiducials in ultrasonic bath for 2 min.
iii. Vortex fiducials for 1 min.
iv. Sonicate fiducials in ultrasonic bath for 2 min.
v. Vortex fiducials for 1 min.
vi. Dilute fiducial stock (0.1%) 1:100 to the working concentration (0.001%) in 2X SSC-T. 250µl is needed for each tissue section.
vii. Routinely vortex fiducial working solution every 30 sec to keep it suspended.

c. Prepare two coplin jars filled with nuclease-free water.
d. After digestion, tap off excess digestion solution and gently dip the tissue section 3 times in nuclease-free water.
e. Repeat by dipping the tissue section in the other jar of nuclease-free water.
f. Tap off excess nuclease-free water and lay the tissue section flat (with the tissue section facing upward) in a clean humid box.
g. Pipette 240µl of fiducial working solution to each tissue section. Ensure that the solution completely covers the tissue and is kept within the incubation frame.
h. Incubate at RT for 5 mins.
i. Prepare four coplin jars filled with 1X PBS, 10% NBF, each and two jars with NBF Stop Buffer.
j. After incubation, tap off excess fiducial solution and transfer the tissue section to 1X PBS and gently wash for 1 min on a belly dancer.
k. Proceed to fixation immediately.

16. Fixation: *Time needed: ~20 mins*

a. Transfer the tissue section to the coplin jar filled with 10% NBF, incubate at RT for 1 min.
b. Stop the fixation by gently washing the slides twice with NBF Stop Buffer at RT for 5 mins each on a belly dancer. ***Note:*** *The 2nd wash should be in a new jar of NBF Stop Buffer.*
c. Remove residual NBF Stop Buffer by washing the tissue sections in a coplin jar of 1X PBS at RT for 5 mins on a belly dancer.
d. Store the tissue section in a new jar of 1X PBS at 4°C until ready for NHS-Acetate blocking.

17. NHS-Acetate blocking: *Time needed: ~40 mins*

a. Prepare 100mM NHS-acetate mix fresh immediately before use as follows:
i. 200µl will be needed per tissue section.
ii. Multiply the weight of the NHS-acetate powder aliquot by 38.5.
iii. Dissolve the NHS-acetate powder in NHS-acetate buffer with the volume calculated.
iv. Carefully pipette to mix thoroughly while minimizing bubbles formation.
b. Remove the tissue section from 1X PBS and gently wipe away excess PBS from the slide.
c. Place the tissue section in a humid box.
d. Pipette 200µl of NHS-acetate solution to each tissue section. Ensure that the solution completely covers the tissue and is kept within the incubation frame.
e. Incubate at RT for 15 mins.
f. While incubating, prepare two coplin jars filled with 2X SSC buffer.
g. After incubation, tap off excess solution and wash tissue sections in 2X SSC buffer twice for 5 mins each on a belly dancer.
h. Store the tissue sections in new 2X SSC buffer at 4°C until ready for probe hybridization.

18. Probe hybridization: *Time needed: 16-18 hours*

a. Turn on and pre-warm the HybEZ Oven to 37°C.
b. 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.*
c. Thaw CosMx RNA Probes and RNAse Inhibitor on ice. Do not vortex. ***Note:*** *Record the lot number of probe used. Do not re-freeze probes, they can be stored at 4°C.*
d. Warm Buffer R to RT.
e. Turn on the thermal cycler and set the lid temperature to 95°C and select "Incubation" at 95°C.
f. Once the probes are thawed, centrifuge briefly and pipette 16µl RNA probes per slide to a PCR tube. ***Note:*** *If custom probes is used, 8µl per custom probe set is needed per slide.*
g. Denature the RNA probes by incubating in the thermal cycler at 95°C for 2 mins.
h. Immediately after, transfer to ice for at least 1 min.
i. Prepare Hybridization buffer as follows:

Reagents	**2 tissue sections**
Denatured probes	32µl
Denatured custom probes (optional) ***Replace with nuclease-free water if no custom probes is used*	16µl
RNAse inhibitor	3.2µl
Buffer R	256µl
Nuclease-free water	12.8µl

Total	320µl

j. Pipette to mix thoroughly without introducing any bubbles.
k. Remove the tissue section from 2X SSC buffer and carefully remove excess solution using a KimWipe.
l. Using tweezers, carefully remove the thick polyester frame backing from the incubation frame to expose top adhesive layer of incubation frame. Ensure that the incubation frame does not lift from the slide when removing the polyester frame backing.
m. Carefully pipette 150µl of Hybridization buffer to the tissue section without introducing any bubbles. Ensure that the solution completely covers the tissue and is kept within the incubation frame.
n. Gently apply the incubation cover on top.
o. Close the lid of the HybEz tray and incubate it in the HybEz oven overnight (16-18 hrs) at 37°C.

Day 5

Note: *Have a clear sense of which FOVs to select based on study design and tissue morphology.*

Time needed: ~1.5 hours

19. Post-hybridization wash:

a. Set warm bath to 37°C.
b. Thaw the Nuclear stain and Segmentation Marker kit on ice.
c. 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. Three coplin jars of 2x SSC buffer.
d. After prewarming the Stringent Wash Buffer, remove the tissue sections from the HybEz Oven.
e. Carefully remove the incubation frame cover with a forceps and immediately dip the slides in 2X SSC buffer.
f. Transfer the tissue section from 2X SSC buffer to the pre-warmed Stringent Wash Buffer and incubate for 25 mins at 37°C.
g. Repeat incubation with the 2nd jar of Stringent Wash Buffer for another 25 mins.
h. Transfer the tissue section to 2X SSC buffer and wash twice for 2 mins each on a belly dancer at low speed.
i. Store the tissue section in a new jar of 2X SSC buffer at 4°C until ready for nuclear and cell segmentation staining.

20. Nuclear and cell segmentation staining:

Time needed: ~1.5 hours

a. Prepare Nuclear Stain as follows:
   i. Vortex the tube for 15 secs and briefly centrifuge it.

Reagent	1 slide
Nuclear Stain (Ch1)	5.5µl
Blocking Buffer	214.5µl
Total	220µl

b. Remove the tissue section from 2X SSC buffer and carefully remove excess solution on the slide using a clean KimWipe.
c. Place the tissue section flat on a humid box.
i. Slowly pipette 200µl of Nuclear Stain on the tissue section. Ensure that the solution completely covers the tissue and is kept within the incubation frame.
j. Incubate at RT for 15 mins in the humid box.
d. While staining, prepare the Segmentation Stain mix as follows:

Reagent	1 slide
Segmentation Mix (CD298/B2M)	8µl
Marker Mix 1 (PanCK/CD45) (Ch3/4)	8µl
Marker Mix 2 (CD68) (Ch5)	8µl
Blocking buffer	176µl
Total	200µl

e. Prepare a coplin jar filled with 1X PBS.
f. After nuclear staining, tap off carefully soak excess stain with a KimWipe.
g. Wash the tissue section in 1X PBS for 5 mins on a belly dancer at low speed.
h. Remove the tissue section from 1X PBS and carefully remove excess solution on the slide using a clean KimWipe.
i. Place the tissue section flat on a humid box.

k. Slowly pipette 200µl of Segmentation Stain on the tissue section. Ensure that the solution completely covers the tissue and is kept within the incubation frame.
j. Incubate at RT for 1 hr in the humidity chamber.
k. While staining, warm NHS-Acetate to RT for the next part steps.
l. After staining, tap off carefully soak excess stain with a KimWipe.
m. Wash the tissue section in 1X PBS thrice for 5 mins each on a belly dancer at low speed.

21. NHS-Acetate blocking: *Time needed: ~40 mins*

l. Prepare 100mM NHS-acetate mix fresh immediately before use as follows:
   v. 200µl will be needed per tissue section.
   vi. Multiply the weight of the NHS-acetate powder aliquot by 38.5.
   vii. Dissolve the NHS-acetate powder in NHS-acetate buffer with the volume calculated.
   viii. Carefully pipette to mix thoroughly while minimizing bubbles formation.
m. Remove the tissue section from 1X PBS and gently wipe away excess PBS from the slide.
n. Place the tissue section in a humid box.
o. Pipette 200µl of NHS-acetate solution to each tissue section. Ensure that the solution completely covers the tissue and is kept within the incubation frame.
p. Incubate at RT for 15 mins.
q. While incubating, prepare two coplin jars filled with 2X SSC buffer.
r. After incubation, tap off excess solution and wash tissue sections in 2X SSC buffer twice for 5 mins each on a belly dancer at low speed.
s. Transfer the tissue sections in new 2X SSC buffer. The tissue sections are now ready for CosMx.
t. Follow instructions as per CosMx SMI.

After sequencing sample QC

22. Perform H&E staining and imaging:

Note: *H&E can be performed with or without CosMx flow cell attached on tissue slide.*

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.