IN-DEPTH: CODEX-XeniumPrime protocol

This protocol outlines the required reagents and step-by-step procedures for performing CODEX imaging followed by XeniumPrime 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 10x Genomics (XeniumPrime). 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
Hematoxylin	StatLab (HXMMHPT)	RT
Blueing Solution	StatLab (HXB00588E)	RT
Eosin Y Solution	StatLab (STE0243)	RT
Xenium Prime 5K Human Pan Tissues & Pathways Panel (2 rxns)	10x Genomics (1000724)	-20ºC
Xenium Prime Sample Preparation Reagents (2 rxns)	10x Genomics (1000720)	-20ºC
Xenium Slide Kit (2 slides)	10x Genomics (1000465)	-20ºC
Xenium Cell Segmentation Staining Reagents (2 rxns)	10x Genomics (1000661)	-20ºC
Xenium Cassette Kit v2	10x Genomics (1000723)	RT
1X TE Buffer (pH 8.0)	Invitrogen (AM9849)	RT
Protease K Solution	Thermo Fisher Scientific (AM2546)	-20ºC
10% NBF Fixation Buffer	EMS Diasum (15740-04)	RT
NBF Stop Buffer (0.1M Tris and 0.1M Glycine in nuclease-free water)	/	RT
10% Tween-20	Thermo Scientific (28320)	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.

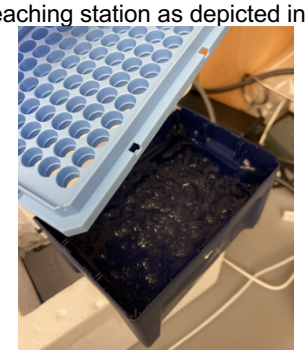
3. **Rehydration:** *Time needed: ~45 mins*
   a. Prepare 95%, 80% and 75% EtOH by diluting 100% EtOH with nuclease-free water.
   b. Examine the tissue sections to ensure all the FFPE wax has melted.
   c. Incubate the tissue sections twice with xylene for 5mins each.
   d. Dip the tissue sections for 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*
    a. After antibody incubation has finished, remove the tissue section from the humid box.
    b. Gently wash the tissue section with S2 buffer in a coplin jar twice for 2 mins each on a belly dancer.
    c. Prepare 200µl of 1.6% PFA in 1X PBS from 16% PFA stock and 10X PBS.
    d. Remove the tissue section from the coplin jar, gently wipe off excess buffer with a Kimwipe.
    e. Place the tissue section in a humid box and add 1.6% PFA, PBS onto the tissue.
    f. Cover the humid box with tin foil and incubate for 10 mins at room temperature.
    g. After incubation has finished, remove the tissue section from the humid box.
    h. Gently remove the excess 1.6% PFA, PBS by soaking with a Kimwipe.
    i. Gently rinse the tissue section with 1X PBS in a coplin jar twice and wash for 2 mins on a belly dancer.
    j. Remove 100% MeOH aliquot from -20°C, fill a slider mailer and place it in ice.
    k. 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.
    l. After incubation has finished, remove the tissue section from the MeOH and gently remove the excess MeOH by soaking with a Kimwipe.
    m. Gently rinse the tissue section with 1X PBS in a coplin jar twice and wash for 2 mins on a belly dancer.
    n. Prepare 150µl fresh final fixative per tissue section by diluting CODEX BS3 final fixative with 1X PBS at 1:50.
    o. Remove the tissue section from the coplin jar, gently wipe off excess buffer with a Kimwipe.
    p. Place the tissue section on a humid box and add 75µl fresh final fixative to each tissue section.
    q. Cover the humid box with tin foil and incubate at room temperature for 20 mins.
    r. At the 10-min mark, add the remaining 75µl fresh final fixative to each tissue section, and continue the incubation.
    s. After incubation has finished, remove the tissue section from the humid box and gently remove the excess fixative by soaking with a Kimwipe.
    t. Gently wash the tissue section with 1X CODEX buffer in a coplin jar twice for 5 mins each.
    u. 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*
    a. 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.
    b. 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.*
    c. Remove flow cell from the case and dispose the plastic cover slide.
    d. Visually inspect to ensure there is no cracks on the flow cell. Use a dust blower to remove any dust on the flow cell.
    e. Peel off the plastic frame that covers the adhesive on the flow cell.
    f. Place the flow cell on the stage of the Assembly Device with the adhesive side facing up.
    g. 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.*
    h. Align the tissue section with the flow cell with the sample facing down on top of the flow cell.
    i. Slide the stage all the way into the Assembly Device and lower the lever in one single action all the way down.
    j. Wait for 30 secs to ensure proper seal.
    k. Raise the lever and transfer the flow cell-assembled tissue section to a coplin jar containing 1X CODEX buffer.
    l. Incubate for at least 10 mins to allow the flow cell to completely adhere to the tissue section.
    m. The tissue section is now ready for CODEX imaging.

12. **CODEX imaging** *Time needed: 1-3 days*
    a. Refer to PhenoCycler Fusion protocol from Akoya Biosciences for detailed procedure on setting up the software.

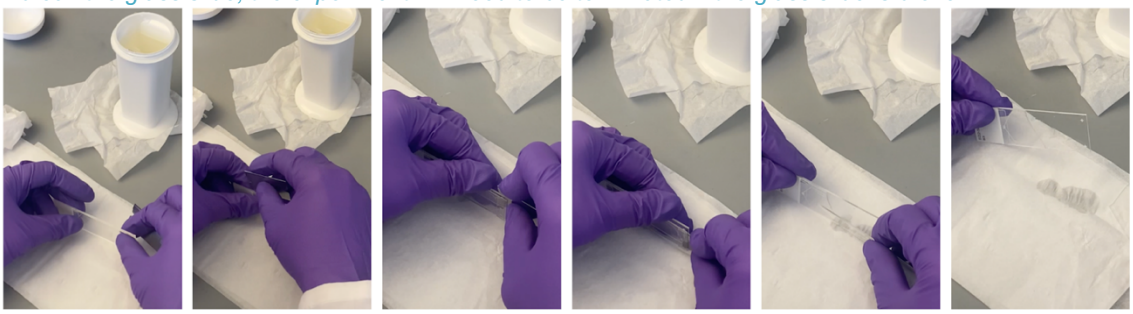
Note: *Exposure times for each antibody markers should be titrated prior to the actual experiment. For reference, please refer to the Supplementary Table 6_Antibody Panel from the original publication "Same-Slide Spatial Multi-Omics Integration Reveals Tumor Virus-Linked Spatial Reorganization of the 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 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 2xSSC buffer.

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 VisiumHD processes.

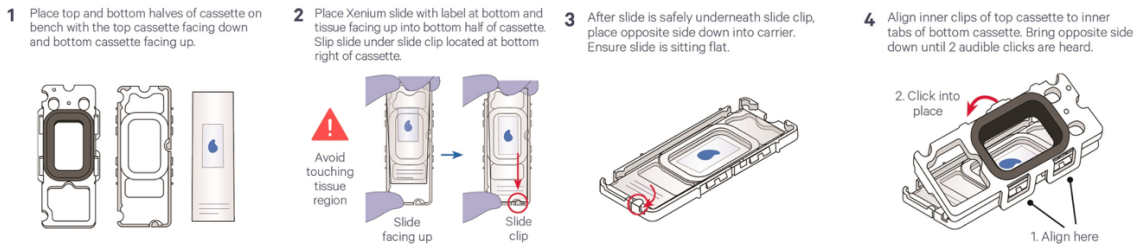
It is strongly recommended to perform VisiumHD tissue preparation and probe hybridization immediately after CODEX imaging to preserve RNA quality.

TISSUE PREPARATION (XeniumPrime)

Note: *There is no turning back once the tissue is processed for Xenium, when ready, proceed to Step.14.*

34. Protease K treatment: *Time needed: ~25 mins*
    a. Prepare 2µg/ml protease K (1:10000) by diluting the stock with 1X PBS (pH 7.4).
    b. Redraw hydrophobic barrier using UV-glue (double layer).
    c. Remove tissue section and gently absorb the excess TE buffer using a Kimwipe.
    d. Incubate tissue section with the 2ug/ml protease K prepared in Step.16a **for 20 mins at 40°C** in a HybEz oven.
    e. After tissue digestion, immediately rinse tissue section by dipping it gently in a jar filled with nuclease-free water for 3 times. Repeat this for two more rounds with a new jar of nuclease-free water each round.
    f. Proceed immediately to NBF fixation.

35. NBF fixation: *Time needed: ~30 mins*
    a. Remove excess nuclease-free water by wiping the tissue section gently with a Kimwipe.
    b. Fix the tissue section by submerging it in a coplin jar filled with 10% NBF buffer. Incubate for 1 min at RT.
    c. Stop the fixation by washing the tissue section with NBF Stop Buffer for 5 mins at RT on a belly dancer. Repeat this for 1 more time using a new jar of NBF Stop Buffer.
    d. Wash the tissue section once with 1X PBS (pH 7.4) for 5 mins at RT on a belly dancer.
    e. Put slides into the Xenium Cassette V2 as follows:



    f. Add 500ul PBS-T (0.05% Tween-20) to the Cassette to keep the tissue hydrated.

Note: *Once cassette is assembled, DO NOT remove slide until after Xenium Analyzer imaging and decoding for optional H&E staining.*

36. <u>Priming hybridization:</u> *Time needed: ~2 hours*

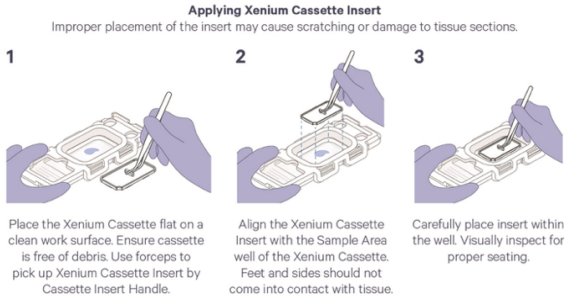
a. Thaw the following reagents:
   i. Priming Hybr Buffer (#2001228): Thaw in a thermomixer at 37C, 300rpm, 5mins, maintain at RT after.
   ii. Post-priming wash buffer (#2001229): Thaw at 37C, 5mins. Vortex and centrifuge briefly, maintain at RT.
   iii. Xenium 5K Hu PTP Priming Oligos (#2001224): Thaw at RT.
b. Prepare the following reagents:
   i. 1X PBS (from 10X PBS, pH 7.4 stock with nuclease-free water), maintain at RT.
   ii. 50ml PBS-T (0.05% Tween-20), maintain at RT.
   iii. 10ml 0.5X SSC-T (0.05% Tween-20), maintain at RT.
   iv. TE buffer, maintain at RT.
c. Briefly centrifuge the priming oligo tube and transfer **52.5ul** per slide (x2 for 2 slides) to a PCR tube.
d. Preheat priming oligo at 95C for 2 mins in a thermal cycler, followed by 1 min on ice.
e. Prepare Priming Hybridization Mix shortly before use as follows, maintain at RT. *(This table is only for pre-designed priming oligos without custom priming oligos)*

Priming Hybridization Mix	**2X + 5%**
Priming Hybr Buffer (#2001228)	189 ul
TE Buffer	21 ul
Xenium 5K Hu PTP Priming Oligos (#2001224)	105 ul
Total	315 ul

f. Pipette mix 10 times and centrifuge briefly, avoid generating bubbles.
g. Prepare a thermal cycler with the following program, **set lid temperature to 50C, 100ul**:

Step	**Temperature**	**Time**
Pre-equilibrate	50C	Hold
Priming hybridization	50C	1 hour 30 mins
Hold	50C	Hold
Post-primming hybridization wash	50C	30 mins
Hold	50C	Hold

h. One slide at a time, remove all PBS-T from the Cassette.
i. Gently place the insert into the Cassette using a forceps as follows:



j. Add **150ul** Priming Hybridization Mix slowly along the cut-out, without introducing bubbles. Make sure the buffer is uniformly covering the tissue.
k. Apply a new Cassette lid to the Cassette and place it to the pre-heated thermal cycler.
l. Close the thermal cycler lid and skip the pre-equilibrate step to initiate Priming Hybridization.
m. After Priming Hybridization is complete, proceed to post-priming wash immediately.

37. <u>Post-priming wash:</u> *Time needed: ~45 mins*

a. Remove Xenium Cassette V2 from the thermal cycler. Do not let Cassette cool down before proceeding to PBS-T washes.
b. Remove Cassette lid (save for later use) and add 200ul PBS-T into the cut-out to float the insert. Remove the insert with a forceps carefully. Discard the insert.
c. Remove all buffers from the Cassette with a pipette.
d. Wash 1: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
e. Wash 2: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
f. Add 500ul Post-priming Wash Buffer to the well.
g. Add the previous Cassette lid to the Cassette and place it back to the thermal cycler.
h. Close the thermal cycler lid and initiate Post-priming Hybridization Wash.
i. After Post-priming Hybridization wash is complete, proceed to RNase-treatment and polishing immediately.

38. <u>RNase-treatment & polishing:</u> *Time needed: ~30 mins*

a. Thaw the following reagents:
   i. 2X RNase Buffer (#2000411): Thaw at RT, vortex and centrifuge briefly, maintain at RT after.
   ii. Polishing Buffer (#20001231): Thaw at RT, vortex and centrifuge briefly, maintain at RT after.
   iii. RNase Enzyme (#3000593): Pipette mix, centrifuge briefly, maintain on ice.
   iv. Polishing Enzyme (#2001230): Pipette mix, centrifuge briefly, maintain on ice.
b. Prepare RNase Mix as follows, maintain on ice:

RNase Mix	**2X + 10%**
Nuclease-free water	539 ul
2X RNase Buffer (#2000411)	550 ul
RNase Enzyme (#3000593)	11 ul
Total	**1100 ul**

c. Remove the Cassette from the thermal cycler.

d. Remove Cassette lid (save for later use) and all buffers.
e. Wash 1: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
f. Wash 2: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
g. Wash 3: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
h. Prepare the thermal cycler with the following program, set lid temperature at 37C, 100ul, and start:

Step	**Temperature**	**Time**
Pre-equilibrate	37C	Hold
RNase Treatment	37C	20 mins
Hold	37C	Hold

i. Add 500ul RNase Mix to the Cassette.
j. Add the previous Cassette lid to the Cassette and place it back to the thermal cycler.
k. Close the thermal cycler lid and initiate Post-priming Hybridization Wash.
l. After RNase treatment is complete, proceed to polishing immediately.

39. Polishing: *Time needed: ~75 mins*

a. Prepare Polishing Mix as follows, maintain on ice:

Polishing Reaction Mix	**2X + 10%**
Polishing Buffer (#20001231)	990 ul
Nuclease-free water	55 ul
Polishing Enzyme (#20001230)	55 ul
Total	**1100 ul**

b. Remove the Cassette from the thermal cycler.
c. Remove Cassette lid (discard) and all buffers.
d. Wash 1: add 500ul 0.5X SSC-T to the Cassette. Incubate 1 min at RT. Remove all 0.5X SSC-T afterwards.
e. Wash 2: add 500ul 0.5X SSC-T to the Cassette. Incubate 1 min at RT. Remove all 0.5X SSC-T afterwards.
f. Wash 3: add 500ul 0.5X SSC-T to the Cassette. Incubate 1 min at RT. Remove all 0.5X SSC-T afterwards.
g. Prepare the thermal cycler with the following program, set lid temperature at 37C, 100ul, and start:

Step	**Temperature**	**Time**
Pre-equilibrate	37C	Hold
Polishing	37C	1 hr
Hold	37C	Hold

h. Add 500ul Polishing Reaction Mix to the Cassette.
i. Add a new Cassette lid to the Cassette and place it back to the thermal cycler.
j. Close the thermal cycler lid and initiate Polishing protocol.
k. After Polishing is complete, proceed to Probe Hybridization immediately.

40. Probe hybridization: *Time needed: ~15 mins then 16-24 hours*

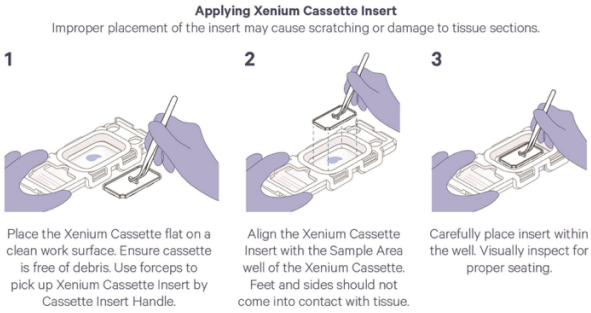
a. Thaw the following reagents:
   i. Probe Hyb Buffer B (#2001232): Thaw in a thermomixer at 37C, 5mins, maintain at RT after.
   ii. Xenium 5K Hu PTP Panel Probes (#2001225): Thaw at RT.
b. Remove the Cassette from the thermal cycler.
c. Remove Cassette lid (save for later) and all buffers.
d. Wash 1: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
e. Wash 2: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
f. Wash 3: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
g. Briefly centrifuge the probe tube and transfer **52.5ul** per slide (x2 for 2 slides) to a PCR tube.
h. Preheat priming oligo at 95C for 2 mins in a thermal cycler, followed by 1 min on ice.
i. Prepare Probe Hybridization Mix shortly before use as follows, maintain at RT. *(This table is only for pre-designed probe panels without custom probes)*

Probe Hybridization Mix	**2X + 5%**
Probe Hybr Buffer B (#2001232)	189 ul
TE Buffer	21 ul
Xenium 5K Hu PTP Panel Probes (#2001225)	105 ul
Total	315 ul

j. Pipette mix 10 times and centrifuge briefly, avoid generating bubbles.
k. Prepare a thermal cycler with the following program, **set lid temperature to 50C, 100ul**:

Step	**Temperature**	**Time**
Pre-equilibrate	50C	Hold
Probe hybridization	50C	Overnight 16-24hrs
Hold	50C	Hold

l. One slide at a time, remove all PBS-T from the Cassette.
m. Gently place the insert into the Cassette using a forceps as follows:

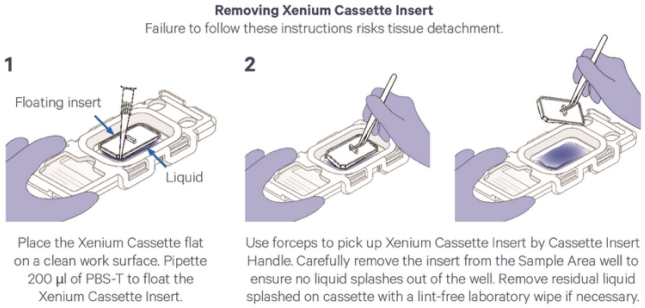


n. Add **150ul** RT Probe Hybridization Mix slowly along the cut-out, without introducing bubbles. Make sure the buffer is uniformly covering the tissue.
o. Apply the previously used Cassette lid to the Cassette and place it to the pre-heated thermal cycler.
p. Close the thermal cycler lid and skip the pre-equilibrate step to initiate Probe Hybridization.
q. After Probe Hybridization is complete, proceed to post-probe hybridization wash immediately.

Day 5

41. <u>Post-hybridization wash:</u> *Time needed: ~20 mins*

a. Thaw the following reagents:
   i. Post-hybridization wash buffer (#2000395): thaw at RT 30mins, vortex and centrifuge briefly, maintain at RT after.
b. Remove the Cassette from the thermal cycler.
c. DO not let Cassette cool down before proceeding to PBS-T washes.
d. Remove Cassette lid (save for later). Carefully add 200ul PBS-T to the Cassette to float the insert.
e. Remove insert with a forceps and discard the insert.



f. Remove all buffers from Cassette.
g. Wash 1: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
h. Wash 2: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
i. Prepare a thermal cycler with the following program, **set lid temperature to 35C, 100ul**:

Step	**Temperature**	**Time**
Pre-equilibrate	35C	Hold
Post-hybridization wash	35C	15 mins
Hold	35C	Hold

j. Add 500ul post-hybridization wash buffer (#2000395) to the Cassette.
k. Apply the previously used Cassette lid to the Cassette and place it to the pre-heated thermal cycler.
l. Close the thermal cycler lid and skip the pre-equilibrate step to initiate post-hybridization wash.
m. After post-hybridization wash is complete, proceed to probe ligation immediately.

42. <u>Probe ligation:</u> *Time needed: ~75 mins*

a. Thaw the following reagents:
   i. Ligation Buffer B (#2001233): Thaw at RT for 15mins, vortex and centrifuge briefly, maintain at RT after.
   ii. Ligation Enzyme A (#2000397): Thaw on ice, pipette mix and centrifuge briefly, keep on ice after.
   iii. Ligation Enzyme B (#2000398): Thaw on ice, pipette mix and centrifuge briefly, keep on ice after.

b. Prepare Ligation Mix shortly before use as follows, maintain at RT.

Ligation Mix	**2X + 10%**
Ligation Buffer B (#2001233)	963 ul
Ligation Enzyme A (#2000397)	27 ul
Ligation Enzyme B (#2000398)	110 ul
Total	1100 ul

c. Remove the Cassette from the thermal cycler.
d. Remove Cassette lid (save for later) and remove all buffers.
e. Wash 1: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
f. Wash 2: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
g. Wash 3: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.

h. Prepare a thermal cycler with the following program, **set lid temperature to 42C, 100ul**:

Step	Temperature	Time
Pre-equilibrate	42C	Hold
Ligation	42C	30 mins
Hold	42C	Hold

i. Add 500ul Ligation Mix to the Cassette.
j. Apply the previously used Cassette lid to the Cassette and place it to the pre-heated thermal cycler.
k. Close the thermal cycler lid and skip the pre-equilibrate step to initiate Ligation.
l. After Ligation is complete, proceed amplification enhancement immediately.

43. Amplification Enhancement:

Time needed: ~2 hours 15 mins

a. Thaw the following reagents:
   i. Amplification Enhancer Buffer (#2001234): Thaw at 37C for 5mins, vortex and centrifuge briefly, maintain at RT after.
   ii. Amplification Enhancer Wash Buffer (#2001236): Thaw at 37C for 5mins, vortex and centrifuge briefly, maintain at RT after.
   iii. Amplification Mix (#2000392): Thaw at RT, vortex and centrifuge briefly, maintain at RT after.
   iv. Amplification Enhancer (#2001235): Transfer to ice immediately before use. Pipette mix 10 times and centrifuge briefly. Maintain on ice.

b. Prepare Amplification Enhancer Master Mix as follows, maintain on ice.

Amplification Enhancer Master Mix	2X + 10%
Amplification Enhancer Buffer (#2001234)	990 ul
Amplification Enhancer (#2001235)	110 ul
Total	1100 ul

c. Remove the Cassette from the thermal cycler.
d. Remove Cassette lid (save for later) and remove all buffers.
e. Wash 1: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
f. Wash 2: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
g. Wash 3: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
h. Prepare a thermal cycler with the following program, **set lid temperature to OFF, 100ul**:

Step	Temperature	Time
Pre-equilibrate	4C	Hold
Amplification Enhancer	4C	2 hours
Hold	4C	Hold

i. Add 500ul Amplification Enhancer Master Mix to the Cassette.
j. Apply the previously used Cassette lid to the Cassette and place it to the pre-heated thermal cycler.
k. Close the thermal cycler lid and skip the pre-equilibrate step to initiate Amplification Enhancer.
l. After Amplification Enhancement is complete, proceed to amplification enhancement wash immediately.
m. Prepare Amplification Master Mix in advance, maintain on ice:

Amplification Master Mix	2X + 10%
Amplification Mix (#2000392)	990 ul
Nuclease-free water	110 ul
Total	1100 ul

44. Post-Amplification Enhancement Wash

Time needed: ~5 mins

a. Remove the Cassette from the thermal cycler.
b. Remove the Cassette lid (save for later) and remove all buffers.
c. Add 500ul Amplification Enhancer Wash Buffer to the Cassette.
d. Incubate 1 min at RT.
e. Proceed to Amplification immediately.

45. Amplification

Time needed: ~1 hours 45 mins

a. Prepare a thermal cycler with the following program, set lid temperature to 30C, 100ul:

Step	Temperature	Time
Pre-equilibrate	30C	Hold
Amplification	30C	1 hour 30 mins
Hold	30C	Hold

b. Remove all buffers from Cassette.
c. Add 500ul Amplification Master Mix to the Cassette.
d. Apply the previously used Cassette lid to the Cassette and place it to the pre-heated thermal cycler.
e. Close the thermal cycler lid and skip the pre-equilibrate step to initiate Amplification.
f. After Amplification is complete, proceed to amplification wash immediately.

46. Post-Amplification wash

Time needed: ~10 mins

a. Remove the Cassette from the thermal cycler.
b. Remove the Cassette lid (save for later) and remove all buffers.
c. Wash 1: add 500ul TE Buffer to the Cassette. Incubate 1 min at RT. Remove all TE Buffer afterwards.
d. Wash 2: add 500ul TE Buffer to the Cassette. Incubate 1 min at RT. Remove all TE Buffer afterwards.
e. Wash 3: add 500ul TE Buffer to the Cassette. Incubate 1 min at RT. Remove all TE Buffer afterwards.
f. Proceed to Cell Segmentation stain.

47. Cell segmentation stain

Time needed: ~15 mins then overnight 16-24 hours11

a. Thaw the following reagents:
   i. Xenium Block and Stain Buffer (#2001083): Thaw at RT for 30mins, vortex and centrifuge briefly, maintain on ice.
   ii. Xenium Multi-Tissue Stain Mix (#2000991): Maintain on ice.
   iii. Xenium Stain Enhancer (#2000992): To be prepared next day, maintain on ice.
   iv. Xenium Cassette insert (#3001885): Remove from -20C and equilibrate to RT.

b. Prepare the following reagents:
    i. 100% Ethanol in 15ml conical tube.
    ii. 70% Ethanol in 15ml conical tube. (10.5ml 100% Ethanol + 4.5ml Nuclease-free water)
c. Prepare 1X Diluted Xenium Block and Stain Buffer as follows:

1X Diluted Xenium Block and Stain Buffer	**2X + 10%**
Nuclease-free water	990 ul
Xenium Block and Stain Buffer (#2002083)	330 ul
Total	1320 ul

d. Remove the Cassette lid and remove all buffers.
e. DO NOT LET TISSUE DRY OUT DURING ETHANOL WASH STEPS.
f. Wash 1: Add 1000ul 70% Ethanol. Incubate for 2 mins at RT. Remove ethanol after.
g. Wash 2: Add 1000ul 100% Ethanol. Incubate for 2 mins at RT. Remove ethanol after.
h. Wash 3: Add 1000ul 100% Ethanol. Incubate for 2 mins at RT. Remove ethanol after.
i. Wash 4: Add 1000ul 70% Ethanol. Incubate for 2 mins at RT. Remove ethanol after.
j. Immediately add 1000ul PBS-T to the Cassette. Incubate 1 min at RT.
k. Add 500ul 1X Diluted Xenium Block and Stain Buffer to the Cassette. (Save the remaining diluted mix).
l. **Incubate 1hr at RT**.
m. Add 220ul 1X Diluted Xenium Block and Stain Buffer to the Xenium Multi-Tissue Stain Mix (#200991).
n. Pipette mix 15 times and centrifuge briefly. Avoid introducing bubbles.
o. Incubate the resuspended Xenium Multi-Tissue Stain Mix for 10mins at 14000xg at 4C. Maintain on ice after.
p. Place the Cassette insert to the Cassette using a forceps.
q. Add 100ul Xenium Multi-Tissue Stain Mix into the cut-out slowly without introducing bubbles.
r. Apply the previously used Cassette lid to the Cassette.
s. Incubate at 4C overnight (16-24 hours).

Day 6

48. Stain Enhancement *Time needed: ~30 mins*

g. Thaw the following reagents:
    i. Xenium Stain Enhancer (#2000992): Thaw at RT for 10mins, centrifuge briefly, ensure the white power is at the bottom of the tube before opening. Add 1100ul 1X PBS. Pipette mix 5 times and centrifuge briefly.
h. Remove the Cassette lid (save for later). Carefully add 200ul PBS-T into the cut-out to float the insert.
i. Remove the insert using a forceps, discard; then remove all buffers from the Cassette.
j. Wash 1: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
k. Wash 2: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
l. Wash 3: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
m. Add 500ul resuspended Xenium Stain Enhancer to the Cassette.
n. Apply the previously used Cassette lid to the Cassette.
o. Incubate **20mins at RT**.
p. Remove Cassette lid (save for later) and remove all buffers.
q. Wash 1: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
r. Wash 2: add 500ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
s. Add 500ul PBS-T to the Cassette.
t. After Cell Segmentation Staining is complete, proceed immediately to Nuclei staining. *Note: We amended the XeniumPrime protocol and skipped Autofluorescence Quenching.*

49. Nuclei Staining: *Time needed: ~5 mins*

a. Thaw the following reagents:
b. Remove Cassette lid (save for later) and remove all buffers.
c. Add 500ul Nuclei Staining Buffer (#200762) to the Cassette.
d. Incubate at **1min at RT in the dark**; then remove all buffers.
e. Wash 1: add 1000ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
f. Wash 2: add 1000ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
g. Wash 3: add 1000ul PBS-T to the Cassette. Incubate 1 min at RT. Remove all PBS-T afterwards.
h. The slides are ready for Xenium Analyzer.

50. If shipping slides to collaborator for Xenium Analyzer

a. Remove all PBS-T, disassemble the Xenium Cassette, and place no more than two slides in a mailer.
b. Ship with ice packs.