Co-Detection of RNA and Protein to Explore Tumor-Immune Interactions Utilizing RNAscope With Imaging Mass Cytometry

Introduction

Imaging Mass Cytometry™ (IMC™) is a proven tool for the study of complex cellular interactions in the tumor microenvironment (TME). It utilizes CyTOF® technology for simultaneous assessment of 40-plus protein markers at subcellular resolution without spectral overlap or background autofluorescence, thus providing unprecedented insight into the organization and function of the TME. Despite this, some protein targets are challenging to include in IMC as they have very few or no commercial antibodies available. Moreover, although cellular identity can easily be deciphered through detection of protein targets, knowledge of the cell's transcriptome improves understanding of cellular function and activation state. This technical note provides a workflow and procedure to achieve RNA and protein co-detection data in IMC experiments. We utilized highly sensitive and specific RNAscope™ technology for RNA detection with the multiplexing capability of IMC to visualize key RNA and protein markers in the same formalin-fixed, paraffin-embedded samples.

Tips for Success

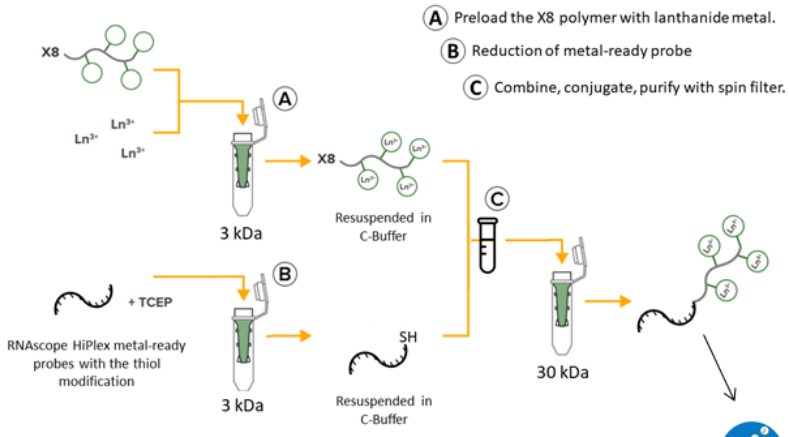
- Before exploring the expression of key RNA targets, we highly recommend the implementation of ACD Positive Control Probes. The RNA quality of different FFPE tissues can vary widely. We suggest using serial sections that are freshly prepared, analyzing the RNA quality on 1 serial section with the RNAscope HiPlex 12 Positive Control Probes – Hs (ACD, Cat. No. 324311), and then proceeding to explore target probes on the other serial sections. The use of Negative Control Probes is also highly encouraged to verify that the RNA staining is specific.
- To optimize detection of RNA targets, ensure that the majority of the detection oligonucleotide is conjugated and the fraction of unconjugated oligonucleotide is negligible. This can be confirmed by using size-exclusion chromatography or through agarose/native polyacrylamide gel electrophoresis.

OBJECTIVES

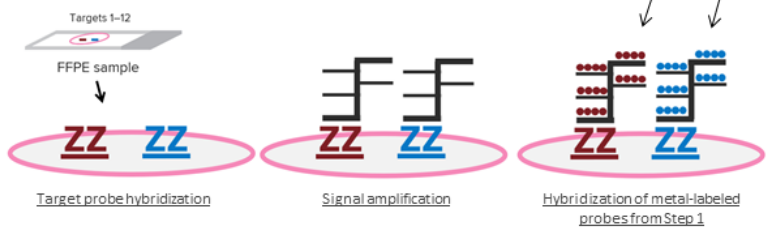
This technical note provides an overview of the procedure to detect both RNA and protein targets in an FFPE sample using IMC. The workflow first (Step 1) describes the procedure for conjugation of metal-ready detection oligonucleotides (purchased from Advanced Cell Diagnostics) with lanthanide metal tags. Next, (Step 2) we adopt a modified RNAscope HiPlex workflow (ACD) utilizing the metal-conjugated oligonucleotides. Finally, (Step 3) we provide guidance on the overnight staining of protein targets with IMC antibodies.

- The addition of antibodies may reduce the RNA signal in IMC. This can be problematic for low-frequency RNA targets. Therefore, it is recommended to use serial sections for RNA alone and for co-detection of RNA and protein. This way, the investigator can easily identify true RNA signal in IMC.
- In the combination workflow, use antibodies that have previously been validated with IMC and have demonstrated good performance. To determine if the antibody performance was impacted by the RNAscope pretreatment protocol, it is recommended to stain a tissue serial section with IMC antibodies alone, following the IMC Staining Protocol (for FFPE sections) (SOP-751R-26). This serial section can be directly compared with your co-detection slide to determine if the staining has been affected. If the antibody performance appears to be impacted by the RNAscope pretreatment protocol, users can consider testing other clones for the problematic antibodies.
- ACD recommends the use of the heat-stable ImmEdge Hydrophobic Barrier PAP Pen (ACD, 310018 or Vector Labs, H-400) for drawing water-repellent barriers around tissue specimens on slides. The use of a heat-tolerant pen is highly recommended as the RNAscope protocol subjects the tissue sample to multiple rounds of heat exposure.
- Do not let the tissue sections dry out during the procedure unless otherwise indicated.

Step 1 – Labeling of Detection Oligonucleotides



Step 2 – Modified RNAscope HiPlex Flex Assay v2



Step 3 – IMC Antibody Staining Overnight

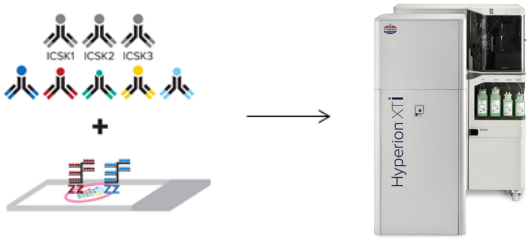


Figure 1. Graphical schematic of the 3-step procedure to acquire RNA and protein co-detection data with IMC

Day	Step	Time
Step 1 – Labeling of detection oligonucleotides		
Day 1	Reduction of metal-ready probe	60 min
	Preload X8 polymer with lanthanide metal.	60 min
	Combine, conjugate, and purify with spin filter.	180 min
Step 2 – Modified RNAscope HiPlex Flex Assay v2		
Day 2	Prepare FFPE tissue sections.	
	Bake slides.	60 min
	Deparaffinize FFPE sections.	20 min
	Prepare pretreatment materials.	30 min
	Perform target retrieval.	15–30 min
	Create a barrier.	15 min
	Optional stopping point	
	Apply RNAscope Protease III.	15–30 min
	Proceed immediately to HiPlex Assay.	240 min
Step 3 – IMC Antibody Staining Overnight		
Day 2	Prepare metal-labeled antibody cocktail.	45 min
	Apply antibody cocktail to FFPE sections overnight.	10 min
Day 3	Slide washing and DNA intercalator stain	45 min
	Dry the slide, then proceed to IMC ablation.	30 min

Table 1. An estimated timeline of the workflow

Summary and Conclusion

In conclusion, this technical note describes a simple 3-step workflow for the combination of Imaging Mass Cytometry and RNAscope. This workflow allows for the co-detection of RNA and protein markers in the same FFPE samples, enabling researchers to gain deeper insights into the complex cellular interactions within the TME. By leveraging the subcellular resolution and multiplexing capabilities of IMC with the high sensitivity and specificity of RNAscope, this integrated approach offers new possibilities for studying previously inaccessible targets and activation states of cells, driving further biological insights within IMC experiments.

Materials

Required Reagents

IMPORTANT: Store reagents as soon as they are received, according to manufacturer's storage recommendations.

IMPORTANT: The Maxpar® X8 Antibody Labeling Kit (available from Standard BioTools™) contains reagents with different storage conditions. Store reagents according to Standard BioTools recommendations. Table 3 outlines the reagents included in the Maxpar X8 Antibody Labeling Kit, which provides the necessary reagents to label 2 metal-ready probes as outlined in this technical note.

Ln Metal Isotope	Part No. (4 Rxn)	Ln Metal Isotope	Part No. (4 Rxn)	Ln Metal Isotope	Part No. (4 Rxn)	Ln Metal Isotope	Part No. (4 Rxn)
141Pr	201141A	151Eu 153Eu	201151A 201153A	161Dy 162Dy 163Dy 164Dy	201161A 201162A 201163A 201164A	169Tm	201169A
142Nd 143Nd 144Nd 145Nd 146Nd 148Nd 150Nd	201142A 201143A 201144A 201145A 201146A 201148A 201150A	155Gd 156Gd 158Gd 160Gd	201155A 201156A 201158A 201160A	165Ho	201165A	171Yb 172Yb 173Yb 174Yb 176Yb	201171A 201172A 201173A 201174A 201176A
147Sm 149Sm 152Sm 154SM	201147A 201149A 201152A 201154A	159Tb	201159A	166Er 167Er 168Er 170Er	201166A 201167A 201168A 201170A	175Lu	201175A

Table 2. Maxpar X8 Antibody Labeling Kits available from Standard BioTools

Product Name	Part No.	Storage
Maxpar Lanthanide Solution—50 mM, 20 µL	1 per kit	4 °C. Do not freeze.
Maxpar R-Buffer—6 mL (1 bottle)	S00001	
Maxpar C-Buffer—5.5 mL (1 bottle)	S00003	
Maxpar W-Buffer—8 mL (1 bottle)	S00005	
Maxpar L-Buffer—1.4 mL (1 tube)	S00007	
Maxpar X8 Polymer—0.1 mg (4 tubes)	S00009	–20 °C. Sealed with desiccant.

Table 3. Contents of the Maxpar X8 Antibody Labeling Kit

Required Consumables

Product Name	Source	Part Number
Amicon® Ultra-0.5 Centrifugal Filter Unit, 0.5 mL V-bottom, 8-pack*	Sigma-Aldrich®	UFC500308 (3 kDa) UFC503008 (30 kDa)
Eppendorf® DNA LoBind Tubes, 1.5 mL, 200 tubes	Sigma-Aldrich	EP0030108310-200EA
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution, pH 7.0 (10 × 1 mL, 0.5 M)	Sigma-Aldrich	646547
ImmEdge Hydrophobic Barrier PAP Pen	ACD Vector Labs	310018 H-400
Xylene	Fisher Scientific™	X3P-1GAL
Cell-ID™ Intercalator-Ir—125 µM	Standard BioTools	201192A
100% alcohol (EtOH)	MasterTech (StatLab)/MLS*	ALREAGAL
10X Phosphate Buffered Saline (PBS)	Fisher Scientific	BP3991
Tissue-Tek® Vertical 24 Slide Racks (or other slide racks or holders)	StatLab/MLS	LWS2124
Paper towel or absorbent paper	MLS	
Forceps, large	MLS	
UltraPure™ DNase/RNase-Free Distilled Water (500 mL)	Fisher Scientific	10977015
HybEZ™ Humidifying Paper	ACD	310015
RNAscope HiPlex Flex Reagent Kit for IMC users (will include all required reagents such as AMP 1,2,3, Protease, Target Retrieval Buffer, Wash Buffer, etc.)	ACD	322725
RNAscope HiPlex metal-ready probe T1	ACD	322711
RNAscope HiPlex metal-ready probe T2	ACD	322712
RNAscope HiPlex metal-ready probe T3	ACD	322713
RNAscope HiPlex metal-ready probe T4	ACD	322714
RNAscope HiPlex metal-ready probe T5	ACD	322715
RNAscope HiPlex metal-ready probe T6	ACD	322716
RNAscope HiPlex metal-ready probe T7	ACD	322717
RNAscope HiPlex metal-ready probe T8	ACD	322718
RNAscope HiPlex metal-ready probe T9	ACD	322719
RNAscope HiPlex metal-ready probe T10	ACD	322720
RNAscope HiPlex metal-ready probe T11	ACD	322721
RNAscope HiPlex metal-ready probe T12	ACD	322722
RNAscope HiPlex metal-ready probes T1-T12	ACD	322710

* MLS = major laboratory supplier

Table 4. Required consumables for this 3-step RNA and protein co-detection procedure

Required Equipment

Product Name	Source	Part Number
2 microcentrifuges capable of 12,000 × *g* with fixed angle rotor compatible with 1.5 mL tubes	MLS*	
Mini-centrifuge compatible with 1.5 mL tubes	MLS	
Water bath capable of 37 ±1.5 °C and compatible with 0.2 mL and 1.5 mL tubes **IMPORTANT** Optional for polymer loading only: You can use a well-calibrated dry heat block if it meets the above criteria. Do not use a metal bead bath with this protocol.	MLS	
Method to assess DNA quantity	MLS	
Pipettes (P10–P1000) and appropriate aerosol barrier (filter) tips	MLS	
Imaging Mass Cytometry system (Hyperion™, Hyperion+™, Hyperion XTi™)	Standard BioTools	108700, 119001, 121701
Oster® Steamer Model 5712, BLACK+DECKER® Steamer HS3000, Braun Multiquick FS 20 Steamer, or Hamilton Beach Steamer		
HybEZ II Oven (110 or 220 VAC)	ACD	321710 or 321720
HybEZ Humidity Control Tray (with lid)	ACD	310012

* MLS = major laboratory supplier

Table 5. Required equipment for this 3-step RNA and protein co-detection procedure

Before you Begin

IMPORTANT: Before using reagents from Standard BioTools or ACD, read and understand the detailed instructions and safety guidelines in this document.

- Vortex and centrifuge all reagents according to manufacturer's instructions.
- Use good laboratory practices to minimize contamination of samples.

Procedure

Step 1 – Metal Labeling of Detection Oligonucleotides

Reduce RNAscope HiPlex metal-ready probe(s)

1. Resuspend HiPlex metal-ready probes (20 nmol) in 100 µL nuclease-free double-distilled water (ddH_2O).
2. Create a 100 mM TCEP stock. Start by taking 15 µL of a 0.5M TCEP aliquot (–80 °C) and add 60 µL of R-Buffer (Maxpar X8 Antibody Labeling Kit, see Table 3) (total volume 75 µL).
3. Reduce metal-ready probe by adding 12.5 µL of 100 mM TCEP to 10 µL of RNAscope HiPlex metal-ready probe(s) (from 20 nmol in 100 µL stock) and add 27.5 µL of nuclease-free ddh20. Final concentrations should be 25 mM TCEP and 2 nmol RNAscope HiPlex metal-ready probe(s) at total volume of 50 µL. Incubate at 37 °C for 60 min.

IMPORTANT: During the 60 min reduction, proceed to the next section. **Preload the X8 polymer with lanthanide metal** to start the X8 polymer and lanthanide metal incubation.

4. During the 60 min reduction, label a new 3 kDa spin filter.
5. Add 400 µL of nuclease-free ddH_2O into the 3 kDa spin filter.
6. After the 60 min reduction, transfer the reduced metal-ready probe into the newly prepared 3 kDa spin filter. Centrifuge at 12,000 *g* for 25 min.
7. Wash 1: Discard the flowthrough and add 400 µL of nuclease-free H_2O. Centrifuge at 12,000 *g* for 25 min.
8. Wash 2: Discard the flowthrough and add 400 µL of C-Buffer (Maxpar X8 Antibody Labeling Kit, see Table 3). Centrifuge at 12,000 *g* for 25 min.
9. Retrieve the final volume from the filter by inverting the filter into a new microcentrifuge tube, and centrifuge for 2 min at 1,500 *g*.

Preload the X8 polymer with lanthanide metal

1. Retrieve X8 polymer from –20 °C. Only retrieve the number of single-use X8 polymer tubes that are required for the experiment, thaw to room temperature (RT) before opening to avoid moisture condensation, and then use immediately. [2 nmol of metal-ready probe requires 0.2 mg of loaded polymer. Each tube contains 0.1 mg of X8 polymer, so use 2 tubes in total (0.2 mg).]
2. Once thawed to RT, centrifuge the X8 polymer tube and the tube containing 50 mM Ln metal chloride solution for 10 sec in a mini-centrifuge to collect contents at the bottom of each tube.

IMPORTANT: Be sure to use the X8 polymer with Ln metal solution. Label the X8 polymer tube with the specific Ln metal isotope.

3. Add 90 µL of L-Buffer (Maxpar X8 Antibody Labeling Kit, see Table 3) to the X8 polymer tube to resuspend the polymer.
4. Mix thoroughly by pipetting until the polymer is completely dissolved (approximately 1 min).
5. Add 10 µL of 50 mM Ln metal solution to the X8 polymer tube.
6. Mix thoroughly by pipetting.
7. Incubate at 37 °C for 30 min in a water bath or dry heat block.
8. After the 30 min metal-polymer incubation is complete, add 300 µL of L-Buffer to a newly labeled 3 kDa filter unit.
9. Wash 1: Retrieve the metal-loaded polymer mixture from Step 7 and transfer all contents (approximately 100 µL each tube) to the 3 kDa filter containing L-Buffer.

   Note: The filter should now contain approximately 400 µL of L-Buffer metal-loaded polymer solution.

10. Use a P100 pipette to mix thoroughly, being careful not to touch the delicate filter.
11. Centrifuge at 12,000 *g* for 25 min at RT. Discard flowthrough.
12. Wash 2: Add 400 µL of C-Buffer to the 3 kDa filter and centrifuge at 12,000 *g* for 25 min at RT.
13. Invert the filter into a new microcentrifuge tube. Retrieve contents by centrifuging for 2 min at 1,500 *g*.

Combine, conjugate, and purify with spin filter

1. Combine the recovered volumes from both the metal-loaded polymer and the reduced metal-ready probe. Increase the total volume to 100 µL if necessary with C-Buffer.
2. Mix gently by pipetting. Incubate at 37 °C for 120 min.
3. The reaction is then purified using a 30 kDa filter column. Add 400 µL of nuclease-free ddH_2O to the 30 kDa filter. Add the conjugation reaction (approximately 100 µL) to the 30 kDa filter.
4. Mix thoroughly by pipette, being careful not to touch the delicate filter. Centrifuge at 12,000 *g* for 10 min. Discard the flowthrough.
5. Wash 1: Add 400 µL of nuclease-free dH_2O to the 30 kDa filter. Centrifuge at 12,000 *g* for 10 min. Discard the flowthrough.
6. Wash 2: Add 400 µL of nuclease-free ddH_2O to the 30 kDa filter. Centrifuge at 14,000 *g* for 10 min. Discard the flowthrough.
7. Add 85 µL of nuclease-free ddH_2O to the filter. Final conjugated oligonucleotides are retrieved by inverting the filter into new microcentrifuge tube and centrifuging for 2 min at 1,500 *g*. Final eluted volume is approximately 100 µL.
8. Use NanoDrop™ measurement for ssDNA to determine concentration. Expected concentration at ssDNA setting is 70–95 ng/ µL. To verify the efficacy of conjugation, we recommend performing either size-exclusion chromatography or agarose/native polyacrylamide gel electrophoresis.
9. Dilute all metal-ready probes to a final 1 µM stock in nuclease-free ddH_2O.

Step 2 – RNAscope HiPlex Flex Assay v2

NOTE: The preparation of FFPE tissue sections is outside the scope of this protocol. Below is a brief workflow for the preparation of FFPE tissue sections with considerations for this protocol in mind.

Prepare FFPE tissue sections

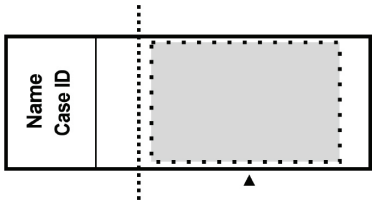
1. Immediately following dissection, fix tissue in 10% neutral-buffered formalin (NBF) for 16–32 hr at RT. Fixation time varies depending on tissue type and size.

IMPORTANT: Fixation for <16 hr or >32 hr impairs the performance of the assay.

2. Wash sample with 1X PBS (Fisher Scientific, BP3991).
3. Dehydrate sample using a standard ethanol series, followed by xylene (Fisher Scientific, X3P-1GAL).

IMPORTANT: Use fresh reagents. Embed samples as quickly as possible to preserve RNA quality.

4. Embed sample in paraffin using standard procedures. Note: Embedded samples may be stored at RT with desiccants. To better preserve RNA quality over a long period (>1 year), storing at 2–8 °C with desiccants is recommended.
5. Trim paraffin blocks as needed and cut embedded tissue into 5 ± 1 µm sections using a microtome.
6. Place paraffin ribbon in a 40–45 °C water bath, and mount sections on SuperFrost™ Plus slides (Fisher Scientific, 12-550-15). Place tissue as shown below (gray box) for optimal staining:



IMPORTANT: Do not mount more than 1 section per slide. Place sections in the center of the slide.

7. Air-dry slides overnight at RT.

OPTIONAL STOPPING POINT. You can store sections with desiccants at RT. Use sectioned tissue within 3 months.

Bake slides

1. Bake slides in a dry oven for 1 hr at 60 °C.

   NOTE: Prepare materials for the next steps while the slides are baking.

Deparaffinize FFPE sections

1. In a fume hood, fill 2 Tissue-Tek Clearing Agent dishes (StatLab, LWS20WH) with approximately 200 mL fresh xylene. Fill 2 Tissue-Tek Staining dishes with approximately 200 mL fresh 100% ethanol.
2. Place slides in a Tissue-Tek Slide Rack (StatLab, LWS2124) and submerge in the first xylene-containing dish in the fume hood.
3. Incubate the slides in xylene for 5 min at RT. Agitate the slides by occasionally lifting the slide rack up and down in the dish.
4. Remove the slide rack from the first xylene-containing dish and immediately place in the second xylene-containing dish in the fume hood.
5. Incubate the slides in xylene for 5 min at RT with agitation.
6. Remove the slide rack from the second xylene-containing dish and immediately place in a dish containing 100% ethanol.
7. Incubate the slides in 100% ethanol for 2 min at RT with agitation.
8. Remove the slide rack from the first ethanol-containing dish and immediately place in the second ethanol-containing dish.
9. Incubate the slides in 100% ethanol for 2 min at RT with agitation.
10. Remove the slides from the rack and place on absorbent paper with the section face up. Dry slides in a drying oven for 5 min at 60 °C (or until completely dry).

Prepare pretreatment materials

1. Turn on the HybEZ Oven and set temperature to 40 °C.
2. Place a Humidifying Paper (ACD, 310015) in the Humidity Control Tray (ACD, 310012) and wet completely with distilled water.
3. Insert covered tray into the oven and close the oven door. Warm the tray for 30 min at 40 °C before use. Keep the tray in the oven when not in use.

4. Prepare 1X Target Retrieval Reagent Buffer (for example, add 180 mL distilled water to 20 mL 10X RNAscope Target Retrieval Buffer and mix well).

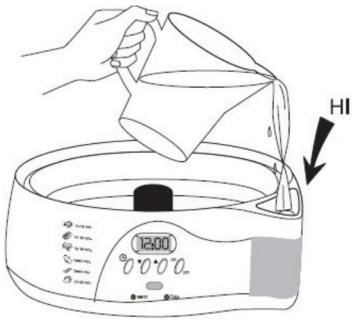
   NOTE: Target Retrieval Buffer must be heated to ≥99 °C before use, but do not boil for more than approximately 15 min before use. See the following procedure.

Perform target retrieval using the steamer

IMPORTANT: Before you begin, make sure you know the pretreatment conditions specific to your sample type from Appendix A – Tissue Pretreatment Recommendations From ACD. We highly recommend using a steamer for target retrieval. For target retrieval using a hot plate, see Appendix B – Manual Target Retrieval.

NOTE: For each steamer, fill the water to the maximum level before starting and do not refill water during the steaming process. Refilling water during the steaming process drops the temperature and interferes with target retrieval.

1. Fill the water reservoir with cold tap water to the MAX fill marking line.



2. Place 2 slide holders in the steam bowl. Fill 1 slide holder with 200 mL of RNAscope 1X Target Retrieval Reagent Buffer. Fill the other slide holder with 200 mL of distilled water.
3. Turn on the steamer. Set the heating time to maximum so that the steamer does not shut off during target retrieval.
4. Insert a digital thermometer through the holes of the lid and into the container containing RNAscope 1X Target Retrieval Reagent Buffer. Allow the temperature to rise to at least 99 °C.
5. Add the slides to the container containing distilled water for 10 sec to acclimate the slides.
6. Remove the slides and move them to the container containing RNAscope 1X Target Retrieval Reagent Buffer. Cover the steamer with the lid.
7. Start the timer for 15 min for mild and standard conditions, and 30 min for extended pretreatment. For pretreatment times, consult Appendix A – Tissue Pretreatment Recommendations From ACD.
8. Remove the slides from the steamer and transfer to a separate rinse container with 200 mL of distilled water (RT). Allow the slides to rinse for 15 sec.
9. Transfer the slides to 100% ethanol for 3 min.
10. Dry the slides in a 60 °C incubator (or at RT) for 5 min.

Create a barrier

1. Draw a barrier 2–4 times around each section with the ImmEdge Hydrophobic Barrier Pen.

IMPORTANT: Do not let the barrier touch the tissue section. An ImmEdge Hydrophobic Barrier Pen is highly recommended. Other pens may cause suboptimal results.

2. Let the barrier dry completely approximately 10 min or overnight at RT. Note: If you need to reapply the hydrophobic barrier during the following procedures, dry the appropriate area of the slide with a Kimwipe®. Do not touch the tissue section.

OPTIONAL STOPPING POINT. Dry slides overnight at RT for use the following day or proceed directly to the next section.

Apply RNAscope Protease III

3. Load the dried slides into the ACD EZ-Batch™ Slide Holder (ACD, 321716) by opening the swing clamp. [Please refer to the ACD HybEZ II Hybridization System and EZ-Batch Slide Processing System User Manual (UM321710-USM) for help and more information.]
4. Add about 5 drops of RNAscope Protease III to entirely cover each section.
5. Place the ACD EZ-Batch Slide Holder in the pre-warmed HybEZ Humidity Control Tray. Close the lid, seal, and insert the tray back into the oven.
6. Incubate at 40 °C for the amount of time specified by the table in Appendix A – Tissue Pretreatment Recommendations From ACD.

   NOTE: If needed, prepare RNAscope Assay materials during this step.

7. Pour at least 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray (ACD, 321717).
8. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
9. Place the ACD EZ-Batch Slide Holder into the clear plastic wash tray containing water. Make sure all the slides are submerged. If needed, carefully add more water. Wash the slides with slight agitation.
10. Repeat the wash step with fresh distilled water.

Proceed to the RNAscope Assay

Workflow

NOTE: No matter how many probes you are using, you must complete all Amp steps.

Step	Time
Prepare the materials.	30 min
Hybridize probes.	120 min
Optional stopping point	
Hybridize RNAscope HiPlex Amp 1.	30 min
Hybridize RNAscope HiPlex Amp 2.	30 min
Hybridize RNAscope HiPlex Amp 3.	30 min
Hybridize metal-conjugated probes.	30 min

Prepare the materials

Prepare 1X Wash Buffer

1. Prepare 3 L of 1X Wash Buffer by adding 2.94 L distilled water to 1 bottle of 50X Wash Buffer (60 mL) in a large carboy. Mix well.

   NOTE: If precipitation occurs in the 50X Wash Buffer, warm it up at 40 °C for 10–20 min before making the 1X Wash Buffer. 1X Wash Buffer may be prepared ahead of time and stored at room temperature for up to 1 month.

Prepare probes

1. Warm RNAscope HiPlex probe stocks and control probes at 40 °C in a water bath or incubator for about 10 min.
2. Warm RNAscope HiPlex diluent at 40 °C in a water bath or incubator for about 10 min.
3. Briefly spin down all 50X probe stocks to collect the liquid at the bottom of the tubes.
4. Mix each unique target probe set by diluting 50X probe stocks with RNAscope HiPlex probe diluent. Dilute probes to 1X by pipetting 1 volume of each stock to 50 volumes of probe diluent. For example, to make 2 mL of solution containing all 12 probes, use 40 µL of each probe stock and add 1,520 µL of RNAscope HiPlex Probe Diluent. Mix well.
5. Mix well by vortexing or invert the tube several times.

   NOTE: Do not mix probes of the same tail assignment. The mixed probes can be stored at 2–8 °C for up to 6 months.

Prepare metal-conjugated probes
(From Step 1 of this procedure)

1. Warm metal-conjugated probes (1 µM stocks) at 40 °C in a water bath or incubator for about 10 min.
2. Warm RNAscope HiPlex diluent at 40 °C in a water bath or incubator for about 10 min.
3. Briefly vortex and spin down all metal-ready probes to collect the liquid at the bottom of the tubes.
4. Dilute each probe to 80 nM using RNAscope HiPlex probe diluent, creating a probe cocktail to be used in the final step of the RNAscope procedure (Hybridize metal-conjugated probes).

Equilibrate reagents

1. Place RNAscope HiPlex Amp 1–3 and metal-ready probes at RT.
2. Ensure that the HybEZ oven and prepared Humidity Control Tray are at 40 °C.

Hybridize target or control probes

IMPORTANT: Do NOT let sections dry out between incubation steps. Work quickly and fill barrier with solutions.

IMPORTANT: Ensure that the probes are prewarmed to 40 °C and cooled to RT prior to use.

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into HybEZ Humidity Control Tray.
2. Add enough of the appropriate probe to entirely cover each section. For example, add 4 drops of the appropriate probe for a 0.75-inch x 0.75-inch barrier.
3. Close the tray and insert into the oven for 2 hr at 40 °C.

4. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
5. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
6. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 min at RT.
7. Repeat the wash step with fresh 1X Wash Buffer for 2 min at RT.

Hybridize RNAscope HiPlex Amp 1

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough RNAscope HiPlex Amp 1 to entirely cover each section.
3. Close the tray and insert into the HybEZ oven for 30 min at 40 °C.
4. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
5. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
6. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 min at RT.
7. Repeat the wash step with fresh 1X Wash Buffer for 2 min at RT.

Hybridize RNAscope HiPlex Amp 2

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough RNAscope HiPlex Amp 2 to entirely cover each section.
3. Close the tray and insert into the HybEZ oven for 30 min at 40 °C.
4. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
5. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
6. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 min at RT.
7. Repeat the wash step with fresh 1X Wash Buffer for 2 min at RT.

Hybridize RNAscope HiPlex Amp 3

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough RNAscope HiPlex Amp 3 to entirely cover each section.
3. Close the tray and insert into the HybEZ Oven for 30 min at 40 °C.
4. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
5. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
6. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 min at RT.
7. Repeat the wash step with fresh 1X Wash Buffer for 2 min at RT.

Hybridize metal-conjugated probes

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough of the diluted metal-conjugated probe cocktail to entirely cover each tissue section.
3. Close the tray and insert into the HybEZ Oven for 30 min at 40 °C.
4. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
5. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
6. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for 2 min at RT.
7. Repeat the wash step with fresh 1X Wash Buffer for 2 min at RT.

Step 3 – IMC Antibody Staining Protocol for FFPE Samples

NOTE: The following procedure is a direct continuation of Step 2 – RNAscope HiPlex Flex Assay v2. In the last step, the slides are washed in 1X Wash Buffer.

1. Remove excess liquid from the slides, then remove the slides from the slide holder rack.
2. Wash the slides in 1X PBS for 2 min at room temperature in a Coplin jar. Repeat the wash in fresh 1X PBS.
3. Remove excess liquid from the slides, and set aside to air-dry as you prepare the following reagents:

Prepare reagents

1. Spin down each antibody at 13,000 x *g* for 2 min and take from the top of the tube to avoid aspirating antibody aggregates that have sedimented.
2. Prepare metal-conjugated antibodies for staining by mixing all antibodies at concentrations specific for the assay in 1X PBS.
3. Calculate the volume of antibody cocktail to prepare for tissue staining.

   NOTE: Approximate volume for a standard section is 100 µL/section. Approximate volume for a tumor microarray (TMA) slide is 150 µL/section.

   NOTE: The antibody cocktail solution must cover the entire tissue.
4. Once the antibody cocktail is prepared, store the antibody cocktail at 4 ˚C and use within 2 hr of preparation.
5. Place slides in a hydration chamber and pipet the antibody master mix onto the tissue section, ensuring full coverage.
6. Incubate overnight with the antibody mixture in hydration chamber at 4 ˚C.

The Next Day:

1. Wash the slides in 0.2% Triton™ X-100 (Thermo Fisher Scientific™, 85111) in PBS for 8 min in a Coplin jar placed on an orbital shaker with gentle agitation (at max setting). Repeat for a total of 2 washes.
2. Wash the slides in 1X PBS for 8 min in a Coplin jar placed on an orbital shaker with gentle agitation (at max setting). Repeat for a total of 2 washes.
3. Prepare Cell-ID Intercalator-Ir to a final concentration of 250 nM (a 2,000X dilution of the 500 µM stock solution) and vortex to mix. Calculate the volume of diluted Intercalator-Ir to prepare for tissue staining.
4. Stain the tissue with 250 nM Cell-ID Ir-Intercalator in DPBS for 30 min at room temperature in a hydration chamber.
5. Wash the slides in Maxpar/Milli-Q® Water for 5 min in a Coplin jar placed on an orbital shaker with gentle agitation (at max setting).
6. Air-dry the slide for at least 20 min at RT.

Proceed to IMC ablation using the Hyperion, Hyperion+, or Hyperion XTi Imaging System.

Appendix A – Tissue Pretreatment Recommendations From ACD

Reagent	Mild	Standard	Extended
RNAscope Target Retrieval Reagents	15 min	15 min	30 min
RNAscope Protease III	15 min	30 min	30 min

Species	Tissue Type	Pathology	Pretreatment Condition
Mouse/rat	Intestine	Normal	Standard
	Intestine	Tumor	Standard
	Embryo	Normal	Standard
	Brain	Normal	Standard
	Spleen	Normal	Mild
	Eye/retina	Normal	Standard
	Liver	Normal	Extended
	Kidney	Normal	Standard
Human	Breast	Tumor	Standard
	Colon	Tumor/normal	Standard
	Lung	Tumor	Standard
		Normal	Standard
	Prostate	Tumor	Standard
		Normal	Standard
	Lymph node	Tumor	Standard
		Normal	Standard
	Tonsil	Normal	Standard
	Pancreas	Normal	Standard
	Cervical	Cancer	Mild
	Cervical dysplasia	Abnormal	Standard

Species	Tissue Type	Pathology	Pretreatment Condition
Human	Brain	Tumor	Extended
		Normal	Standard
	Head	Normal	Standard
	Neck	Cancer	Standard
	Liver	Cancer	Standard
	Kidney	Normal	Standard
	Melanoma	Tumor	Standard
	Nevus	Benign	Standard
	Placenta	Normal	Standard
	Brain	Normal	Standard
	Skin (TMA)	Normal	Standard
	Breast (TMA)	Normal	Standard
	Melanoma (TMA)	Normal	Standard
	Nevus (TMA)	Benign	Standard
	Stomach (TMA)	Tumor	Standard
		Normal	Standard
	Cell pellets, fixed with 10% NBF		Mild
	HeLa cells, fixed with 10% formaldehyde/ PBS (ACD control)	Normal	10 min Target Retrieval; 30 min Protease III

Appendix B – Manual Target Retrieval

Required Materials

- Prepared slides
- Distilled water
- Glass beaker (1 or 2 L)
- Paper towel or absorbent paper
- Hot plate, Isotemp®
- Aluminum foil
- Thermometer
- Forceps, large
- Tissue-Tek Slide Rack
- Tissue-Tek Staining Dish
- ImmEdge Hydrophobic Barrier Pen
- 100% ethanol
- RNAscope 10X Target Retrieval Reagents

IMPORTANT: Do NOT boil the 1X RNAscope Target Retrieval Reagent Buffer more than 15 min before use.

1. Prepare 700 mL of fresh RNAscope 1X Target Retrieval Reagent Buffer by adding 630 mL distilled water to 1 bottle (70 mL) 10X Target Retrieval Reagent in the beaker. Mix well.
2. Place the beaker containing RNAscope 1X Target Retrieval Reagents on the hot plate. Cover the beaker with foil, and turn the hot plate on high for 10–15 min.
3. Once the 1X RNAscope Target Retrieval Reagent Buffer reaches a mild boil (98–102 °C), turn the hot plate to a lower setting to maintain the correct temperature. Check the temperature with a thermometer.
4. With a pair of forceps, very slowly submerge the slide rack containing the slides into the mildly boiling RNAscope 1X Target Retrieval Reagent Buffer solution. Cover the beaker with foil and boil the slides for the amount of time specified by the table in Appendix A – Tissue Pretreatment Recommendations From ACD.
5. Use the forceps to immediately transfer the hot slide rack from the RNAscope 1X Target Retrieval Reagent Buffer to the staining dish containing distilled water. Do not let the slides cool in the Target Retrieval Reagent Buffer.
6. Wash slides 3–5 times by moving the Tissue-Tek Slide Rack up and down in the distilled water.
7. Wash slides in fresh 100% alcohol, and allow the slides to dry completely at 60 °C for 5 min.
8. Draw the hydrophobic barrier and continue with Apply RNAscope Protease III in Step 2.

References

1. IMC Staining Protocol (for FFPE sections) (SOP-751R-26)
2. Wang, F. et al. “RNAscope: a novel *in situ* RNA analysis platform for formalin-fixed, paraffin-embedded tissues.” *The Journal of Molecular Diagnostics* 14 (2012): 22–29.

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FLDM-01182 Rev 01