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Title: Dual-modality Molecular Cartography: Integrating Multiplex mRNA Detection with Protein Imaging Mass Cytometry

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all donr**

Videographer: Please film the computer screen for all shots labelled as SCREEN

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   0.25 miles

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **N/A**

Current Protocol Length
Number of Steps: 20
Number of Shots: 44

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

1.1. **Sammy Ferri-Borgogno:** We focused on spatial biology to better understand the molecular mechanism of ovarian cancer in the hopes of finding targeted therapies or predictors for survival.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 4.1.1*.

~~What are the current experimental challenges?~~

1.2. **Sammy Ferri-Borgogno:** Some of the current IMC challenges include detection sensitivity for low-abundance proteins, the labor of antibody validation and titration, as well as the complex, time-consuming pixel classification during analysis.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What advantage does your protocol offer compared to other techniques?~~

~~1.3. **Angelique Lin:** RNAscope with IMC lets researchers simultaneously detect 12 RNA and multiple protein targets, overcoming fluorescence limitations such as spectral overlap, which enables higher-plex for antibodies.~~

~~1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.~~

NOTE: Authors chose to skip 1.3

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Review Board (IRB) at the University of Texas MD Anderson Cancer Center

Protocol

2. Hybridizing RNA-ISH Amplifier 3 to Ovarian Cancer FPPE Sections

Demonstrator: Basant T. Gamal

2.1. Begin by removing excess liquid from the prepared FPPE slides **[1-TXT],** take the humidity control tray out of the oven **[2],** and place the slide holder into the tray **[3]**.

2.1.1. WIDE: Talent removing excess liquid from the slide. **TXT: Perform RNA-ISH Amplifier 1 and 2 hybridizations in advance**

2.1.2. Talent opening the oven and removing the humidity control tray.

2.1.3. Talent placing the slide holder securely into the tray.

2.2. Then, vortex RNA-ISH *(R-N-A-Ish)* Amplifier 3 thoroughly **[1]** and add enough reagent to completely cover each tissue section on the slides **[2]**. Close the tray **[3]** and insert it into the hybridization oven for 30 minutes at 40 degrees Celsius **[4]**.

2.2.1. Talent vortexing the RNA-ISH Amplifier 3.

2.2.2. Talent adding Amplifier 3 to each slide, ensuring full coverage of each tissue section.

2.2.3. Talent closing the tray lid.

2.2.4. Talent placing the closed tray into the hybridization oven.

2.3. During incubation, prepare the metal oligonucleotide mix by diluting each oligo at a 1 to 15 ratio **[1]**. Vortex the metal oligo mix thoroughly and leave it at room temperature until further use **[2]**. Now, remove the humidity control tray from the oven **[3],** take the slide holder out of the tray **[4]**, and place the tray back into the oven **[5]**.

2.3.1. Talent adding metal oligonucleotides to a tube.

2.3.2. Talent vortexing the prepared metal oligo mix and keeps it on the bench.

2.3.3. Talent removing the humidity control tray from the oven.

2.3.4. Talent lifting the slide holder out of the tray.

2.3.5. Talent placing the empty tray back into the oven.

2.4. Then, place the slides into a clear slide holder that has been pre-washed with nuclease-free water **[1]**. Wash the slides in wash buffer under slight agitation for 2 minutes **[2-TXT]**.

2.4.1. Talent transferring slides into the clear slide holder.

2.4.2. Talent adds a wash buffer to the slide and shakes it. **TXT: Repeat 1x**

3. Hybridizing Metal Oligos to the Tissue Sections

3.1. After removing excess liquid from the slides, take the humidity control tray out of the oven **[1]** and place the slide holder into the tray **[2]**. Add the mixed metal oligonucleotides prepared earlier to fully cover each tissue section **[3]**.

3.1.1. Talent opening the oven and removing the humidity control tray.

3.1.2. Talent placing the slide holder into the tray.

3.1.3. Talent pipetting the metal oligo mix onto each tissue section to ensure full coverage.

3.2. Now, close the tray completely **[1]** and insert it into the hybridization oven for 45 minutes at 40 degrees Celsius **[2]**.

3.2.1. Talent closing the tray lid securely.

3.2.2. Talent placing the tray into the hybridization oven.

3.3. Remove the humidity control tray from the hybridization oven **[1]**, take out the slide holder **[2]**, and return the empty tray to the oven **[3-TXT]**.

3.3.1. Talent removing the humidity control tray from the oven.

3.3.2. Talent taking the slide holder out of the tray.

3.3.3. Talent placing the empty tray back into the oven. **TXT: Wash the slides 2x**

4. Hybridizing Metal Conjugated Antibodies to the Tissue Sections
Demonstrator: Sammy Ferri-Borgogno

4.1. To block non-specific binding sites, add a sufficient volume of blocking buffer to fully cover each tissue section on the slide **[1]** and incubate the slides at room temperature for 30 minutes **[2]**.

4.1.1. Talent applying blocking buffer to each slide to cover the tissue sections.

4.1.2. Talent placing the slides on a flat surface.

4.2. While the slides are incubating, prepare the antibody mix by diluting the antibodies using the antibody diluent buffer **[1]**.

4.2.1. Talent pipetting antibodies into a tube containing antibody diluent buffer.

4.3. Once the incubation is over, discard the buffer from the tissue slides **[1]** and dispense enough of the previously prepared antibody mix to completely cover the tissue sections **[2]**.

4.3.1. Talent removing the blocking buffer from the slides.

4.3.2. Talent pipetting the antibody mix onto each slide to ensure complete coverage.

4.4. Then, place the slides into a humidity chamber **[1]** and incubate them overnight at 4 degrees Celsius **[2]**.

4.4.1. Talent places slides carefully into the humidity chamber.

4.4.2. Talent placing the chamber into a refrigerator set to 4 degrees Celsius and closing the door.

5. DNA Staining to Prepare Tissue Sections for Imaging

5.1. To prepare a fresh Iridium-Intercalator solution, make a 1 to 2000 dilution from a 500 micromolar stock solution using RNase-free TBS **[1-TXT]**.

5.1.1. Talent pipetting 500 micromolar Iridium-Intercalator into RNase-free TBS to prepare the dilution. **TXT: Aliquot and store the working solution at - 20 °C**

5.2. Place the slides into a clear slide holder that has been pre-washed with nuclease-free water **[1]** and wash the slides in TBS-T buffer using slight agitation for 5 minutes **[2-TXT]**.

5.2.1. Talent transferring slides into the pre-washed clear slide holder.

5.2.2. Talent washing the slides in TBS-T buffer on a rocker or shaker. **TXT: Repeat wash 1x**

5.3. Then, to stain the slides, add the prepared Iridium working solution and incubate for 5 minutes at room temperature **[1]**.

5.3.1. Talent adding Iridium working solution to the slides and keeping it aside.

5.4. After that, place the slides into a clear slide holder that has been pre-washed with nuclease-free water **[1]**. Wash the slides two times in TBS-T buffer with slight agitation for 5 minutes **[2-TXT]**.

5.4.1. Talent placing slides into the clear, pre-washed slide holder.

5.4.2. Talent washing the slides in TBS-T buffer with agitation. **TXT: Wash 2x with TBS buffer**

5.5. Then, dip the slides quickly in double-distilled water to prevent salt crystallization from

TBS on the tissue **[1]**.

5.5.1. Talent dipping each slide swiftly into double-distilled water.

5.6. Dry the slides under a chemical hood for 10 minutes **[1]** and store them at 4 degrees Celsius until image acquisition **[2]**.

5.6.1. Talent placing slides under a chemical hood.

5.6.2. Talent placing the slides into a refrigerator set at 4 degrees Celsius.

6. Image Acquisition

Demonstrator: Angelique J. Lin

6.1. Load the prepared slide into the ablation chamber of the imaging mass cytometry system **[1]**. Capture a panorama image of the slide to identify the region of interest **[2]**.

6.1.1. SCREEN: 68616_SCREENSHOT_6.1.1.MP4 00:05-00:10, 01:55-02:10

6.1.2. SCREEN: 68616_SCREENSHOT_6.1.2.MP4 00:19-00:24,00:32-00:38, 00:47-00:52, 03:43-03:50

Videographer: Please film the computer screen for all shots labelled as SCREEN

6.2. Using the image acquisition software, mark the desired regions of interest on the panorama image **[1]**. Apply the acquisition template in the image acquisition software and adjust the laser power based on the sample type **[2].**

6.2.1. SCREEN: 68616_SCREENSHOT_6.2.1.MP4 00:06-00:23

6.2.2. SCREEN: 68616_SCREENSHOT_6.2.1.MP4 00:49-01:03, 01:10-01:14

6.3. Now, start the laser ablation process **[1]**. Measure the quantity of each isotope using the detector, which converts the data into digital form for analysis **[2]**.

6.3.1. SCREEN: 68616_SCREENSHOT_6.3.1.MP4 00:21-00:30

6.3.2. SCREEN: 68616_SCREENSHOT_6.3.2.MP4 00:05-00:10, 00:30-00:50

Results

7. Results

7.1. The activation status of CD8 T cells was assessed using co-detection of Granzyme B at both mRNA and protein levels, along with Interferon Gamma positivity **[1].**

7.1.1. LAB MEDIA: Figure 2A, B, and C. *Video editor: Highlight the merged panel*.

7.2. A cancer-associated fibroblast was shown to express MFAP5 *(M-Fap-Five)* protein and POSTN *(Post-N)* mRNA, both markers of cancer-associated fibroblast aggressiveness **[1].** A CD4 T cell was shown to co-express IL17a *(I-L-Seventeen-A)* and IL6 *(I-L-Six)* mRNA, consistent with Th17 *(T-H-Seventeen)* cell identity **[2].**

7.2.1. LAB MEDIA: Figure 3A**.** *Video editor: Highlight the merged panel showing co-expression of MFAP5 protein and POSTN mRNA in the same cell.*

7.2.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the merged panel showing CD4 T cell with IL17a and IL6 mRNA signals.*

7.3. CD4 T cells were observed expressing IL-13 mRNA **[1].** Keratin-positive, CD47 (*C-D-Forty-Seven)*-positive tumor cells were shown near a CD8 T cell **[2].** An activated CD8 T cell was shown to express CD25 -Five *(C-D-Twenty-Five)* protein **[3].**

7.3.1. LAB MEDIA: Figure 3C**.** *Video editor: Highlight the merged panel showing IL-13 mRNA signal within CD4 T cells.*

7.3.2. LAB MEDIA: Figure 3D**.** *Video editor: Highlight merged panel where Keratin+ CD47+ tumor cells are adjacent to a CD8+ T cell.*

7.3.3. LAB MEDIA: Figure 3E**.** *Video editor: Highlight the merged panel showing CD8 T cell co-expressing CD25 protein.*

7.4. Comparable mRNA staining of THBS2 *(T-H-B-S-two)* and CD47 was observed between metal-conjugated **[1]** and fluorescent-labelled protocols **[2].**

7.4.1. LAB MEDIA: Figure 4A. *Video editor: Highlight merged panel showing THBS2 and CD47 signals using metal-conjugated protocol.*

7.4.2. LAB MEDIA: Figure 4B. *Video editor: Highlight merged panel showing THBS2 and CD47 signals using fluorescent-labelled protocol.*

Pronunciation List

1. RNAscope
   Pronunciation link: https://www.howtopronounce.com/rnascope
   IPA: /ˌɑːrɛnˈeɪˌskoʊp/
   Phonetic Spelling: ar-en-ay-skohp
2. FFPE
   Pronunciation link: No confirmed link found
   IPA: /ˌɛf ɛf piː ˈiː/
   Phonetic Spelling: eff-eff-pee-ee
3. Oligonucleotide
   Pronunciation link: https://www.merriam-webster.com/dictionary/oligonucleotide
   IPA: /ˌɒlɪˌgəʊnjuːˈkleətaɪd/ (American: /ˌɑːlɪˌgoʊnjuːˈkleətaɪd/)
   Phonetic Spelling: ol-ih-go-noo-klee-uh-tide
4. Humidity
   Pronunciation link: https://www.merriam-webster.com/dictionary/humidity
   IPA: /hjuːˈmɪdɪti/
   Phonetic Spelling: hyoo-mid-ih-tee
5. Incubation
   Pronunciation link: https://www.merriam-webster.com/dictionary/incubation
   IPA: /ˌɪnkjuˈbeɪʃən/
   Phonetic Spelling: in-kyoo-bay-shun
6. Agglutination
   Pronunciation link: https://www.merriam-webster.com/dictionary/agglutination
   IPA: /əˌgluːtɪˈneɪʃən/
   Phonetic Spelling: uh-gloo-tih-nay-shun
7. Titration
   Pronunciation link: https://www.merriam-webster.com/dictionary/titration
   IPA: /taɪˈtreɪʃən/
   Phonetic Spelling: ty-tray-shun
8. Intercalator
   Pronunciation link: No confirmed link found
   IPA: /ˌɪntərˈkeɪleɪtər/
   Phonetic Spelling: in-ter-kay-lay-ter
9. Panorama
   Pronunciation link: https://www.merriam-webster.com/dictionary/panorama
   IPA: /ˌpænəˈrɑːmə/ (American: /ˌpænəˈræmə/)
   Phonetic Spelling: pan-uh-rah-muh
10. Ablation

Pronunciation link: https://www.merriam-webster.com/dictionary/ablation
IPA: /əˈbleɪʃən/
Phonetic Spelling: uh-blay-shun

11. Isotope
    Pronunciation link: https://www.merriam-webster.com/dictionary/isotope
    IPA: /ˈaɪsəˌtoʊp/
    Phonetic Spelling: eye-suh-tohp
12. Microinjection
    Pronunciation link: https://www.merriam-webster.com/dictionary/microinjection
    IPA: /ˌmaɪkroʊɪnˈdʒɛkʃən/
    Phonetic Spelling: my-kroh-in-jek-shun
13. Phenotype
    Pronunciation link: https://www.merriam-webster.com/dictionary/phenotype
    IPA: /ˈfiːnəˌtaɪp/
    Phonetic Spelling: fee-nuh-type
14. Transcriptome
    Pronunciation link: No confirmed link found
    IPA: /trænˈskrɪptoʊm/
    Phonetic Spelling: tran-skrip-tohm
15. Proteome
    Pronunciation link: No confirmed link found
    IPA: /ˈproʊtiˌoʊm/
    Phonetic Spelling: proh-tee-ohm