Submission ID #: 68617
Scriptwriter Name: Pallavi Sharma
Project Page Link: https://review.jove.com/account/file-uploader?src=20928233

Title: MicroRNA Amplification and Recognition through Locked-nucleic-acid In Situ Hybridization as A Novel Detection and Quantification Method

Authors and Affiliations:
Basant T. Gamal[1], Danielle L. Stolley[2], Christopher D. Pacheco[2], Akshay Basi[2], Anna K. Casasent[2], Jared K. Burks[2], Sammy Ferri-Borgogno[1]

[1]Department of Gynecologic Oncology and Reproductive Medicine, The University of Texas MD Anderson Cancer Center
[2]Department of Hematopoietic Biology & Malignancies, The University of Texas MD Anderson Cancer Center

Corresponding Authors:
Jared K. Burks (jburks@mdanderson.org)
Sammy Ferri-Borgogno (SFerri@mdanderson.org)

Email Addresses for All Authors:
(BTGamal@mdanderson.org)
(DStolley@mdanderson.org)
(CDPacheco@mdanderson.org)
(AVBasi@mdanderson.org)
(AKCasasent@mdanderson.org)
(jburks@mdanderson.org)
(SFerri@mdanderson.org)

Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **NO**

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **09/15/25**

When you are ready to submit your video files, please contact our Content Manager, Utkarsh Khare.

Current Protocol Length

Number of Steps: 23
Number of Shots: 49

Introduction

1.1. **Basant Gamal:** The scope of our research was to develop and validate an assay that simultaneously detects and quantifies miRNAs and multiple proteins within FFPE tissue to better spatially observe the relationship between both within the TME.
   1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 1*

What advantage does your protocol offer compared to other techniques?

1.2. **Basant Gamal:** It overcomes common limitations of existing molecular, sequencing, and traditional ISH by providing: direct quantification, colocalization and simultaneous spatial detection of miRNA and proteins without destroying the tissue and preserving cell and subcellular localization.
   1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What new scientific questions have your results paved the way for?

1.3. **Basant Gamal:** This technology potentially paves the way for both basic research into regulatory networks, disease mechanisms, and translational or clinical studies seeking better biomarkers and therapeutic targets.
   1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

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. Tissue Preparation for Immunofluorescence Hybridization Assay

Demonstrator: Basant Gamal

2.1. Begin by taking out formalin-fixed paraffin-embedded samples of human ovarian tumor kept overnight at 60 degrees Celsius **[1]**. Using RNAse-free water, clean all equipment and tools thoroughly **[2-TXT]**.

2.1.1. WIDE: Talent removing the FFPE slides from an oven.

2.1.2. Talent wipes down lab tools and equipment. **TXT: Ensure the sections are around 5 µm thick**

2.2. Next, to perform deparaffinization, immerse the samples in xylene three times for 5 minutes each **[1]**. Rehydrate the tissues with 10 rounds of sequential ethanol series, each for 3 minutes **[2],** and then wash the samples once in TBS for 2 minutes **[3]**.

2.2.1. Talent placing slides into xylene tube.

2.2.2. Talent transferring slides through a graded ethanol series arranged in labeled beakers.

2.2.3. Talent places slides in a beaker of tris-buffered saline.

2.3. Apply a 3 percent hydrogen peroxide solution to the samples for 10 minutes **[1]**. Wash the samples twice for 2 minutes each with TBS **[2]**. Then, apply a 3.7 percent paraformaldehyde solution for 10 minutes **[3-TXT]**.

2.3.1. Talent applying hydrogen peroxide solution to slides.

2.3.2. Talent places the slides in two fresh containers of tris-buffered saline.

2.3.3. Talent adding paraformaldehyde solution to slides. **TXT: Wash 2x with TBS for 2 min**

2.4. After that, begin the first round of antigen retrieval using the EZ *(E-Z)*-Retriever infrared system. Submerge the slides in fresh EZ-AR1 *(E-Z-A-R-One)* buffer **[1]** and microwave at 95 degrees Celsius for 15 minutes **[2-TXT]**.

2.4.1. Talent placing slides in EZ-Retriever IR system with buffer

2.4.2. Talent placing the slides in the microwave and selecting settings for 95 degrees Celsius and 15 minutes. **TXT: Cool the samples at RT for 20 min**

2.5. Then, rinse the samples in TBS for 2 minutes, followed by two 2-minute washes in TBS-T **[1].**

2.5.1. Talent places the slides in a beaker of tris-buffered saline.

3. Protein Application for Dual-Antibody Multiplex Immunofluorescence

3.1. To begin, draw a barrier around each tissue section using a hydrophobic barrier pen **[1]**. Add antibody blocking solution **[2]** and incubate the slides for 30 minutes at room temperature in a humidity chamber **[3]**.

3.1.1. Talent outlining tissue sections on slides with a hydrophobic barrier pen

3.1.2. Talent pipetting blocking solution onto the slide.

3.1.3. Talent places the slide inside a humidity chamber with the lid closed.

3.2. Now, apply the primary antibody diluted in the same blocking solution to fully cover the tissue section **[1]** and incubate for 1 hour at room temperature in a humidity chamber **[2]**.

3.2.1. Talent applying the antibody mixture to the tissue section.

3.2.2. Talent placing the slide in the humidity chamber.

3.3. After washing the slides three times in TBS-T, add the Anti-Mouse and Rabbit horseradish peroxidase solution to cover the tissue **[1]** and incubate in the humidity chamber at room temperature for 10 minutes **[2-TXT]**.

3.3.1. Talent pipetting HRP solution onto the slide.

3.3.2. Talent placing the slide into the humidity chamber. **TXT: Wash 3x in TBS-T for 2 min**

3.4. Now, dilute the first fluorophore at a 1 to 100 ratio with amplification dilutant **[1]**. Apply this mixture to the tissue and incubate for 10 minutes at room temperature in a humidity chamber, avoiding direct light exposure **[2-TXT]**.

3.4.1. Talent using a pipette to mix the fluorophore with amplification dilutant in a tube.

3.4.2. Talent applying the fluorophore mix to the slides using back light and places them inside the humidity chamber. **TXT: Wash 3x for 2 min each in TBS-T**

3.5. After performing the second round of antigen retrieval and blocking as shown previously, dilute the second primary antibody with the same blocking solution **[1]**. Apply the diluted antibody to cover the entire tissue section **[2]** and incubate overnight

for at least 16 hours at 4 degrees Celsius inside a humidity chamber **[3]**.

3.5.1. Talent mixing the second primary antibody with blocking solution in a microcentrifuge tube.

3.5.2. Talent applying the antibody mix to the slide placed in the humidity chamber.

3.5.3. Talent placing the chamber into a refrigerator.

3.6. The next day, wash the slides three times for 2 minutes each in TBS-T and incubate with Anti-Mouse and Rabbit horseradish peroxidase solution as shown earlier **[1-TXT]**. Then, apply the diluted second fluorophore to the slides and incubate for 10 minutes at room temperature in the humidity chamber **[2]**.

3.6.1. Talent rinsing the slides in TBS-T. **TXT: Wash 3x for 2 min in 1x TBS-T**

3.6.2. Talent applies the diluted fluorophore to the slide placed in the humidity chamber and keeping it aside.

4. Probe Application for Antigen Detection

4.1. For probe application, wash the slides three times for 2 minutes each in TBS-T **[1]**.

4.1.1. Talent places the slides sequentially in fresh containers of TBS-T.

4.2. To begin the third and final antigen retrieval, submerge the slides in EZ-AR1 Elegance buffer **[1]** and microwave at 107 degrees Celsius for 15 minutes **[2]**. Cool the slides at room temperature for 20 minutes **[3]** before washing them once for 3 minutes in TBS **[4]**.

4.2.1. Talent placing slides into the EZ-Retriever system filled with EZ-AR1 Elegance buffer.

4.2.2. Talent setting the microwave to 107 degrees Celsius for 15 minutes.

4.2.3. Talent removes the slide from the microwave and keeps it on the bench for cooling.

4.2.4. Talent immersing the slides in a beaker of TBS.

4.3. Apply enough RNAscope *(R-N-A-Scope)* Protease Plus to cover the tissue area **[1]** and incubate at 40 degrees Celsius for 30 minutes using an oven **[2-TXT]**.

4.3.1. Talent pipetting RNAscope Protease Plus onto the slide.

4.3.2. Talent places the slide into a 40 degrees Celsius oven. **TXT: Wash 2x with TBS for 5 min**

4.4. Then, heat the microRNA *(Micro-R-N-A)* locked nucleic acid probe to 90 degrees Celsius for 4 minutes to denature it **[1]**. Immediately dilute the denatured probe with microRNA in situ hybridization buffer **[2]**.

4.4.1. Talent placing an aliquot of the probe in a dry bath at 90 degrees Celsius for 4 minutes.

4.4.2. Talent adding ISH buffer to the denatured probe tube.

4.5. After vortexing and spinning the prepared probe, add it to cover the entire tissue area **[1]** and incubate for 1 hour at 60 degrees Celsius in the oven **[2]**. Wash the slides once for 5 minutes in 5 times SSC *(S-S-C)* buffer **[3-TXT]**.

4.5.1. Talent applying probe solution to the slides.

4.5.2. Talent places the slide in the oven set at 60 degrees Celsius.

4.5.3. Talent rinsing the slides in a beaker filled with 5 times saline sodium citrate buffer. **TXT: SSC: Saline Sodium Citrate**

4.6. Perform five stringent washes at 60 degrees Celsius using SSC buffer for 5 minutes each **[1-TXT]**.

4.6.1. Talent transferring the slides through to labeled beakers prewarmed to 60 degrees Celsius. **TXT: 1 round 5x SSC, 2 rounds of 1x SSC, and 2 rounds of 0.2x SSC**

4.7. Then, wash the slides once for 5 minutes in 0.2 times SSC at room temperature **[1]**. To prepare the blocking buffer, combine TBS and 0.5 percent blocking reagent powder **[2-TXT]**. Apply the blocking buffer and incubate for 30 minutes at room temperature in a humidified chamber **[3]**.

4.7.1. Talent immersing slides in a beaker of 0.2 times SSC at room temperature.

4.7.2. Talent adding powder to TBS in a beaker and stirring. **TXT: Vortex and spin the solution before use**

4.7.3. Talent applying blocking buffer onto slides and placing them in a humidified chamber.

4.8. Next, apply Anti-Digoxigenin Mouse horseradish peroxidase conjugate and incubate for 30 minutes at room temperature in a humidified chamber **[1]**.

4.8.1. Talent pipetting the diluted HRP conjugate onto the slides and placing them inside the humidified chamber.

4.9. After three TBS-T washes, apply 1 to 50 dilutions of the Tyramide Signal Amplification Plus Cyanine 3 to the slides for 10 minutes at room temperature in a humidity chamber **[1]**.

4.9.1. Talent applying TSA+Cy3 fluorophore to the slides inside the humidity chamber under low lighting conditions.

4.10. Wash the slides three more times for 5 minutes each in TBS-T **[1]**.

4.10.1. Talent immersing the slides in TBS-T container.

5. Nuclear Staining and Mounting for Imaging

5.1. Apply enough DAPI *(Dapi)* working solution, diluted 1 to 1000 in TBS, to fully cover the tissue section **[1]**. Incubate the slides for 5 minutes at room temperature in a humidified chamber with ambient and uncontrolled humidity **[2]**.

5.1.1. Talent pipetting DAPI working solution onto the slide surface.

5.1.2. Talent placing the slide inside a humidified chamber.

5.2. Wash the slides three times for 5 minutes each in TBS-T **[1]**. Apply coverslips using mounting media and let the slides dry for 10 minutes at room temperature **[2]**. Store the dried slides at 4 degrees Celsius in the dark for 24 to 72 hours before imaging or up to three months for longer-term storage **[3]**.

5.2.1. Talent rinsing the slides in TBS-T.

5.2.2. Talent carefully placing coverslips over the stained slides using a pipette to dispense mounting media.

5.2.3. Talent placing dried slides in a labeled slide box inside a 4 degrees Celsius refrigerator.

Results

6. Results

6.1. The miRNA *(M-I-R-N-A)* expression of miR-181c-3p *(Mir-One-Eight-One-C-Three-P)* was consistently detected in both control **[1]** and antibody-stained slides **[2]**, with visibly stronger signals concentrated in the tumor cell regions **[3]**.

6.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight panels A and B* .

6.1.2. LAB MEDIA: Figure 2. *Video editor: Highlight panels C and D.*

6.1.3. LAB MEDIA: Figure 2. *Video editor: Highlight panels C and D and Zoom in on the dense red areas in the center of the tissue*.

6.2. Application of the Anti-EpCAM *(Anti-Ep-Cam)* antibody with a 620-nanometer emission fluorophore stained the tumor cells in the HGSC *(H-G-S-C)* sample **[1]**. Application of the Anti-CD8 *(C-D-Eight)* antibody with a 620-nanometer emission fluorophore distinctly marked CD8-positive T cells **[2]**.

6.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight panels E and F.*

6.2.2. LAB MEDIA: Figure 2. *Video editor: Highlight panels G and H.*

6.3. Combined protein and miRNA detection enabled spatial localization of miR-181c-3p relative to EpCAM-positive tumor cells and CD8-positive immune cells **[1]**.

6.3.1. LAB MEDIA: Figure 2. *Video editor: Highlight panels I and J.*

6.4. The positive control probe targeting U6 small nuclear RNA produced strong fluorescent signals across all tissue types, confirming probe effectiveness and tissue integrity **[1].** The negative control probe, which used a scrambled miRNA sequence, showed no detectable signal in any tissue type, indicating no nonspecific binding **[2].**

6.4.1. LAB MEDIA: Figure 4. *Video editor: Highlight panels A to D*

6.4.2. LAB MEDIA: Figure 4. *Video editor: Highlight panels E to H*

6.5. The target probe for miR-181c-3p showed strong expression in the high-grade serous carcinoma tissue **[1],** with no signal in normal ovarian tissue **[2]** or normal fallopian tube tissue, demonstrating the specificity and accuracy of the probe and assay **[3].**

6.5.1. LAB MEDIA: Figure 4I-L. *Video editor: Highlight the bright pink stained areas in panels I and J*

6.5.2. LAB MEDIA: Figure 4I-J. *Video editor: Highlight the panel K*

6.5.3. LAB MEDIA: Figure 4I-L. *Video editor: Highlight the panel K*

1. **Paraffin**
   Pronunciation link: https://www.merriam-webster.com/dictionary/paraffin
   IPA: /ˈpærəfɪn/
   Phonetic spelling: pa-ruh-fin
2. **Deparaffinization**
   Pronunciation link: https://www.merriam-webster.com/dictionary/deparaffinization
   IPA: /diːˌpærəˌfɪnɪˈzeɪʃən/
   Phonetic spelling: dee-pa-ruh-fin-i-ZAY-shun
3. **Retrieval**
   Pronunciation link: https://www.merriam-webster.com/dictionary/retrieval
   IPA: /rɪˈtrivəl/
   Phonetic spelling: ri-TREE-vuhl
4. **Fluorophore**
   Pronunciation link: https://www.merriam-webster.com/dictionary/fluorophore
   IPA: /ˈflʊəroʊfɔːr/
   Phonetic spelling: FLU-oh-for
5. **Hydrogen**
   Pronunciation link: https://www.merriam-webster.com/dictionary/hydrogen
   IPA: /ˈhaɪdrədʒən/
   Phonetic spelling: HY-dro-jen
6. **Protease**
   Pronunciation link: https://www.merriam-webster.com/dictionary/protease
   IPA: /ˈproʊtiˌeɪz/
   Phonetic spelling: PRO-tee-ayz
7. **Denature**
   Pronunciation link: https://www.merriam-webster.com/dictionary/denature
   IPA: /diˈneɪtʃər/
   Phonetic spelling: di-NAY-cher
8. **Amplification**
   Pronunciation link: https://www.merriam-webster.com/dictionary/amplification

IPA: /ˌæmpləfɪˈkeɪʃən/
Phonetic spelling: am-pli-fi-KAY-shun