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Title: Precise Visualization of Insulin Receptors A and B in Murine Brain with an RNA In Situ Hybridization Assay

Authors and Affiliations:
Qin Yao, Caio H. Mazucanti, Patrícia A. Gomes, Qingrong Liu, Josephine M. Egan

Diabetes Section/Laboratory of Clinical Investigation, National Institute on Aging, National Institutes of Health

Corresponding Authors:

Josephine M. Egan eganj@grc.nia.nih.gov

Email Addresses for All Authors:

Qin Yao qin.yao@nih.gov
Caio H. Mazucanti caio.mazucanti@nih.gov
Patrícia A. Gomes patricia.amorimgomes@nih.gov
Qingrong Liu qingrong.liu@nih.gov
Josephine M. Egan eganj@grc.nia.nih.gov

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? **No.**

3. **Filming location:** Will the filming need to take place in multiple locations? **No.**

Current Protocol Length
Number of Steps: 25
Number of Shots: 51

Introduction

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

1.1. **Qin Yao:** My research bridges neuroscience, metabolism, and aging, focusing on brain insulin signaling. I investigate how insulin receptor isoforms IR-A and IR-B are differentially expressed across brain regions and their roles in neurodevelopment, synaptic function, and age-related diseases like Alzheimer's.

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

What are the most recent developments in your field of research?

1.2. **Qin Yao:** A key development is recognizing the brain as insulin-sensitive, with distinct roles for IR-A and IR-B. My work advances this by introducing a high-resolution RNA-based assay to map isoform expression, illuminating their roles in aging, diabetes, and cognition.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: 3.10*

What technologies are currently used to advance research in your field?

1.3. **Patrícia Gomes:** Advanced tools like RNAscope, spatial transcriptomics, single-cell RNA-seq, and 3D imaging now enable isoform-specific, spatially resolved analysis of insulin signaling in the brain, linking receptor function to neurodegeneration, cognition, and metabolism through integrated molecular, behavioral, and in vivo models.

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

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

Ethics Title Card

This research has been approved by the National Institute on Aging (NIA), which is fully accredited by the American Association for Accreditation of Laboratory Animal Care

Protocol

2. Section Pretreatment Before Duplex Detection Assay

Demonstrator: Qin Yao

2.1. To begin, take the formalin-fixed brain tissue slides **[1]** and wash them in PBS for 5 minutes while gently moving the slide rack up and down to remove any residual optimal cutting temperature compound **[2]**.

2.1.1. WIDE: Talent at the working bench with the slides placed in front of him.

2.1.2. Talent immersing slide rack in a container filled with PBS and moving it up and down slowly.

2.2. Place the slides in an oven and bake at 60 degrees Celsius for 30 minutes **[1]**.

2.2.1. Talent placing the washed slides into the oven.

2.3. Then, place the slides into a container with prechilled 4 percent paraformaldehyde in PBS **[1]** and incubate for 15 minutes at 4 degrees Celsius **[2]**.

2.3.1. Talent placing the slide rack into a container with 4 percent paraformaldehyde.

2.3.2. Talent places the slide rack at 4 degrees Celsius.

2.4. After dehydrating the slides in graded ethanol and drying them, apply 2 to 4 drops of hydrogen peroxide to each tissue section on the slide and incubate for 10 minutes at room temperature **[1]**. Rinse the slides once with distilled water by gently pouring or dipping **[2]**.

2.4.1. Talent adding 2 to 4 drops of hydrogen peroxide onto each slide section and keeping it aside.

2.4.2. Talent rinsing the slides with distilled water.

2.5. Next, prepare 700 milliliters of fresh Target Retrieval solution in a beaker, cover it with aluminum foil **[1],** and bring the solution to a consistent boil at 99 to 100 degrees Celsius **[2]**.

2.5.1. Talent pouring 700 milliliters of 1 times Target Retrieval solution into a beaker and covering it

2.5.2. Talent placing the beaker on a hot plate until it reaches a rolling boil.

2.6. Using forceps, carefully immerse the slide rack in the boiling Target Retrieval solution and incubate for 5 minutes **[1-TXT]**. Immediately transfer the hot slide rack into a dish containing distilled water and move the rack up and down 3 to 5 times **[2-TXT]**.

2.6.1. Talent lowering the slide rack into the beaker with boiling Target Retrieval solution. **TXT: Maintain the temperature between 98 °C and 102 °C**

2.6.2. Talent quickly transferring the hot slide rack into a container with distilled water and agitating it 3 to 5 times. **TXT: Repeat with fresh distilled water**

2.7. Then, rinse the slides in fresh 100 percent ethanol and move the slide rack up and down 3 to 5 times **[1]**.

2.7.1. Talent immersing the slides in fresh 100 percent ethanol and agitating them gently.

2.8. After air drying the slides, use a hydrophobic barrier pen to carefully draw around each tissue section 2 to 3 times **[1-TXT]**. Then, place the slides into the Batch Slide Tray **[2]**. Add protease to each section, ensuring full tissue coverage **[3]**. Incubate the tray in an oven at 40 degrees Celsius for 15 minutes **[4]**.

2.8.1. Talent circling each tissue section on the slide using a hydrophobic barrier pen. **TXT: Allow the slides to dry at RT**

2.8.2. Talent positioning the slides in the Batch Slide Tray.

2.8.3. Talent pipetting protease solution over each tissue section until fully covered.

2.8.4. Talent placing the slide tray into a preheated oven set to 40 degrees Celsius.

2.9. Remove the slide tray from the oven and transfer it to a dish of distilled water **[1].** Gently move the slides up and down for 2 minutes **[2-TXT]**.

2.9.1. Talent removing the tray from the oven and placing it into a container with distilled water.

2.9.2. Talent moving the slide up and down. **TXT: Repeat 2x with dH_2O**

3. Duplex Detection Assay

Demonstrator: Patrícia Gomes

3.1. Prepare two additional slide sections for positive and negative control probes **[1-TXT].**

3.1.1. Shot of the additional slide sections marked as positive and negative. **TXT: Select control probes with the same number of ZZ pairs as the target probe**

3.2. Before use, equilibrate all probes at 40 degrees Celsius for 10 minutes **[1]**, and bring AMP1 *(A-M-P-One)* through AMP12 *(A-M-P-Twelve)* reagents to room temperature **[2]**.

3.2.1. Talent placing probe tubes in a heat block at 40 degrees Celsius.

3.2.2. Talent removing AMP1 through AMP12 reagents from cold storage and leaving them on the bench.

3.3. After briefly spinning the C2 probe, mix it with the C1 probe in a 1 to 50 ratio **[1]**. Apply probe mix to fully cover each tissue section **[2]** and incubate the slides in an oven at 40 degrees Celsius for 2 hours **[3]**. Wash the slides two times for 2 minutes each in Wash Buffer at room temperature **[4]**.

3.3.1. Talent mixing C2 with C1 at the specified ratio.

3.3.2. Talent pipetting the probe mix onto each slide section, ensuring full coverage.

3.3.3. Talent placing the slides into an oven set at 40 degrees Celsius.

3.3.4. Talent performing two 2-minute washes in Wash Buffer at room temperature.

3.4. Now, remove excess wash buffer from the slides **[1]** and apply AMP1 reagent to cover each tissue section completely **[2]**. Incubate the slides at 40 degrees Celsius for 30 minutes and wash as shown earlier **[3]**. Similarly, incubate the slides with AMP3 through AMP8 reagent under the given conditions **[4].**

3.4.1. Talent blotting off the remaining wash buffer.

3.4.2. Talent pipetting AMP1 reagent onto the sections.

3.4.3. Talent placing the slides into an oven at 40 degrees Celsius.

3.4.4. **TEXT ON A PLAIN BACKGROUND**

AMP3: 40 °C, 15 min

AMP4: 40 °C, 15 min

AMP5: 40 °C, 30 min

AMP6: 40 °C ,15 min

AMP7: RT, 30 min

AMP8: RT, 15 min

3.5. For red signal detection, briefly centrifuge the Fast Red-B reagent **[1],** then mix it with Fast Red-A in a 1 to 60 ratio **[2]**.

3.5.1. Talent spinning down the Fast Red-B tube.

3.5.2. Talent combining Fast Red-A and B to prepare the red signal detection mix.

3.6. Next, apply 100 to 200 microliters of the prepared red signal detection solution to each slide section **[1]**. Cover the tray **[2]** and incubate in the dark at room temperature for 10 minutes **[3]**.

3.6.1. Talent pipetting the red mix onto the slides.

3.6.2. Talent covering the tray to protect it from light.

3.6.3. Talent placing the tray in a dark area for incubation at room temperature.

3.7. Then, wash the slides two times in Wash Buffer at room temperature **[1]**.

3.7.1. Talent rinsing the slides in two fresh containers of 1 times Wash Buffer.

3.8. After removing excess buffer from the slides, apply AMP9 reagent to fully cover each tissue section **[1]**. Incubate the slides in the oven at 40 degrees Celsius for 15 minutes **[2]**.

3.8.1. Talent adding AMP9 reagent after draining the wash buffer.

3.8.2. Talent placing the slides in an oven set to 40 degrees Celsius.

3.9. Then, wash the slides twice for 2 minutes each in Wash Buffer at room temperature **[1]**. Similarly, incubate the slides with AMP10, 11, and 12 under the given conditions **[2].**

3.9.1. Talent washing the slides in fresh Wash Buffer.

3.9.2. **TEXT ON A PLAIN BACKGROUND**

AMP10: 40 °C, 15 min

AMP11: RT, 30 min

AMP12: RT, 15 min

3.10. To detect a green signal, first centrifuge the Fast Green-B tube, then mix it with Fast Green-A in a 1 to 50 ratio **[1]**. Apply the green signal detection solution to each tissue section **[2]**. Cover the slide tray and incubate for 10 minutes at room temperature **[3]**.

3.10.1. Talent combining Fast Green-B with Fast Green-A to make the green detection mix.

3.10.2. Talent pipetting the green detection mix over the slides.

3.10.3. Talent covering the tray and placing it on the bench for a 10-minute room temperature incubation.

3.11. Wash the slides in Wash Buffer for 5 minutes, followed by a brief rinse in distilled water **[1]**.

3.11.1. Talent soaking the slides in Wash Buffer for 5 minutes.

4. Counterstaining and Mounting of the Slides for Imaging

Demonstrator: Qin Yao

4.1. Immerse the slides in 50 percent hematoxylin staining solution for 1 minute at room temperature until the tissue sections appear purple **[1]**.

4.1.1. Talent placing the slides into a container of hematoxylin stain.

4.2. Rinse the slides in tap water 3 to 5 times by moving them up and down **[1]**. Repeat this with fresh tap water until the background clears and the sections remain purple **[2]**.

4.2.1. Talent rinsing the slides in tap water by dipping them repeatedly.

4.2.2. Talent inspecting the sections for clarity.

4.3. Then, dip the slides in 0.02 percent ammonia water 3 times until the tissue sections turn blue **[1]**.

4.3.1. Talent immersing and agitating the slides in ammonia water while observing the color change to blue.

4.4. After rinsing the slides, completely dry the slides in a dry oven set to 60 degrees Celsius for approximately 15 minutes **[1]**. Dip the dried slides in fresh xylene to clear them **[2]**.

4.4.1. Talent placing the slides in a drying oven and setting the timer to 15 minutes.

4.4.2. Talent immersing the dried slides in a container of fresh xylene.

4.5. Apply mounting medium onto each slide **[1]** and gently place a coverslip over the section, taking care to avoid forming bubbles **[2]**. Air-dry the slides for at least 5 minutes at room temperature **[3-TXT]**.

4.5.1. Talent pipetting mounting medium onto the tissue section.

4.5.2. Talent carefully lowering a coverslip without trapping air bubbles.

4.5.3. Talent placing the mounted slides on a flat surface and allowing them to air-dry.
TXT: Image using a bright field microscope at 20x or 40x magnification

Results

5. Results

5.1. In the choroid plexus of the third ventricle, IR-A *(I-R-A)* was more abundantly expressed than IR-B *(I-R-B)*, with IR-A signals concentrated near the nuclei of epithelial cells **[1],** while fewer IR-B signals were detected in the same region **[2].**

5.1.1. LAB MEDIA: Figure 1A. *Video editor: Zoom in on the area with dense black arrows*

5.1.2. LAB MEDIA: Figure 1A. *Video editor: Highlight the red dots near the red arrows*

5.2. In the lateral ventricle, cells displayed similar expression patterns, with IR-A more prevalent than IR-B **[1]**, and several cells co-expressed both isoforms **[2].**

5.2.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the area where multiple black arrows point to clusters of teal dots*

5.2.2. LAB MEDIA: Figure 1B. *Video editor: Emphasize the red arrows pointing to cells containing both red and teal dots.*

5.3. The positive control confirmed successful RNA detection with widespread BaseScope *(Base-Scope)* signal across the tissue **[1]**, while the negative control showed minimal to no signal, indicating low background noise **[2].**

5.3.1. LAB MEDIA: Figure 1C.

5.3.2. LAB MEDIA: Figure 1D.

5.4. Immunofluorescence analysis demonstrated insulin receptor protein localization along the apical surface of epithelial cells in both the third **[1]** and lateral ventricles, confirming the presence at the protein level **[2].**

5.4.1. LAB MEDIA: Figure 1E. *Video editor: emphasize the green colored cells*

5.4.2. LAB MEDIA: Figure 1F. *Video editor: emphasize the green colored cells*

Pronunciation Guide:
1. isoform
Pronunciation link:
https://www.merriam-webster.com/dictionary/isoform
IPA: /ˈaɪ.səˌfɔːrm/
Phonetic spelling: *EYE-suh-form*

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2. RNAscope
(This is a branded assay name, so not typically in standard dictionaries.)
Pronunciation link: No confirmed link found
IPA: /ɑːr.en.ˈeɪ.skəʊp/
Phonetic spelling: *ar-en-A-scope*

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3. transcriptomics
Pronunciation link: No confirmed link found (not in Merriam-Webster; also specialized)
IPA: /ˌtræn.skɹɪpˈtoʊ.mɪks/
Phonetic spelling: *trans-kript-OH-miks*

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4. hybridization
Pronunciation link: https://www.merriam-webster.com/dictionary/hybridization
IPA: /hʌˌbrɪd.əˈzeɪ.ʃən/
Phonetic spelling: *huh-BRID-uh-ZAY-shun*

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5. protease
Pronunciation link: https://www.merriam-webster.com/dictionary/protease
IPA: /ˈproʊ.ti.eɪs/
Phonetic spelling: *PROH-tee-ayss*

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6. hematoxylin
Pronunciation link: https://www.merriam-webster.com/dictionary/hematoxylin
IPA: /ˌhiː.məˈtɒk.sə.lɪn/ (though the American may tend toward /ˌhi.məˈtɑksɪ.lɪn/)
Phonetic spelling: *hee-muh-TOKS-uh-lin*

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7. choroid (as in choroid plexus)
Pronunciation link: https://www.merriam-webster.com/dictionary/choroid
IPA: /ˈkɔːr.ɔɪd/ or American often /ˈkɔːrɔɪd/
Phonetic spelling: *KOR-oyd*

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8. plexus
Pronunciation link: https://www.merriam-webster.com/dictionary/plexus
IPA: /ˈplek.səs/
Phonetic spelling: *PLEK-suhs*

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9. paraformaldehyde
Pronunciation link: https://www.merriam-webster.com/dictionary/paraformaldehyde
IPA: /ˌpær.əˌfɔːrˈmæl.dəˌhaɪd/
Phonetic spelling: *PAR-uh-for-MAL-duh-hyd*

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10. ammonia
Pronunciation link: https://www.merriam-webster.com/dictionary/ammonia
IPA: /əˈmoʊ.njə/
Phonetic spelling: *uh-MOH-nyuh*