FINAL SCRIPT: APPROVED FOR FILMING

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Title: Isolation and Quantification of Axonal mRNAs Using Porous Membrane Inserts and RTddPCR

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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 done**

   If **Yes**, we will need you to record using screen recording software.

   We recommend using the screen capture program OBS. JoVE's tutorial for using OBS Studio is provided at this link: https://review.jove.com/v/5848/screen-capture-instructions-for-authors?status=a7854k

   As these files are necessary for finalizing your script, please upload all screen-captured video files to your project page as soon as possible: **https://review.jove.com/account/file-uploader?src=21228353**

Videographer: Please film the instrument screen as backup for step 2.13

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

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. **No**

Current Protocol Length
Number of Steps: 15
Number of Shots: 40

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. **Pabitra Sahoo:** Our research studies how local protein synthesis is regulated and their roles in neural repair, development, and degeneration.

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

Videographer's Note : Renamed to 1.3

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

1.2. **Shruti Ghumra:** Recent advances include high-sensitivity mRNA detection systems like ddPCR for identifying low-abundance axonal mRNAs in compartmentalized neuronal cultures.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.
Videographer's Note : Renamed to 1.4

CONCLUSION:

~~What significant findings have you established in your field?~~

1.3. **Pabrita Sahoo:** We have shown the presence of stress granule-like structures in the axons under physiological conditions, which inhibit local protein synthesis.

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

~~What research gap are you addressing with your protocol?~~

1.4. **Manasi Agrawal:** Our protocol offers a reliable and consistent method for isolating neuronal compartments and detecting low-abundant mRNAs.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. ~~*Suggested B.roll:3.5*~~

~~What questions will future research focus on?~~

1.5. **Meghal Desai:** Future research will focus on isolating and characterizing distinct axonal subdomains, such as growth cones and shafts, beyond bulk analyses.

1.5.1. 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.

Protocol

2. Differential Collection of Soma and Neurite Lysates for Gene Expression Studies

Demonstrator: Manasi Agrawal; Shruti Ghumra

2.1. To begin, aliquot 250 microliters of TRIzol *(Tri-zol)* into a 1.5-milliliter microcentrifuge tube corresponding to each insert and to one well or insert of a six-well plate **[1]**. Keep tubes aside until needed **[2]**.

2.1.1. WIDE: Talent pipetting 250 microliters of TRIzol into each labeled 1.5 milliliter microcentrifuge tube.
Videographer's Note: Filmed as a WIDE shot and a CU shot

2.1.2. Shot of filled tubes.

2.2. Add 2 milliliters of sterile PBS into each well of a second six-well plate, ensuring the number of wells or plates matches the number of inserts **[1]**. Pipette out the culture media from both the top and bottom of the insert **[2]**and transfer the insert into the six-well plate containing PBS **[3]**.

2.2.1. Talent pipetting 2 mL sterile PBS into each well of the second six-well plate.

2.2.2. Talent aspirating culture media from the top and bottom of the insert.

2.2.3. Talent transferring the insert into a well containing PBS.
Videographer's Note: clip B112_B096_111186_001 includes both steps

2.3. Now, using forceps, gently place the insert **[1].** Then add 2 milliliters of PBS on top of it **[2]**. Aspirate the PBS from both sides and repeat to wash twice **[3]**. Then, leave the insert in fresh PBS **[4]**.

2.3.1. Talent placing the insert using forceps.

2.3.2. Talent pipetting PBS on top.

2.3.3. Talent aspirating PBS from both the top and bottom of the insert during second wash.

2.3.4. Shot of the insert in PBS.

2.4. Next, use a sterile cell scraper to scrape the whole neuron fraction from the top of the insert **[1-TXT].** Collect the soma lysate from the insert **[2].** Transfer it into a 1.5-milliliter microcentrifuge tube **[3]**. Centrifuge the tube at 10,000 to 15,000 *g* for 2 minutes **[4]**.

2.4.1. Talent scraping the top of the insert with a sterile cell scraper using gentle

pressure. **TXT: Apply gentle pressure so that cells are removed without breaking membrane**
Videographer's Note: Rename 2.4.1 to 2.4.2

2.4.2. Talent pipetting out the soma lysate from the top of the insert.
Videographer's Note: Rename 2.4.2 to 2.4.3

2.4.3. Shot of the lysate being transferred into a labeled 1.5-milliliter microcentrifuge tube.
Videographer's Note: Rename 2.4.3 to 2.4.4

2.4.4. Talent placing the microcentrifuge tube into the centrifuge and starting the spin at the specified speed and time.
Videographer's Note: Rename 2.4.4 to 2.4.5

2.5. Discard the supernatant **[1]** and resuspend the pellet in 250 microliters of TRIzol **[2]**. Label this tube as the whole neuron fraction **[3]**.

2.5.1. Talent pipetting out the supernatant.

2.5.2. Talent resuspending the pellet by adding 250 microliters of TRIzol.

2.5.3. Talent labeling the microcentrifuge tube clearly as "whole neuron fraction".
Videographer's Note: Please move 2.5.3 before 2.4.1. Rename 2.5.3 to 2.4.1

2.6. To collect the neurite fraction, move one end of a sterile cotton swab slowly in a zig-zag pattern from top to bottom on the whole neuron side of the insert **[1]**. Rotate the insert 90 degrees and repeat using the other end of the swab **[2],** then discard the swab **[3]**.
Videographer's Note: 2.6.1-2.6.3 filmed in one clip but also filmed a (PU) pickup shot for just 2.6.

2.6.1. Talent using a sterile swab to make a slow zig-zag motion across the surface of the insert.

2.6.2. Talent rotating the insert and repeating the motion.

2.6.3. Talent discarding the swab into a biohazard container.

2.7. Use a new swab and move in concentric circles starting from the center of the insert outward, making sure to clean the circumference as well **[1]**.

2.7.1. Shot of the swab being moved on the insert in circular motion from center outward, reaching all edges and walls.

2.8. Invert the insert so the neurite side faces up **[1]**. ~~While holding it with forceps **[2],**~~ Cut the membrane using a new sterile scalpel blade **[3-TXT]**.

2.8.1. Talent inverting the insert.

~~2.8.2. Talent holding the insert with forceps.~~
Note: Removed as per videographer's note

2.8.3. Talent cutting the membrane with a scalpel blade while leaving the edge intact.

TXT: Leave 2 – 3 mm in the periphery
Videographer's Note: Rename 2.8.3 to 2.8.2

2.9. Place the cut membrane into the six-well plate containing TRIzol with the neurite side facing down **[1].** Ensure that the membrane is submerged **[2]**.

2.9.1. Talent placing the cut membrane into the well with TRIzol.

2.9.2. Shot of the submerged membrane.

2.10. Now collect the TRIzol containing the neurite lysate from the well **[1].** Transfer it into a 1.5-milliliter microcentrifuge tube **[2]**.

2.10.1. Shot of the TRIzol being pipetted out.

2.10.2. Talent pipetting the TRIzol lysate into a labeled microcentrifuge tube.

2.11. Proceed with RNA isolation or store the soma and neurite lysates at minus 80 degrees Celsius for later processing **[1]**.

2.11.1. Talent placing labeled tubes into a -80 degrees Celsius freezer rack.

2.12. Prepare reverse-transcription droplet digital PCR reactions using droplet digital PCR ready-to-use universal mix and target-specific primers with appropriate complementary DNA **[1]**. Pipette the reaction mixture into the droplet generation cartridge **[2].** Then add the generation oil into the designated wells of the cartridge **[3].**

2.12.1. Shot of prepared droplet mix, primers, and complementary DNA.

2.12.2. Talent pipetting the reaction mixture into the droplet generation cartridge.

2.12.3. Talent adding droplet generation oil to the designated wells of the cartridge.

2.13. Now seal the cartridge with the gasket **[1].** Generate the droplets using the droplet generator **[2]**.

2.13.1. Talent sealing the cartridge with the gasket.

2.13.2. Talent placing the cartridge into the droplet generator and starting the droplet generation.

2.14. Once the droplets have been generated, transfer them into a 96-well PCR plate **[1]** and seal with foil **[2]** before placing the plate in the thermocycler **[3].**

2.14.1. Talent transferring the generated droplets into a 96-well PCR plate.

2.14.2. Talent sealing with foil.

2.14.3. Talent placing the 96-well PCR plate in the thermocycler

~~2.15. Read the endpoint fluorescence with the droplet reader **[1]** and analyze the data using the built-in software **[2].** Manually inspect the droplet fluorescence amplitude plots to verify automatic thresholding accuracy **[3].**~~

~~*Videographer: Please film the instrument screen as backup for this step*~~

~~2.15.1. SCREEN: Shot of the instrument screen where the endpoint fluorescence is being seen.~~

~~2.15.2. SCREEN: Shot of the instrument screen where the built in software is being opened to view data.~~

~~2.15.3. SCREEN: Talent looking at amplitude plots and adjusting threshold if necessary.~~

NOTE: This step has been elaborated in 2.16. as per the SC file

2.16. Open the QX Manager software to begin the analysis **[1].** Click on the **Browse** option and open the file to be analysed **[2].**

2.16.1. SCREEN: 69601_screenshot_1.mp4 00:00-00:07

2.16.2. SCREEN: 69601_screenshot_1.mp4 00:07-00:14

Video Editor: Please blur the file names at 00:14

2.17. When the file has loaded, the dashboard view appears on the left panel **[1]**. Select the wells of interest by holding the control key while clicking **[2]**.

2.17.1. SCREEN: 69601_screenshot_1.mp4 00:19-00:25

2.17.2. SCREEN: 69601_screenshot_1.mp4 00:19-00:25

2.18. Click on **1D Amplitude** to visualize a dot graph **[1].** Select **Threshold Multiple Wells. to** apply the same threshold to more than one well **[2]**. Enter the threshold value based on where a clear separation is seen between positive and negative droplets **[3].**

2.18.1. SCREEN: 69601_screenshot_1.mp4 00:27-00:31

2.18.2. SCREEN: 69601_screenshot_1.mp4 00:31-00:38

2.18.3. SCREEN: 69601_screenshot_1.mp4 00:38-00:39

2.19. Now view the data section well showing the sample description, concentration values, number of accepted droplets, as well as positive and negative droplet counts **[1].** Click **Save** to record the thresholding results **[2].**

2.19.1. SCREEN: 69601_screenshot_1.mp4 00:39-00:50

2.19.2. SCREEN: 69601_screenshot_1.mp4 00:50-00:53

Results

3. Results

3.1. RNA quantification using the RiboGreen *(Ry-Boh-Green)* assay revealed that whole neuron fractions yielded 212.85 nanograms of RNA per insert **[1]**, while neurite fractions yielded 42.75 nanograms **[2]**.

3.1.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the row "Whole neuron"*

3.1.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the row "Neurite"*

3.2. PCR validation identified primer set 1 as the most specific for the *Gap43 (Gap-forty-three)* transcript, showing a distinct single band **[1]**.

3.2.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the Gap43 gel panel*

3.3. Ambiguous PCR results for *Acty (Act-gamma)* prompted a temperature gradient test using primer set 2, which showed strongest amplification at 55 degrees Celsius **[1]**.

3.3.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the leftmost lane under "55 °C" showing the brightest band.*

3.4. RTddPCR amplitude plots showed clear droplet separation for *Acty* in both whole neuron **[1]** and neurite samples, confirming reliable transcript detection **[2]**.

3.4.1. LAB MEDIA: Figure 4A. *Video editor: Emphasize the cluster of blue droplets for "Acty".*

3.4.2. LAB MEDIA: Figure 4C. *Video editor: Highlight both rows for "Acty"*

3.5. Normalized to total RNA, *Gap43* transcript levels were higher in neurite fractions, with 2,740 copies per nanogram of RNA **[1]** compared to 11,660 in whole neurons **[2]**.

3.5.1. LAB MEDIA: Figure 4C. *Video editor: Highlight the Gap43 in the "Neurite" row*

3.5.2. LAB MEDIA: Figure 4C. *Video editor: Highlight the same column in the "Whole neuron" row showing 11,660.*

Pronunciation Guide:
mRNA
Pronunciation link: No confirmed link found
IPA: /ˌɛm.ɑːr.enˈeɪ/
Phonetic Spelling: em·ahr·en·ay
RTddPCR
Pronunciation link: No confirmed link found
IPA: /ˌɑr.tiː.diː.diː.piː.siːˈɑːr/
Phonetic Spelling: ar-tee·dee·dee·pee-see-ar
Axonal
Pronunciation link: https://www.merriam-webster.com/dictionary/axonal
IPA: /ækˈsəʊ.nəl/ (AmE: /ækˈsoʊ.nəl/)
Phonetic Spelling: ak·soh·nuhl
Compartmentalized
Pronunciation link: https://www.merriam-webster.com/dictionary/compartmentalized
IPA: /kəmˈpɑːrt.mənˌtaɪzd/ (AmE: /kəmˈpɑːrt·mən·ˌtaɪzd/)
Phonetic Spelling: kuhm·paar·tuh·men·tyzd
Transcriptome
Pronunciation link: https://www.merriam-webster.com/dictionary/transcriptome
IPA: /trænsˈkrɪpˌtoʊm/
Phonetic Spelling: trans·krip·tohm
Neurite
Pronunciation link: https://www.merriam-webster.com/dictionary/neurite
IPA: /ˈnjʊəˌraɪt/ (AmE often /ˈnʊrˌaɪt/)
Phonetic Spelling: nur·ite