FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 69095
Scriptwriter Name: Pallavi Sharma
Project Page Link: https://review.jove.com/files_upload.php?src=21074023

Title: Tracking Single Proteins in Lipid Bilayers Using Fluorescence Microscopy

Authors and Affiliations:

Eric P. Jacobo[1], Michael J. Martinez[1], Alessia Memeo[2], Barbara Barile[2], Grazia Paola Nicchia[2], James A. Brozik[1]

[1]Department of Chemistry, Washington State University
[2]Department of Biosciences, Biotechnology and Environment, University of Bari Aldo Moro

Corresponding Authors:
James A. Brozik (brozik@wsu.edu)

Email Addresses for All Authors:

Eric P. Jacobo (eric.jacobo@wsu.edu)
Michael J. Martinez (michael.j.martinez@wsu.edu)
Alessia Memeo (alessia.memeo@uniba.it)
Barbara Barile (barbara.barile@uniba.it)
Grazia Paola Nicchia (graziapaola.nicchia@uniba.it)
James A. Brozik (brozik@wsu.edu)

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**

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

   WITHIN THE SAME BUILDING, ROUGHLY 500 FT APART.

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: 28
Number of Shots: 52

Introduction

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

Videographer's Note: Audio file *Scene_1.1_1.2_1.3_1.4_1.5_Take1* is to sync with video file *DSC_1564.MOV*.
I had some technical issues with the shotgun picking up some mystery interference so I used a lav connected with my phone.

INTRODUCTION:

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

1.1. **James A. Brozik:** Proteins operate in a complex state-space, much of which is hidden. We use single-molecule imaging to reveal these states.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. ~~*Suggested B.roll:6.1.1*~~

~~What are the current experimental challenges?~~

1.2. **James A. Brozik:** When tracking single membrane proteins in lipid layers, the main challenges are sample preparation, photobleaching, and time resolution.

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

CONCLUSION:

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

1.3. **James A Brozik:** In this video, we have identified key states important to AQP4 regulation and their associated thermodynamic forces.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. ~~*Suggested B.roll:6.1.2*~~

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

1.4. **James A. Bozik:** This protocol eliminates guesswork in creating biomimetic membranes with purified transmembrane proteins that are suitable for time-lapse single-molecule fluorescence experiments.

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

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

1.5. **James A Brozik:** Protein machines operate at inflection points. Time-lapse single-protein tracking enables direct observation of stochastic pathways, branches, and dead ends.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. ~~*Suggested B.roll:5.3*~~

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

Protocol

2. Preparation of Small Unilamellar Vesicles

Demonstrator: Eric Jacobo

2.1. To begin, remove the lipid stock solutions from the freezer and allow them to reach room temperature **[1]**.

2.1.1. WIDE: Talent removing vials of lipid stock solutions from the freezer and placing them on the benchtop.

2.2. Take a clean 10-milliliter flask out of the oven and let it cool to room temperature **[1]**.

2.2.1. Talent retrieving a 10 milliliter round-bottom flask from the oven using protective gloves and setting it on the benchtop.

2.3. Add 900 microliters of spectroscopic-grade chloroform and 100 microliters of spectroscopic-grade methanol to the cooled round-bottom flask **[1]**. Then, add the appropriate amounts of each lipid to the flask and swirl to mix thoroughly **[2]**.

2.3.1. Talent using a micropipette to dispense chloroform and methanol into the round-bottom flask.

2.3.2. Talent adding lipid stock solutions one by one into the solvent-filled flask and swirling the flask gently.

2.4. Now, place a vacuum distillation connector on top of the round-bottom flask and attach it to the nitrogen drying line **[1]**. Then, turn on the nitrogen gas and adjust the pressure until the flow is just barely felt on the back of the hand **[2-TXT]**.

2.4.1. Talent fitting a vacuum distillation connector onto the flask and connecting it to a nitrogen gas line.

2.4.2. Talent opening the nitrogen gas valve and adjusting the flow while placing the back of the hand near the outlet to feel the gas. **TXT: Allow the sample to dry for 3 h to fully remove residual solvent**

2.5. Pipette 1 milliliter of buffer for every 5 micromoles of total lipid into the round-bottom flask **[1]**. Place a glass stopper on the flask and seal it with paraffin film **[2]**.

2.5.1. Talent pipetting the buffer into the round-bottom flask according to the lipid concentration.

2.5.2. Talent inserting a glass stopper into the flask opening and wrapping the neck of the flask with paraffin film.

2.6. Position the flask in a clamp on a ring stand and submerge its bottom in a 60-degree Celsius sonicator bath **[1-TXT]**. Confirm that the solution becomes opaque and that no lipids remain attached to the inner wall of the flask **[2]**.

2.6.1. Talent setting the flask in a clamp and lowering it into a warm sonicator bath. **TXT: Incubate 1 h, swirl every 15 min to form multilamellar liposomes**

2.6.2. Talent inspecting the flask and confirming an opaque solution with no visible lipid residue on the glass.

2.7. After the 1-hour incubation, turn on the sonicator and set the amplitude to its highest level **[1]**. Move the flask around within the bath to locate the spot with the strongest agitation where the solution visibly bumps and sprays inside the flask **[2]**. Sonicate the solution for 30 minutes, observing the transition from opaque to transparent and slightly opalescent **[3]**.

2.7.1. Talent switching on the sonicator and adjusting the amplitude setting.

2.7.2. Talent shifting the flask within the bath to find the point of maximum agitation.

2.7.3. Talent observing the change in solution appearance as sonication progresses.

2.8. Remove the lipid solution from the flask and transfer it to a microcentrifuge tube **[1]**. Centrifuge the tube at 100,000 *g* for 1 hour at 4 degrees Celsius **[2]**. After centrifugation, remove and discard the supernatant from the tube **[3-TXT]**.

2.8.1. Talent pouring the lipid solution into a labeled microcentrifuge tube.

2.8.2. Talent placing the tube into a centrifuge and initiating the run at specified settings.

2.8.3. Talent carefully removing and discarding the supernatant from the centrifuged tube. **TXT: Use SUVs fresh or store at 4 °C (1 week) / −80 °C with trehalose**

3. Preparation of Quartz Coverslips

Demonstrator: Michael Martinez

3.1. Place the 25-millimeter quartz coverslips into a beaker **[1]** and add equal volumes of nanopure water, 30 percent hydrogen peroxide, and concentrated nitric acid **[2]**.

3.1.1. WIDE: Talent placing multiple coverslips into a clean beaker.

3.1.2. Talent pouring in measured amounts of nanopure water, hydrogen peroxide,

and nitric acid.

3.2. Heat the coverslips in the prepared solution for 30 minutes until bubbling begins **[1]**.

3.2.1. Talent placing the beaker on a hot plate and heat until bubbles form in the solution.

3.3. Check the solution every 10 minutes and swirl gently to prevent the coverslips from sticking together **[1]**. Observe the coverslips sliding apart and separating during swirling **[2]**. Once separated, gradually reduce the swirling speed to maintain their separation and allow the bubbling solution to coat the coverslips evenly **[3]**.

3.3.1. Talent swirling the beaker gently at regular intervals while monitoring the coverslips.

3.3.2. Shot of coverslips shifting and sliding apart during swirling.

3.3.3. Talent slowing the swirling motion and letting the bubbles rise around the separated coverslips. **TXT: After 30 min, allow the solution to cool**

3.4. Then, rinse the coverslips thoroughly with purified water while gently swirling to remove all chemical residue **[1]**.

3.4.1. Talent pouring out the used solution and rinsing the coverslips multiple times with purified water using a swirling motion.

3.5. Alternatively, clean the quartz coverslips using n-hexane followed by methanol, wiping with lens tissue for each solvent **[1]**. Place the coverslips inside a UV ozone cleaner with the surface to be treated facing the lamp **[2]**.

3.5.1. Talent folding lens tissue, applying n-hexane to it, and cleaning the coverslips.

3.5.2. Talent positioning the coverslips into the UV ozone cleaner with treated surfaces oriented toward the light source.

3.6. Allow oxygen to flow into the chamber at 5 pounds per square inch for 5 minutes **[1]**, then turn off the oxygen flow **[2]**.

3.6.1. Shot of the gas control system with oxygen flow set to 5 psi and the timer set for 5 minutes.

3.6.2. Talent turning off the oxygen supply.

3.7. Now, turn on the ultraviolet lights for 15 minutes, then allow the coverslips to rest for at least 10 minutes to permit the ozone to dissipate **[1-TXT]**.

3.7.1. Shot of the UV ozone cleaner interface as the lamp is turned on with a visible 15-minute countdown. **TXT: Use freshly cleaned coverslips immediately**

4. Bilayer Formation and Incorporation of AQP4 into a Bilayer Membrane

Demonstrator: Eric Jacobo

4.1. Place a freshly cleaned coverslip into a 25-millimeter sample holder using a one-quarter inch SM1 *(S-M-One)* lens tube **[1]**. Cut an 8-millimeter diameter, double-layered parafilm gasket **[2]** and position it in the center of the sample holder **[3]**.

4.1.1. Talent mounting the coverslip into the sample holder using a lens tube.

4.1.2. Talent trimming a parafilm gasket to size.

4.1.3. Talent placing it precisely in the sample holder's center.

4.2. Apply a 50-microliter droplet of the small unilamellar vesicles to the center of the 8-millimeter gasket **[1]**. After sealing the chamber, incubate at 37 degrees Celsius for 1 hour **[2]**.

4.2.1. Talent pipetting the vesicle droplet onto the gasket.

4.2.2. Talent placing the setup it in a 37 degree Celsius incubator.

4.3. Post incubation, use a pipette to rinse away the solution **[1]**. Add 50 microliters of fresh buffer to the bilayer and repeat this process a total of 10 times **[2]**.

4.3.1. Talent pipetting out the solution from the sample holder.

4.3.2. Talent pipetting in fresh buffer to the same spot.

4.4. After the final rinse, remove the buffer from the sample holder **[1]**. Add a 50-microliter aliquot of the desired protein in detergent, ensuring the concentration is at or below the critical micellar concentration **[2]**. Incubate the sample at 37 degrees Celsius for at least 1 hour to allow protein incorporation **[3]**.

4.4.1. Talent using a pipette to remove the last buffer wash.

4.4.2. Talent adding the protein-detergent mixture carefully to the bilayer.

4.4.3. Talent placing the sample back into the 37 degree Celsius incubator.

4.5. Rinse the sample with buffer to remove any unincorporated protein and detergent **[1-TXT]**.

4.5.1. Talent using a pipette to wash the sample gently with buffer. **TXT: The sample is ready for imaging**

5. Data Collection and Tracking Analysis

Demonstrator: Michael Martinez

5.1. Set the vertical pixel shift speed to approximately 600 nanoseconds and increase the vertical clock voltage using overclock mode **[1]**. Then, configure the horizontal pixel readout to its maximum speed **[2]**. Adjust the pre-amplifier gain to 2, set the amplifier output for electron multiplication, and set the electron multiplier gain to its highest level **[3]**.

5.1.1. SCREEN: 69095_screenshot_2.mp4 00:03-00:13

5.1.2. SCREEN: 69095_screenshot_2.mp4 00:13-00:20

5.1.3. SCREEN: 69095_screenshot_2.mp4 00:20-00:40

5.2. Then, set the exposure time to 25 milliseconds. Confirm that the actual frame rate exceeds the exposure time slightly **[1]**.

5.2.1. SCREEN: 69095_screenshot_2.mp4 00:47-01:04

5.3. Now adjust the laser power until the signal clearly stands out from the background noise **[1]**. Collect enough imaging data to yield a minimum of 1,000 tracks per sample **[2]**.

5.3.1. SCREEN: 69095_screenshot_2.mp4 01:10-01:20

5.3.2. SCREEN: 69095_screenshot_2.mp4 01:22-01:33

5.4. For tracking analysis, crop all data sets to a consistent size and perform background correction using ImageJ **[1]**.

5.4.1. SCREEN: 69095_screenshot_3.mov 00:17-00:30, 01:10-01:21

5.5. In FIJI *(Fiji),* drag and drop the movie or stacked TIFF file that needs to be analyzed into the interface **[1]**. To enter the pixel calibration details, go to the **Analyze** tab, select **Set Scale**, and apply the pixel-to-distance calibration specific to the instrument **[2]**.

5.5.1. SCREEN: 69095_screenshot_1.mov 00:00-00:13

5.5.2. SCREEN: 69095_screenshot_1.mov 00:30-00:56

5.6. Then, save a copy of the dataset to preserve the original and track any changes made

[1].

5.6.1. SCREEN: 69095_screenshot_1.mov 00:59-01:10

5.7. Subtract the background from a selected region of interest and apply it to the saved copy of the original data. Go to the **Process** tab and choose **Subtract Background [1]**.

5.7.1. SCREEN: 69095_screenshot_1.mov 01:28-01:50

5.8. In the **Plugins** tab, select **Tracking** followed by **TrackMate** to launch the TrackMate dialog window for tracking analysis **[1]**.

5.8.1. SCREEN: 69095_screenshot_1.mov 02:09-02:34

Results

6. Results

6.1. The degree of labeling for AQP4 *(A-Q-P-Four)* tetramers was calculated to be 4.12 using UV-Visible spectrometry **[1],** and the Poisson distribution analysis indicated that 98% of tetramers carried at least one fluorescent dye molecule **[2].**

6.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight the central bar in the histogram at "4" on the x-axis labeled "Number of Fluorescent Labels"*

6.1.2. LAB MEDIA: Figure 2. *Video editor: Zoom in on the height of the bars between 1 and 8 fluorescent labels*

6.2. The histogram shows a wide distribution of step sizes, consistent with diffusion of differently sized orthogonal arrays of particles **[1],** and the calculated average diffusion coefficient was 0.0143 square micrometers per second **[2].**

6.2.1. LAB MEDIA: Figure 5.

6.2.2. LAB MEDIA: Figure 5. *Video editor: Zoom in on the diffusion coefficient value "0.0143 µm²/s" displayed prominently in the upper center of the plot*

Pronunciation Guide:

 Fluorescence
Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescence
IPA: /flʊˈrɛsəns/
Phonetic Spelling: floor·eh·suhns
 Microscopy
Pronunciation link: https://www.merriam-webster.com/dictionary/microscopy
IPA: /maɪˈkrɑːskəpi/
Phonetic Spelling: my·krah·skuh·pee
 Lipid
Pronunciation link: https://www.merriam-webster.com/dictionary/lipid
IPA: /ˈlɪpɪd/
Phonetic Spelling: lih·pid
 Bilayers
Pronunciation link: https://www.merriam-webster.com/dictionary/bilayer
IPA: /ˈbaɪˌleɪər/
Phonetic Spelling: by·lay·er
 Unilamellar
Pronunciation link: https://www.merriam-webster.com/dictionary/unilamellar
IPA: /ˌjuːnɪləˈmɛlər/
Phonetic Spelling: yoo·nih·luh·meh·ler
 Vesicles
Pronunciation link: https://www.merriam-webster.com/dictionary/vesicle
IPA: /ˈvɛsɪkəl/
Phonetic Spelling: veh·sih·kuhl
 Chloroform
Pronunciation link: https://www.merriam-webster.com/dictionary/chloroform
IPA: /ˈklɔːrəˌfɔːrm/
Phonetic Spelling: klor·uh·form
 Methanol
Pronunciation link: https://www.merriam-webster.com/dictionary/methanol
IPA: /ˈmɛθəˌnɔːl/
Phonetic Spelling: meh·thuh·nawl
 Sonicator
Pronunciation link: https://www.merriam-webster.com/dictionary/sonicator
IPA: /ˈsɑːnɪˌkeɪtər/
Phonetic Spelling: sah·nih·kay·ter
 Centrifuge
Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛntrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj

 Ozone
Pronunciation link: https://www.merriam-webster.com/dictionary/ozone
IPA: /ˈoʊˌzoʊn/
Phonetic Spelling: oh·zohn
 Aquaporin
Pronunciation link: https://www.merriam-webster.com/dictionary/aquaporin
IPA: /ˌɑːkwəˈpɔːrɪn/
Phonetic Spelling: ah·kwuh·por·in
 AQP4
Pronunciation link: No confirmed link found
IPA: /ˌeɪ.kjuːˈpiː fɔːr/
Phonetic Spelling: ay·kyoo·pee·for
 Tetramers
Pronunciation link: https://www.merriam-webster.com/dictionary/tetramer
IPA: /ˈtɛtrəˌmər/
Phonetic Spelling: teh·truh·mer
 Photobleaching
Pronunciation link: https://www.merriam-webster.com/dictionary/photobleaching
IPA: /ˌfoʊtoʊˈbliːtʃɪŋ/
Phonetic Spelling: foh·toh·blee·ching
 Thermodynamic
Pronunciation link: https://www.merriam-webster.com/dictionary/thermodynamic
IPA: /ˌθɝːmoʊdaɪˈnæmɪk/
Phonetic Spelling: thur·moh·dy·na·mik
 Spectrometry
Pronunciation link: https://www.merriam-webster.com/dictionary/spectrometry
IPA: /spɛkˈtrɑːmətri/
Phonetic Spelling: spek·trah·muh·tree
 Poisson
Pronunciation link: https://www.merriam-webster.com/dictionary/Poisson
IPA: /ˈpwɑːsɑːn/
Phonetic Spelling: pwah·sahn
 Diffusion
Pronunciation link: https://www.merriam-webster.com/dictionary/diffusion
IPA: /dɪˈfjuːʒən/
Phonetic Spelling: dih·fyoo·zhuhn
 Micrometers
Pronunciation link: https://www.merriam-webster.com/dictionary/micrometer
IPA: /maɪˈkrɑːmɪtər/
Phonetic Spelling: my·krah·mih·ter