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Scriptwriter Name: Sulakshana Karkala

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Title: Localization of Plasma Membrane and Intracellular Neuronal Nicotinic Acetylcholine Receptors Using Quantitative Imaging in Mammalian Cells

Authors and Affiliations:

Lahra Weber¹, Sarah M. Suarez¹, Maegan M. Weltzin^{1, 2}

¹Department of Chemistry and Biochemistry, University of Alaska Fairbanks

²Institute of Arctic Biology, University of Alaska Fairbanks

Corresponding Authors:

Maegan M. Weltzin (mmweltzin@alaska.edu)

Email Addresses for All Authors:

Lahra Weber (lweber7@alaska.edu)

Sarah M. Suarez (smsuarez@alaska.edu)

Maegan M. Weltzin (mmweltzin@alaska.edu)

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? **Yes**

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=21158203>

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: **After 2/16/2026 but before 3/1**

When you are ready to submit your video files, please contact our Content Manager, [Utkarsh Khare](#).

Current Protocol Length

Number of Steps: 31

Number of Shots: 56

Introduction

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

INTRODUCTION:

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

- 1.1. **Maegan Weltzin**: Understanding nicotinic acetylcholine receptor trafficking to mammalian plasma membranes is crucial for investigating chaperone-mediated regulation and evaluating target-selective therapeutics.
 - 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. **Sarah Suarez**: A primary advantage of pH-sensitive probes combined with live-cell confocal microscopy is the high spatial resolution of subunit location.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

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

- 1.3. **Lahra Weber**: Using the presented protocol, we characterize novel chaperone effects on nicotinic receptor expression and trafficking, and evaluate new target-selective therapeutics.
 - 1.3.1. 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?~~

- 1.4. **Sarah Suarez**: An advantage of our protocol is its adaptability for homomeric and heteromeric nicotinic receptors, enabling quantification of relative cellular location.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How will your findings advance research in your field?~~

- 1.5. **Maegan Weltzin:** Our approach enables rapid, robust nicotinic receptor plasma membrane expression, aiding chaperone-mediated trafficking analysis and drug discovery efforts.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Transfection of Neuro2a Cells with Nicotinic Acetylcholine Receptor Subunits Using Lipid-Based Delivery

Demonstrators: Lahra Weber, Sarah Suarez

2.1. To begin, prewarm reagents to 37 degrees Celsius [1]. Thaw the nAChR (*N-A-C-H-R*) subunit DNAs completely and bring the transfection reagent to room temperature [2-TXT]. Clean all reagents with 70 percent ethanol before placing them inside the biosafety cabinet [3].

2.1.1. Talent placing tubes in a water bath to prewarm.

2.1.2. Close-up of thawed DNA vials and transfection reagent at RT. **TXT: nAChR: neuronal Nicotinic Acetylcholine Receptors**

2.1.3. Talent wiping down reagent bottles with ethanol before moving to the cabinet.

2.2. To express alpha7 nAChRs, in a microcentrifuge tube, dilute alpha7 and NACHO (*nacho*) plasmid DNAs into reduced serum medium [1]. Mix gently by pipetting after DNA addition [2].

2.2.1. Talent pipetting DNA into reduced serum medium.

AND

TEXT ON PLAIN BACKGROUND:

4 µg of α7 (1 µg/µL)

1 µg of NACHO (1 µg/µL) plasmid DNAs

Add dropwise into reduced serum medium

Final volume: 250 µL

Video Editor: Please play both shots side by side in a split screen

2.2.2. Talent pipetting the contents of microcentrifuge tube gently. **NOTE: Included with shot 2.2.1 in one clip.**

2.3. Alternatively, to express alpha4beta2 (*Alpha-four-beta-two*) receptors, dilute 4 micrograms of each subunit DNA into a total of 250 microliters of reduced serum medium and mix gently [1-TXT].

2.3.1. Talent pipetting subunit DNAs into the medium.

TXT: Adjust the subunit ratios to optimize expression of specific isoforms

2.4. For alpha4beta2 receptors with an additional plasmid, add equal amounts of each plasmid DNA [1].

2.4.1. Talent adding three equal DNA volumes into a microcentrifuge tube.

AND

TEXT ON PLAIN BACKGROUND:

The blue fluorescent protein mTagBFP2³¹ in pcDNA3.1(+) serves as a control plasmid and can be replaced with any $\alpha\beta 2$ chaperone

Video Editor: Please play both shots side by side in a split screen

2.5. Next, in a new microcentrifuge tube, dilute 8 microliters of transfection reagent dropwise into 250 microliters of reduced serum medium [1].

2.5.1. Talent adding reagent dropwise into a tube with medium.

2.6. After a 5-minute incubation at room temperature, combine the diluted DNAs with the diluted transfection reagent dropwise into a tube [1]. Mix gently by pipetting and incubate for 20 minutes at room temperature [2].

2.6.1. Talent combining two tubes by dropwise addition.

2.6.2. Talent mixing and placing the tube on a bench. **NOTE: Included with shot 2.6.1 in one clip.**

2.7. While complexes form, replace the medium in the culture dishes with 1 milliliter of serum-free EMEM [1].

2.7.1. Talent aspirating old medium from the culture dishes and adding serum-free medium.

2.8. Add 500 microliters of the DNA-transfection reagent complex dropwise to each dish [1]. Mix gently by rocking the plate to distribute evenly, especially over the glass-bottom surface [2], and return the dish to the incubator [3-TXT].

2.8.1. Talent adding transfection mix dropwise to the dish.

2.8.2. Talent gently rocking the plate in a circular motion **NOTE: Included with shot 2.8.1 in one clip.**

2.8.3. Talent walking back to the incubator to place cells inside. **TXT: Incubation: 37°C, 24 h; Replace medium after 4 - 6 h**

3. Confocal Imaging of pH-Sensitive Fluorescent Signals in Transfected Neuro2a Cells Using Live Buffer Exchange

Demonstrator: Sarah Suarez

3.1. Remove the old medium by suction [1]. Rinse the dish three times with 2 milliliters of PBS for 10 minutes each at room temperature [2]. Then add 2 milliliters of pH 7.4 imaging buffer to the dish [3].

- 3.1.1. Talent removing the medium from the imaging dish using suction.
- 3.1.2. Talent adding 2 milliliters PBS to the dish. **NOTE: Included with shot 3.1.1 in one clip.**
- 3.1.3. Talent adding 2 milliliters of pH 7.4 imaging buffer to the dish.
- 3.2. Insert the imaging dish into the slide holder of a confocal microscope and place the holder into the prewarmed environmental chamber [1]. Connect the peristaltic pump tubing to the imaging dish holder and close the environmental chamber [2].
 - 3.2.1. Talent placing the dish into the slide holder and inserting the holder into the environmental chamber.
 - 3.2.2. Talent attaching tubing from the peristaltic pump to the dish holder and securing the chamber.
- 3.3. Now launch the imaging software and select lasers for the fluorophores of interest [1].
 - 3.3.1. SCREEN: Imaging_3.3.1-3.4.1.avi: 00:05-00:07, 00:47-00:52
- 3.4. Scan the dish to locate cells for imaging using a 10 to 60X objective. Increase magnification if desired and adjust the focus depth as needed [1].
 - 3.4.1. SCREEN: Imaging_3.3.1-3.4.1.avi: 00:50-00:58, 02:16-02:23
- 3.5. Select the preferred resolution and capture a single image using the desired objective, [1]. Record the location on the dish map to relocate the same cells after buffer change [2-TXT].
 - 3.5.1. SCREEN: Imaging_3.5.1-3.5.2.Avi: 00:03-00:15
 - 3.5.2. SCREEN: Imaging_3.5.1-3.5.2.Avi00:23-00:31, 01:00-01:05

**TXT: Repeat imaging for several areas;
Use the same capture settings for all images**
- 3.6. Keeping only the outlet line connected to the peristaltic pump, slowly suction out the pH 7.4 buffer from the dish into a waste container [1]. Then transfer the inlet line to the pH 5.5 quenching buffer [2].
 - 3.6.1. Talent disconnecting the inlet tube and slowly removing the buffer using the outlet tubing.
 - 3.6.2. Talent placing the inlet tube into the container with pH 5.5 buffer.
- 3.7. Turn on the peristaltic pump to add pH 5.5 buffer at a flow rate of 1.5 milliliters per minute, ensuring that the cells remain attached [1]. After 20 minutes, turn off the pump and incubate the cells in the buffer for an additional 20 minutes to allow equilibration [2]. After incubation, repeat imaging of the saved locations [3-TXT]. **NOTE: The VO is edited for the additional shot**

- 3.7.1. Talent turning on the peristaltic pump and observing flow into the imaging dish.
- 3.7.2. Talent switching off the pump and leaving the dish to incubate.
- 3.7.3. **Added shot: SCREEN: Imaging_3.7.3.avi: 00:04-00:13 TXT: Repeat imaging at same locations**

4. Quantitative Analysis of Fluorescent Cell Images Using ImageJ to Calculate Corrected Total Cell Fluorescence (CTCF)

Demonstrator: Sarah Suarez

- 4.1. To begin image quantification, open ImageJ and load the image to be analyzed. The image will appear as a stack with each captured channel displayed in the same frame [1].
 - 4.1.1. SCREEN: Analysis-_4.1.1.-4.7.1.mp4: 00:08-00:21
- 4.2. Navigate to the **Phase** channel. If the image appears overly bright, select **Process** and choose **Enhance Contrast**, then click **OK** [1].
 - 4.2.1. SCREEN: Analysis-_4.1.1.-4.7.1.mp4: 00:21-00:35
- 4.3. Now, use the **magnifying glass** tool to zoom in on one cell [1]. Use the **Freehand selection** tool to trace the outline of the cell in the Phase channel [2].
 - 4.3.1. SCREEN: Analysis-_4.1.1.-4.7.1 00:39-00:47
 - 4.3.2. SCREEN: Analysis-_4.1.1.-4.7.1: 00:47-00:57
- 4.4. Then, switch to the fluorescent channel that corresponds to the fluorophore of interest [1]. Set the desired measurements by selecting **Analyze** and clicking on **Set Measurements**. Ensure that **Area**, **Mean Gray Value**, and **Integrated Density** are checked [2].
 - 4.4.1. SCREEN: Analysis-_4.1.1.-4.7.1.mp4: 01:11-01:14
 - 4.4.2. SCREEN: Analysis-_4.1.1.-4.7.1.mp4: 00:58-01:11
- 4.5. To measure the selected region, choose **Analyze** and then click **Measure** [1]. Take three background measurements by tracing empty areas near the selected cell [2].
 - 4.5.1. SCREEN: Analysis-_4.1.1.-4.7.1.mp4: 01:14-01:20
 - 4.5.2. SCREEN: Analysis-_4.1.1.-4.7.1.mp4: 01:20-01:31, 01:47-01:51
- 4.6. After performing measurements for all desired cells, copy and paste the measurement data into Excel for analysis. Then, calculate the average background mean from the three background measurements [1].
 - 4.6.1. SCREEN: Analysis-_4.1.1.-4.7.1.mp4: 01:50-02:00

4.6.2. ~~SCREEN: Show background values in Excel and the formula used to compute the average.~~ **NOTE: merged with 4.6.1**

4.7. Compute the corrected total cell fluorescence or CTCF (*C-T-C-F*) value for each cell using the provided formula [1]. Using the same workflow, analyse images acquired after buffer change [2]. Export all results to data analysis software for statistical processing [3].

4.7.1. SCREEN: Analysis-_4.1.1.-4.7.1.mp4: 01:58-02:00 *Video editor: Use freeze frame and emphasize CTCF*

AND

TEXT ON PLAIN BACKGROUND:

CTCF=Integrated density–(Area of cell x Mean background fluorescence)

Repeat for all cells and images across all groups

Video Editor: Please play both shots side by side in a split screen

4.7.2. SCREEN: Analysis-_4.1.1.-4.7.1.mp4: 02:20-02:30

4.7.3. -SCREEN: Analysis_4.7.2.mp4: 00:45-00:57

Results

5. Results

- 5.1. Weak α BTX-AF647 (*alpha-B-T-X-A-F-Six-Forty-Seven*) staining was observed in non-transfected N2a cells [1], while robust punctate labeling was detected in cells transfected with α 7 (*Alpha-seven*) DNA [2]. Quantification confirmed that plasma membrane expression of α 7 nAChRs was significantly higher in α 7-transfected N2a cells than in non-transfected controls [3].
 - 5.1.1. LAB MEDIA: Figure 2A,A'. *Video editor: Highlight the panel A.*
 - 5.1.2. LAB MEDIA: Figure 2B,B'. *Video editor: Highlight the panel B.*
 - 5.1.3. LAB MEDIA: Figure 2C. *Video editor: Highlight the blue bar labeled "+ α 7"*
- 5.2. At pH 7.4, total α 7-pHuji (*Alpha-seven-fuji*) fluorescence was visible at the plasma membrane in transfected N2a cells [1]. At pH 5.5, α 7-pHuji fluorescence was reduced, revealing internal receptor localization [2]. The external α 7-pHuji receptor fluorescence accounted for the majority of receptor expression [3].
 - 5.2.1. LAB MEDIA: Figure 3A,A'. *Video editor: Highlight panel A.*
 - 5.2.2. LAB MEDIA: Figure 3B,B'. *Video editor: Highlight panel B*
 - 5.2.3. LAB MEDIA: Figure 3C,C'. *Video editor: Highlight panel C*
- 5.3. Quantification showed that 82.6% of α 7-pHuji receptors were located on the plasma membrane, while 17.4% were internal [1]. Fluorescence was restored upon return to pH 7.4, confirming pHuji signal reversibility [2].
 - 5.3.1. LAB MEDIA: Figure 3D. *Video editor: Highlight the taller "External" bar*
 - 5.3.2. LAB MEDIA: Figure 3E,E'. *Video editor: Highlight panel E*
- 5.4. At pH 7.4, N2a cells expressing α 4-SEP (*Alpha-Four-S-E-P*) and β 2-pHuji showed robust membrane fluorescence for both subunits [1]. At pH 5.5, fluorescence was quenched, indicating internal localization of some α 4-SEP and β 2-pHuji subunits [2]. Quantification showed that 76.5% of α 4-SEP was at the plasma membrane and 23.4% was internal [3].
 - 5.4.1. LAB MEDIA: Figure 4A–D. *Video editor: Please sequentially highlight the images*
 - 5.4.2. LAB MEDIA: Figure 4E–H. *Video editor: Please sequentially highlight the images*
 - 5.4.3. LAB MEDIA: Figure 4I. *Video editor: Highlight the green " α 4 External" and " α 4 Internal" bars*
- 5.5. In triple-transfected cells, α 4-SEP (*alpha-four-S-E-P*) displayed 82.8% membrane localization and 17.2% internal expression [1]. In the same cells, β 2-pHuji subunits showed 63.4% on the membrane and 36.6% internal [2].

- 5.5.1. LAB MEDIA: Figure 5K. *Video editor: Highlight the green “α4 External” and “α4 Internal” bars*
- 5.5.2. LAB MEDIA: Figure 5K. *Video editor: Highlight the red “β2 External” and “β2 Internal” bars .*

1. nAChR
Pronunciation link: No confirmed link found
IPA: /ˌɛn.ɛɪ.sɪː.ɛɪtʃˈɑːr/
Phonetic Spelling: en·ay·see·aych·ar
2. Nicotinic
Pronunciation link: <https://www.merriam-webster.com/dictionary/nicotinic>
IPA: /ˌnɪk.əˈtɪn.ɪk/
Phonetic Spelling: nik·uh·tin·ik
3. Acetylcholine
Pronunciation link: <https://www.merriam-webster.com/dictionary/acetylcholine>
IPA: /ə.sɪː.təlˈkoʊ.liːn/
Phonetic Spelling: uh·see·tuhl·koh·leen
4. Transfection
Pronunciation link: <https://www.merriam-webster.com/dictionary/transfection>
IPA: /trænsˈfɛk.ʃən/
Phonetic Spelling: tranz·fek·shuhn
5. Biosafety
Pronunciation link: <https://www.merriam-webster.com/dictionary/biosafety>
IPA: /ˌbaɪ.oʊˈseɪf.ti/
Phonetic Spelling: by·oh·sayf·tee
6. Microcentrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/microcentrifuge>
IPA: /ˌmaɪ.kroʊˈsen.trɪ.fjuːdʒ/
Phonetic Spelling: my·kroh·sen·truh·fyooj
7. Plasmid
Pronunciation link: <https://www.merriam-webster.com/dictionary/plasmid>
IPA: /ˈplæz.mɪd/
Phonetic Spelling: plaz·mid
8. Isoforms
Pronunciation link: <https://www.merriam-webster.com/dictionary/isoform>
IPA: /ˈaɪ.souˌfɔːrm/
Phonetic Spelling: eye·soh·formz

9. Incubation
 Pronunciation link: <https://www.merriam-webster.com/dictionary/incubation>
 IPA: /,ɪn.kjuːˈbeɪ.ʃən/
 Phonetic Spelling: in·kyoo·bay·shuhn
10. EMEM
 Pronunciation link: No confirmed link found
 IPA: /,iː.em.iːˈem/
 Phonetic Spelling: ee·em·ee·em
11. Confocal
 Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>
 IPA: /kɑːnˈfoʊ.kəl/
 Phonetic Spelling: kon·foh·kuhl
12. Fluorophores
 Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorophore>
 IPA: /ˈflɒr.əˌfɔːr/
 Phonetic Spelling: floor·uh·forz
13. Peristaltic
 Pronunciation link: <https://www.merriam-webster.com/dictionary/peristaltic>
 IPA: /,per.əˈstɔːl.tɪk/
 Phonetic Spelling: peh·ruh·staal·tik
14. Equilibration
 Pronunciation link: <https://www.merriam-webster.com/dictionary/equilibration>
 IPA: /ɪˌkwɪːləˈbreɪ.ʃən/
 Phonetic Spelling: ih·kwee·luh·bray·shuhn
15. Quantification
 Pronunciation link: <https://www.merriam-webster.com/dictionary/quantification>
 IPA: /ˌkwɑːn.təˈfeɪ.ʃən/
 Phonetic Spelling: kwon·tuh·fuh·kay·shuhn
16. Magnification
 Pronunciation link: <https://www.merriam-webster.com/dictionary/magnification>
 IPA: /ˌmæɡ.nəˈfeɪ.ʃən/
 Phonetic Spelling: mag·nuh·fuh·kay·shuhn
17. Integrated Density
 Pronunciation link: <https://www.howtopronounce.com/integrated-density>
 IPA: /ˈm.tɪ.greɪ.tɪd ˈden.sə.ti/
 Phonetic Spelling: in·tuh·gray·tuhd den·suh·tee
18. Corrected Total Cell Fluorescence
 Pronunciation link: No confirmed link found
 IPA: /kəˈrek.tɪd ˈtoʊ.təl sel flʊˈres.əns/
 Phonetic Spelling: kuh·rekt·id toh·tuhl sel floo·reh·suhns
19. Quenching
 Pronunciation link: <https://www.merriam-webster.com/dictionary/quenching>
 IPA: /ˈkwentʃɪŋ/
 Phonetic Spelling: kwench·ing

20. Neuro2a
Pronunciation link: No confirmed link found
IPA: /'nɒr.oo tu: eɪ/
Phonetic Spelling: noor·oh two·ay
21. Environmental
Pronunciation link: <https://www.merriam-webster.com/dictionary/environmental>
IPA: /ɪnˌvaɪ.rən'men.təl/
Phonetic Spelling: in·vy·ruhn·men·tuhl
22. Tubing
Pronunciation link: <https://www.merriam-webster.com/dictionary/tubing>
IPA: /'tu:.bɪŋ/
Phonetic Spelling: too·bing
23. Reversibility
Pronunciation link: <https://www.merriam-webster.com/dictionary/reversibility>
IPA: /rɪˌvɜː.sə'bɪl.ə.ti/
Phonetic Spelling: rih·vur·suh·bil·uh·tee
24. Localization
Pronunciation link: <https://www.merriam-webster.com/dictionary/localization>
IPA: /ˌloʊ.kə.lə'zeɪ.ʃən/
Phonetic Spelling: loh·kuh·luh·zay·shuhn
25. Subunits
Pronunciation link: <https://www.merriam-webster.com/dictionary/subunit>
IPA: /'sʌb.ju:.nɪt/
Phonetic Spelling: sub·yoo·nitz