

Submission ID #: 69853

Scriptwriter Name: Poornima G

Project Page Link: <https://review.jove.com/account/file-uploader?src=21305213>

Title: Single-Molecule Localization Microscopy of Membrane Proteins Using Single-Antibody Labeling

Authors and Affiliations:

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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**

- 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. **Yes**

Current Protocol Length

Number of Steps: 24

Number of Shots: 45 (35 SC)

Introduction

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

Authors: Answers are slightly edited in accordance with journal guidelines.

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

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Neal T Ramseier:** My research focuses on the membrane of T cells and aims to reveal new nanoscale insights relevant to therapeutic targeting.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Neal Ramseier:** Existing imaging methods do not typically directly visualize nanoscale antibody–epitope interactions in cells; this protocol overcomes that limitation by enabling direct observation within the cellular environment.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Neal Ramseier:** We can observe antibodies interacting with their membrane epitopes in a cellular environment, providing insight relevant to therapeutic antibodies.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Neal Ramseier:** The antibody concentration, non-illumination interval, and photobleaching steps are critical to optimize. Sub-optimal parameters will compromise the results.

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

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Neal Ramseier**: Single-Antibody Labeling can be extended to simultaneously evaluate membrane protein binding by multiple antibodies within the same cellular environment.

- 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.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

What is the key takeaway you hope viewers remember after watching your video article?

- 1.6. **Neal Ramseier, PhD Candidate**: (authors will present their testimonial statements live)

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

How does the video format of your article help you to share your work with the broader scientific community?

- 1.7. **Neal Ramseier, PhD Candidate**: (authors will present their testimonial statements live)

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

Protocol

2. Image Acquisition After Sample Preparation

Demonstrator: Neal Ramseier

2.1. After mounting the sample on the microscope and depositing gold colloids onto the surface, use brightfield illumination to verify that enough colloids have been deposited within the region of interest [1]. Confirm that the ideal density of 5 to 10 gold colloids is present within the region of interest before proceeding [2].

2.1.1. **WIDE:** Talent adjusting the microscope to brightfield illumination.

2.1.2. Talent viewing the sample.

2.2. For the optical configuration of the antibody probe fluorophore, open the imaging software and access the optical configuration settings [1]. Select the appropriate dichroic mirror [2] and set the laser excitation wavelength and power density [3]. Adjust the camera integration time and select the emission filter [4], then set the camera pixel binning to achieve a pixel size of 150 to 160 nanometers [5].

2.2.1. SCREEN: 2.2.1.

2.2.2. SCREEN: 2.2.2.

2.2.3. SCREEN: 2.2.3.

2.2.4. SCREEN: 2.2.4.

2.2.5. SCREEN: 2.2.5.

2.3. Now, create an optical configuration for the photobleaching step by selecting a photobleaching mode in the imaging software [1]. Set the laser power to 100% [2] and adjust the camera integration time to 1 second [3].

2.3.1. SCREEN: 2.3.1.

2.3.2. SCREEN: 2.3.2.

2.3.3. SCREEN: 2.3.3.

2.4. Set the imaging parameters in the image acquisition software by defining the non-illuminating interval, number of frames, and frequency of the photobleaching step [1].

2.4.1. SCREEN: 2.4.1. *Video editor: Please speed up*

2.5. Next, prepare the imaging buffer by diluting the CD81 antibody to a concentration of 1 microgram per milliliter in 200 microliters of 5 percent BSA made in DPBS [1]. The final recommended antibody concentration is 0.5 nanomolar for Jurkat T cells or 2 nanomolar for U2OS cells [2].

2.5.1. Talent pipetting the CD81 antibody into a tube with bovine serum albumin in Dulbecco's phosphate-buffered saline.

2.5.2. Close-up of the prepared imaging buffer labeled with the appropriate final antibody concentration.

2.6. Add the prepared imaging buffer to the well containing the cells [1] and start the image acquisition in the software to begin data collection [2].

2.6.1. Talent gently adding the imaging buffer to the well.

2.6.2. SCREEN: 2.6.2.

3. Imaging Parameter Optimization

3.1. Use the live-view function in the imaging software configured for membrane single-antibody labeling to monitor antibody binding in real time [1].

3.1.1. SCREEN: 3.1.1.

3.2. Configure the imaging parameters in the image acquisition software to obtain at least a 10-frame acquisition and set the non-illuminating interval to 5 seconds [1].

3.2.1. SCREEN: 3.2.1-3.2.2.

3.3. Start the acquisition [1] and evaluate the sparsity of single-molecule localizations in the recorded movie [2].

3.3.1. SCREEN: 3.3.1.

3.3.2. SCREEN: 3.3.2.

3.4. If multiple overlapping antibody-binding events are observed immediately after adding the imaging buffer or if single-molecule localizations accumulate over time, abort the acquisition [1]. Reduce the antibody concentration by 2-fold [2] and repeat the

acquisition on a new sample [3]. Continue reducing the antibody concentration by 2-fold and reevaluating the number of events per unit area until the desired single-molecule localization density is achieved [4].

3.4.1. SCREEN: 3.4.1.

3.4.2. Talent mixing new buffer.

3.4.3. Talent adding the adjusted antibody solution to a new sample well.

3.4.4. SCREEN: 3.4.4.

3.5. If the event density is sparse, increase the antibody concentration in the imaging buffer by 2-fold [1]. Repeat the acquisition on a new sample and continue increasing the antibody concentration by 2-fold while reevaluating the number of binding events per unit area until the desired single-molecule localization density is achieved [2].

3.5.1. Talent viewing the data appearing on the screen.

3.5.2. SCREEN: 3.5.2.

3.6. For non-illuminating interval optimization, configure the imaging parameters in the image acquisition software to obtain a 50-frame acquisition [1].

3.6.1. Talent accessing the image acquisition settings in the imaging software.

3.7. After adding the antibody and starting acquisition, evaluate the density and sparsity of single-molecule localizations in the recorded movie [1]. Determine the lowest non-illuminating interval that yields an optimal single-molecule event density [2].

3.7.1. SCREEN: 3.7.1.

3.7.2. SCREEN: 3.7.2.

3.8. Finally, if spatially overlapping binding events occur as the image acquisition progresses, introduce a photobleaching step at regular intervals [1]. Adjust the photobleaching frequency so that fluorescence from bound antibodies is removed before overlapping events accumulate [2].

3.8.1. SCREEN: 3.8.1.

3.8.2. SCREEN: 3.8.2

4. Data and Cluster Analysis

- 4.1. Set the filepath in the MATLAB software to the folder that contains the reconstructed mSAL comma-separated values file [1].
 - 4.1.1. SCREEN: 4.1.1.
- 4.2. Run the code by clicking **Run** in the MATLAB toolbar. When prompted, select the **mSAL.CSV** (*M-sal C-S-V*) file and click **Open** to load it into the program [1].
 - 4.2.1. SCREEN: 4.2.
- 4.3. Run the analysis using the previously defined inputs as a starting point [1]. Evaluating the scatter plot containing the unclustered localization data [2], choose a minimum point value that is higher than the number of noise localizations but lower than the number of localizations on the cell [3-TXT].
 - 4.3.1. SCREEN: 4.3.1.
 - 4.3.2. SCREEN: 4.3.2.
 - 4.3.3. SCREEN: 4.3.3-4.3.4. **TXT: Set epsilon to capture target localizations**
- 4.4. Evaluate the window that depicts the clustered data. Confirm that clusters are retained on the cell with the expected phenotype while localizations outside the cell are minimized [1].
 - 4.4.1. SCREEN: 4.4.
- 4.5. If the clustering parameters are too restrictive, decrease the minimum points value and increase epsilon [1]. If the clustering parameters are too inclusive, increase the minimum points value and decrease epsilon [2].
 - 4.5.1. SCREEN: 4.5.1.
 - 4.5.2. SCREEN: 4.5.2.
- 4.6. Finally, after adjusting the minimum points value and epsilon, rerun the code to obtain optimal clustering [1].
 - 4.6.1. SCREEN: 4.6.1.

Results

5. Results

5.1. Jurkat T cells were immobilized with sufficient spacing to preserve membrane integrity and allow antibody access to membrane epitopes [1].

5.1.1. LAB MEDIA: Figure 1A.

5.2. Gold colloids were visualized and used as fiducial markers for lateral drift correction during mSAL image acquisition [1].

5.2.1. LAB MEDIA: Figure 2A AND B. *Video editor: Highlight the white arrows pointing to small round gold particles.*

5.3. Optimization of the non-illumination interval showed that a 5-second interval produced antibody binding events with minimal spatial overlap compared to shorter or longer intervals at 1 nanomolar antibody concentration [1].

5.3.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the panels labeled 5s.*

5.4. Application of drift correction improved the reconstructed mSAL image compared to the uncorrected reconstruction [1].

5.4.1. LAB MEDIA: Figure 4B. *Video editor: Show the reconstructed image after correction and zoom into the boxed region.*

5.5. Density-based clustering of mSAL localizations reduced low-density background interactions and highlighted clustered CD81 binding events [1].

5.5.1. LAB MEDIA: Figure 5. *Video editor: Focus on the black box in C.*

1. Colloids

Pronunciation link: <https://www.merriam-webster.com/dictionary/colloid>

IPA: /ˈkɑːləɪd/

Phonetic Spelling: kaa·loyd

2. Brightfield

Pronunciation link: <https://www.merriam-webster.com/dictionary/bright-field>

IPA: /ˈbraɪtˌfiːld/

Phonetic Spelling: brayt·feeld

3. Dichroic
Pronunciation link: <https://www.merriam-webster.com/dictionary/dichroic>
IPA: /daɪˈkroʊɪk/
Phonetic Spelling: dye·kroh·ik
4. Fluorophore
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorophore>
IPA: /ˈflʊr.əˌfɔːr/, /ˈflɔːr.əˌfɔːr/
Phonetic Spelling: floo·ruh·for
5. Excitation
Pronunciation link: <https://www.merriam-webster.com/dictionary/excitation>
IPA: /ˌɛk.səɪˈteɪ.ʃən/
Phonetic Spelling: ehk·sy·tay·shun
6. Nanometers
Pronunciation link: <https://www.merriam-webster.com/dictionary/nanometer>
IPA: /ˈnæn.oʊ.miː.tər/
Phonetic Spelling: nan·oh·mee·ter
7. Photobleaching
Pronunciation link: <https://www.merriam-webster.com/dictionary/photobleaching>
IPA: /ˈfoʊ.toʊˌbliː.tʃɪŋ/
Phonetic Spelling: foh·toh·blee·ching
8. Jurkat
Pronunciation link: <https://www.howtopronounce.com/jurkat>
IPA: /ˈjɜːrkæt/
Phonetic Spelling: jur·kat
9. U2OS
Pronunciation link: No confirmed link found
IPA: /ˌjuːˈtuː.əʊˈɛs/
Phonetic Spelling: yoo·too·oh·es
10. Bovine
Pronunciation link: <https://www.merriam-webster.com/dictionary/bovine>
IPA: /ˈboʊ.vain/
Phonetic Spelling: boh·vyn
11. Dulbecco's
Pronunciation link: <https://www.howtopronounce.com/dulbecco>
IPA: /dɒlˈbɛk.oʊz/
Phonetic Spelling: duhl·bek·ohz
12. Phosphate-buffered saline
Pronunciation link: <https://www.howtopronounce.com/phosphate-buffered-saline>
IPA: /ˈfas.feɪt ˈbʌ.fərd ˈseɪ.liːn/
Phonetic Spelling: foss·fayt·buh·ferd·say·leen
13. Localization
Pronunciation link: <https://www.merriam-webster.com/dictionary/localization>
IPA: /ˌloʊ.kə.ləˈzeɪ.ʃən/
Phonetic Spelling: loh·kuh·luh·zay·shun

14. MATLAB

Pronunciation link: <https://www.howtopronounce.com/matlab>

IPA: /'mæt.læb/

Phonetic Spelling: mat·lab

15. Epsilon

Pronunciation link: <https://www.merriam-webster.com/dictionary/epsilon>

IPA: /'ɛp.sɪ.lɑːn/, /'ɛp.sɪ.lən/

Phonetic Spelling: ep·sih·lon

16. Phenotype

Pronunciation link: <https://www.merriam-webster.com/dictionary/phenotype>

IPA: /'fiː.nəˈtaɪp/

Phonetic Spelling: fee·nuh·type

17. Fiducial

Pronunciation link: <https://www.merriam-webster.com/dictionary/fiducial>

IPA: /fɪˈduː.ʃəl/, /fɑːˈduː.ʃəl/

Phonetic Spelling: fih·doo·shul

18. mSAL

Pronunciation link: No confirmed link found

IPA: /ɛmˈsæl/

Phonetic Spelling: em·sal

19. Antibody

Pronunciation link: <https://www.merriam-webster.com/dictionary/antibody>

IPA: /'æn.tɪˈbɑː.di/

Phonetic Spelling: an·tee·bah·dee

20. Cluster

Pronunciation link: <https://www.merriam-webster.com/dictionary/cluster>

IPA: /'klʌs.tər/

Phonetic Spelling: kluh·ster