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Title: Peroxisome Staining in Mammalian Cells Using Peroxisome-Specific Probes

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? **No**

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

Two floors in the same building. The lab is located on the 3rd floor, and the microscope room and the quiet room for the interview are located on the 5th floor.

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: 12

Number of Shots: 17

Introduction

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

Authors: The answers are edited in accordance with journal guidelines. Each answer can be around 20-25 words long.

INTRODUCTION:

NOTE: 1.1 and 1.2 were self-filmed by the authors and rest were filmed by the videographer

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

- 1.1. **Charlotte Howman:** Our research investigates peroxisome regulation in health and disease. We develop fluorescent probes and staining protocols for live and fixed cells to visualize peroxisomes across systems. **NOTE: 1.1 and 1.2 answers were changed by the authors**

1.1.1. LAB MEDIA: 20260204_124049000_iOS.MP4 00:00-00:14

~~What are the current experimental challenges?~~

- 1.2. **Charlotte Howman:** Key experimental challenges include noninvasive peroxisome labeling, limited antibodies, real-time visualization, and obtaining functional readouts, though PeroxiSPY variability enables distinguishing distinct peroxisome functional states.

1.2.1. LAB MEDIA: 20260204_124401000_iOS.MP4 00:00-00:14

CONCLUSION:

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

- 1.3. **Tamar Cohen:** We have developed the PeroxiSPY dye that can be used to visualize peroxisomes in both live and fixed animal cells.

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. **Tamar Cohen:** Our fluorescent probe can offer a simple and quick method to study peroxisome dynamics, heterogeneity, and dysfunction in ways that were previously limited.

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. **Tamar Cohen:** This protocol will enable imaging-based peroxisome research in diverse cell types, including patient-derived cells with peroxisomal disorders.

- 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. Peroxisome Staining in Cells with PeroxiSPY

Demonstrator: Tamar Cohen

- 2.1. To begin, split the cells into a plate with coverslips to achieve a 30 to 70% confluency the next day [1].
 - 2.1.1. Talent walking into the room with the culture plate.
- 2.2. Dilute the probe with fresh media in a light-sensitive microcentrifuge tube to achieve a 4 micromolar final concentration [1].
 - 2.2.1. Talent pipetting 2 microliters of probe into a light-sensitive microcentrifuge tube containing 0.5 milliliters of media.
- 2.3. Select the desired well to stain and label the outside using a marker pen [1].
 - 2.3.1. Talent identifying a well and labeling it clearly.
- 2.4. Aspirate 0.5 milliliters of media from the selected well [1] and replace it with the prepared probe media drop by drop along the wall of the well [2].
 - 2.4.1. Talent removing media from the well.
 - 2.4.2. Shot of dispensing probe media dropwise into the well.
- 2.5. After dispensing, incubate the cells at 37 degrees Celsius for 10 to 15 minutes [1]. From this point forward, shield the cells from light using aluminum foil [2].
 - 2.5.1. Talent placing the imaging plate in the incubator.
 - 2.5.2. Talent covering the plate with aluminum foil.
- 2.6. Post-incubation, carefully remove the stained media and wash the cells twice with PBS [1]. Then, place the plate under a chemical hood, add 4 percent paraformaldehyde in PBS to the cells [2] and incubate at room temperature for 20 minutes [3].
 - 2.6.1. Talent aspirating stained media and washes with PBS. **Authors:** We can only show one wash in the video, and mention twice in the narration

- 2.6.2. Talent placing the plate inside the chemical hood and adding 4% paraformaldehyde to the wells.
- 2.6.3. Talent placing the plate aside and starting a timer.
- 2.7. After fixation, remove the paraformaldehyde into a designated waste container [1] and wash the cells once with PBS [2-TXT].
- 2.7.1. Talent aspirating paraformaldehyde and discarding it into a labeled waste.
- 2.7.2. Talent adding the cells with PBS. **TXT: Remove the PBS completely after the wash**
- 2.8. Next, add 0.5 percent Triton X in PBS to permeabilize the cells and incubate for 2.5 minutes [1-TXT].
- 2.8.1. Talent adding Triton X solution and setting the plate aside. **TXT: Any other suitable detergent can be used as required**
- 2.9. After incubation, remove the Triton X and wash the cells twice with PBS for 10 minutes each [1].
- 2.9.1. Talent aspirating Triton X and adding PBS into the well.
- 2.10. To block the cells, incubate them with 5 percent bovine serum albumin in PBS overnight at 4 degrees Celsius [1].
- 2.10.1. Talent placing plate in the refrigerator. **TXT: Alternatively, incubate at RT; 20 min**
- 2.11. Perform the immunofluorescence staining procedure following standard protocol [1].
- 2.11.1. Talent removing the plate from the fridge.
- 2.12. For live cell imaging, split the cells and incubate them directly with the dye [1]. Finally, perform microscopy to acquire images of both fixed and live cells [2].
- 2.12.1. TEXT ON 2.12.1's BACKGROUND:
- Start confocal software and set laser/imaging parameters
 - Prepare 1 μ M probe solution
 - Stain the cells and incubate for 10 min
 - Focus cells and position for imaging

- Optimize settings and acquire .nd2 images

2.12.2. Two talents watching the fixed cells on the screen in the microscope room and talent setting up the microscope. **NOTE: If possible, show both the talents in this scene**

Results

3. Results

3.1. Live-cell staining with PeroxiSPY (*peroxy-spy*) probes resulted in ATP Binding Cassette Subfamily D-dependent import into peroxisomes, producing a high peroxisome to cytoplasm fluorescence ratio in metabolically active cells [1].

3.1.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the WT “peroxiSPY555” image.*

3.2. In wild-type HEK293T cells expressing GFP-SKL, PeroxiSPY555 staining showed distinct co-localization with peroxisomes [1], whereas in PEX19 knockout cells, that lack peroxisomes, the staining was absent [1].

3.2.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the merge image of WT.*

3.2.2. LAB MEDIA: Figure 1B. *Video editor: Highlight the merge image of PEX19 KO.*

3.3. In human fibroblasts, PeroxiSPY staining co-localized with the peroxisomal marker GFP-SKL, confirming peroxisome targeting [1].

3.3.1. LAB MEDIA: Figure 1C. *Video editor: Highlight the merged image .*

3.4. Peroxisome density quantification revealed significant variation among different cell types [1].

3.4.1. LAB MEDIA: Figure 1D.

3.5. Optimized staining conditions in COS-7 (*cos-7*) cells using 250 nanomolar PeroxiSPY for 20 minutes significantly increased the peroxisome to cytoplasm fluorescence ratio [1] compared to the original 1 micromolar for 10 minutes protocol [2].

3.5.1. LAB MEDIA: Figure 2A. *Video editor: Highlight “20 min 250 nM” image and data points in the graph.*

3.5.2. LAB MEDIA: Figure 2A. *Video editor: Highlight “10 min 1 μ M” image and data points in the graph.*

3.6. Extended incubation of HEK293T cells with PeroxiSPY for 24 hours led to an improved peroxisome to cytoplasm fluorescence intensity ratio compared to 15 minutes [1], despite reduced peroxisome staining [2].

3.6.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the violin plot for ‘24 hours’ in the*

plot on the extreme right showing 'peroxisome to cytoplasm FI, au' on Y-axis.

- 3.7. A fixation protocol enabled successful antibody co-staining, demonstrated by preserved PeroxiSPY labeling and anti-PEX14 (*pex-14*) immunostaining in Huh7 (*H-U-H-7*) and HEK293T cells [1].

3.7.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the merged images for top 2 rows.*

- 3.8. Quantification showed that fixation and permeabilization procedures affected peroxisome fluorescence intensity [1] when compared to live cell staining [2].

3.8.1. LAB MEDIA: Figure 2D. *Video editor: Highlight the violin plots for "Fixed", and "Permeabilized" conditions.*

3.8.2. LAB MEDIA: Figure 2D. *Video editor: Highlight the violin plots for "Live" condition.*

1. Confluency
Pronunciation link: <https://www.merriam-webster.com/dictionary/confluency>
IPA: /'kɑ:n.flu.ən.si/
Phonetic Spelling: kahn·floo·uhn·see
2. Coverslips
Pronunciation link: <https://www.merriam-webster.com/dictionary/coverslip>
IPA: /'kʌv.ər.slɪp/
Phonetic Spelling: kuh·ver·slip
3. Microcentrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/microcentrifuge>
IPA: /,maɪ.kroʊ'sɛn.trəˌfjuːdʒ/
Phonetic Spelling: my·kroh·sen·truh·fyooj
4. Micromolar
Pronunciation link: <https://www.merriam-webster.com/dictionary/micromolar>
IPA: /,maɪ.kroʊ'moʊ.lər/
Phonetic Spelling: my·kroh·moh·ler
5. Microliters
Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>
IPA: /'maɪ.kroʊ.liː.tər/
Phonetic Spelling: my·kroh·lee·ter
6. Aspirate
Pronunciation link: <https://www.merriam-webster.com/dictionary/aspirate>
IPA: /'æs.pəˌreɪt/
Phonetic Spelling: ass·puh·rayt

7. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /'m.kjəˌbeɪt/
Phonetic Spelling: in·kyuh·bayt
8. Paraformaldehyde
Pronunciation link: <https://www.merriam-webster.com/dictionary/paraformaldehyde>
IPA: /ˌpɛr.əˌfɔːrˈmæl.dəˌhaɪd/
Phonetic Spelling: pair·uh·for·mal·duh·hyde
9. Phosphate-buffered saline
Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphate-buffered%20saline>
IPA: /'fɑːs.feɪt ˈbʌf.ə.d sə'liːn/
Phonetic Spelling: fahs·fayt buhf·erd suh·leen
10. Triton X
Pronunciation link: <https://www.merriam-webster.com/dictionary/Triton>
IPA: /'traɪ.tən/
Phonetic Spelling: try·tahn
11. Permeabilize
Pronunciation link: <https://www.merriam-webster.com/dictionary/permeabilize>
IPA: /'pɜː.mi.əˌbaɪ.laɪz/
Phonetic Spelling: per·mee·uh·bye·lize
12. Immunofluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/immunofluorescence>
IPA: /ɪˌmjuː.nouˌfluˈres.əns/
Phonetic Spelling: ih·myoo·noh·floor·ess·uhns
13. Confocal
Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>
IPA: /kɑːnˈfoʊ.kəl/
Phonetic Spelling: kahn·foh·kuhl
14. Peroxisome
Pronunciation link: <https://www.merriam-webster.com/dictionary/peroxisome>
IPA: /pəˈrɑːk.sɪˌsoʊm/
Phonetic Spelling: puh·rahk·suh·sohm
15. Cytoplasm
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytoplasm>
IPA: /'saɪ.təˌplæz.əm/
Phonetic Spelling: sigh·tuh·plaz·uhm
16. PeroxiSPY
Pronunciation link: No confirmed link found
IPA: /pəˈrɑːk.sɪˌspaɪ/
Phonetic Spelling: puh·rahk·see·spy
17. PeroxiSPY555
Pronunciation link: No confirmed link found
IPA: /pəˈrɑːk.sɪˌspaɪ faɪv faɪv faɪv/
Phonetic Spelling: puh·rahk·see·spy five·five·five

18. ATP
 Pronunciation link: <https://www.merriam-webster.com/dictionary/ATP>
 IPA: /,eɪ.tiːˈpiː/
 Phonetic Spelling: ay·tee·pee
19. HEK293T
 Pronunciation link: No confirmed link found
 IPA: /hek tuː nɑːm θriː tiː/
 Phonetic Spelling: heck two·nine·three·tee
20. GFP-SKL
 Pronunciation link: No confirmed link found
 IPA: /,dʒiː.ɛfˈpiː ɛs keɪ ɛl/
 Phonetic Spelling: gee·ef·pee ess·kay·ell
21. PEX19
 Pronunciation link: No confirmed link found
 IPA: /peks nɑːnˈtiːn/
 Phonetic Spelling: pex nine·teen
22. Fibroblasts
 Pronunciation link: <https://www.merriam-webster.com/dictionary/fibroblast>
 IPA: /ˈfaɪ.broʊˌblæst/
 Phonetic Spelling: fy·broh·blast
23. COS-7
 Pronunciation link: No confirmed link found
 IPA: /kɑːz ˈseɪv.ən/
 Phonetic Spelling: kahz sev·uhn
24. Nanomolar
 Pronunciation link: <https://www.merriam-webster.com/dictionary/nanomolar>
 IPA: /,næ.nouˈmoʊ.lər/
 Phonetic Spelling: nan·oh·moh·ler
25. Violin plot
 Pronunciation link: <https://www.merriam-webster.com/dictionary/violin%20plot>
 IPA: /ˈvaɪ.əlɪn plɑːt/
 Phonetic Spelling: vy·uh·lin plot
26. PEX14
 Pronunciation link: No confirmed link found
 IPA: /peks fɔːrˈtiːn/
 Phonetic Spelling: pex four·teen
27. Huh7
 Pronunciation link: No confirmed link found
 IPA: /hʌ seɪv.ən/
 Phonetic Spelling: huh sev·uhn