FINAL SCRIPT: APPROVED FOR FILMING

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Scriptwriter Name: Poornima G
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Title: Fluorescence-Based Calcium Imaging in Primary Human Airway Epithelial Cultures Using Automated Cell Segmentation

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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**

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: 26
Number of Shots: 55 (29 SC)

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. **Chiara D'Addario:** Single-cell transcriptomics reveal heterogeneity in cystic fibrosis airway epithelium. We intend to understand cell-type specific functions and changes affecting disease pathogenesis.

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

~~What are the current experimental challenges?~~

1.2. **Abdelkader Daoud:** The current challenge is to accurately measure single cell proteomics and functions. This still requires technical development.

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. **Abdelkader Daoud:** Our studies will help in understanding distinct cell types responses to their environment, which will aid in the development of targeted therapeutics.

1.3.1. 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?~~

1.4. **Chiara D'Addario:**Our findings have the potential to zero in on distinct cell types to assign certain live cell responses to environmental stimuli.

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

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

1.5. **Abdelkader Daoud:** In future, we intend to explore how cell-type specific signaling in diseased and healthy cells is altered to determine the types are responsible.

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. Calu-3 Cell Culturing for Live Calcium Imaging

Demonstrator: Chiara D'Addario

2.1. To begin, maintain Calu-3 *(Kal-you-3)* cells in T-25 flasks containing Eagle's minimum essential medium culture media supplemented with 20% FBS and 1% penicillin-streptomycin **[1-TXT]**. When the cells reach approximately 70 percent confluence, wash the cells with 5 milliliters of PBS **[2]**.

2.1.1. WIDE: Talent placing a T-25 flask containing Calu-3 cells in an incubator. **TXT: Replace the media on alternating days (5 mL/flask)**

2.1.2. Talent aspirating media from a T-25 flask containing adherent cells and adding PBS.

2.2. After washing, add 1 milliliter of TrypLE *(tryp-L-E)* directly onto the cells **[1]** and place the flask in an incubator set to 37 degrees Celsius and 5 percent carbon dioxide for 10 to 15 minutes **[2]**.

2.2.1. Talent adding TrypLE to the T-25 flask.

2.2.2. Talent placing the flask into the incubator.

2.3. Then, neutralize the TrypLE by adding 4 milliliters of fresh complete culture media and mix the cell suspension thoroughly **[1]**. Plate the cells into a 96-well plate at approximately 90 to 100 percent confluence, corresponding to about 40,000 cells per well **[2-TXT]**.

2.3.1. Talent adding fresh culture media to the flask to neutralize TrypLE and pipetting up and down.

2.3.2. Talent dispensing cell suspension into individual wells of a 96-well plate using a pipette. **TXT: 200 µL cell suspension/well**

2.4. Allow the cells to adhere to the 96-well plate overnight **[1]**. Change the media every day and let the cells remain for 2 to 5 days after reaching confluence **[2]**.

2.4.1. Talent placing the seeded 96-well plate into the incubator.

2.4.2. Talent removing media in a 96-well plate using a multichannel pipette.

3. Loading Calu-3 Cells and the Primary Airway Cells with Cal-520 AM or Fluo-4 AM

Demonstrator: Abdelkader Daoud

3.1. On the experiment day, wash the Calu-3 cells and incubate them with appropriate dyes **[1 and 2]**.

3.1.1. Talent adding dye to the cells and mixing by pipetting.

3.1.2. TEXT ON 3.1.1's BACKGROUND:

- **Wash (2x):** 200 µL/well prewarmed calcium buffer
- **Incubation with Cal-520 AM:**
  - Dye cocktail: 3 µM Cal-520 AM, 2.5 mM Probenecid, 1:5000 Hoechst in calcium buffer
  - 200 µL/well dye cocktail
  - 1 h at 37 °C, 5% CO_2
- **For Fluo-4 AM experiments**: Use 3 µM Fluo-4 AM instead of Cal-520 AM
- **Wash (2x):** 200 µL/well prewarmed calcium buffer

3.2. Next, add 100 microliters per well of 2.5 millimolar probenecid diluted in calcium buffer to maintain the dye within the cytoplasmic space **[1]** and wrap the plate in aluminum foil to protect it from light until imaging **[2]**.

3.2.1. Talent dispensing probenecid solution into each well of the plate.

3.2.2. Talent wrapping the 96-well plate in aluminum foil.

3.3. For primary airway cells, remove the basolateral media from the transwell and wash the apical side by adding 300 microliters of calcium buffer **[1]** and then, wash the basolateral side by adding 750 microliters of calcium buffer **[2]**. Remove the buffer by pipetting and repeat the wash once more on both sides **[3]**.

3.3.1. Talent removing the media and adding calcium buffer to the apical compartment of the insert.

3.3.2. Talent adding calcium buffer to the basolateral compartment.

3.3.3. Talent aspirating buffer from both apical and basolateral compartments.

3.4. Now, incubate the cells with 200 microliters of dye solution on the apical side and 600 microliters of the dye solution on the basolateral side **[1-TXT]**.

3.4.1. Talent pipetting dye solution into the apical compartment of the transwell insert. **TXT: 1 h; 37 °C; 5% CO_2**

3.5. During the incubation, prepare thapsigargin diluted in calcium buffer as a three times intermediate stock **[1]**. Adjust the concentration to 6 micromolar to achieve a final concentration of 2 micromolar in the well **[2]**.

3.5.1. Talent pipetting thapsigargin into a labeled tube containing calcium buffer.

3.5.2. Close-up shot of a tube being labeled "3x intermediate stock".

3.6. After incubation, remove the dye solution from both apical and basolateral compartments by pipetting **[1]**. Wash the cells twice with 300 microliters of calcium buffer to the apical side **[2]** and 750 microliters of calcium buffer to the basolateral side **[3]**, then remove the buffer by pipetting **[4]**.

3.6.1. Talent aspirating dye solution from the transwell insert.

3.6.2. Talent adding calcium buffer to the apical compartment.

3.6.3. Talent adding calcium buffer to the basolateral compartment.

3.6.4. Talent aspirating wash buffer from both compartments.

3.7. Next, add 100 microliters of 2.5 millimolar probenecid in calcium buffer to the apical compartment **[1]** and add 500 microliters of the same solution to the basolateral compartment **[2]**.

3.7.1. Talent dispensing probenecid solution into the apical side of the transwell.

3.7.2. Talent dispensing probenecid solution into the basolateral side.

3.8. Keeping the transwell insert within the plate, wrap the entire plate in aluminum foil to protect it from light **[1]** and set the wrapped plate aside for imaging **[2-TXT]**.

3.8.1. Talent wrapping the transwell plate with aluminum foil.

3.8.2. Talent placing the foil-wrapped transwell plate on the bench. **TXT: Acquire a continuous live calcium video before and after adding thapsigargin**

4. Calcium Imaging Analysis Using the Fiji Software

Demonstrator: Chiara D'Addario

4.1. Open the Fiji application **[1]**. Select **File** and choose **Open** to upload the video file **[2]**. In the resulting pop-up window, keep all default settings and confirm the selection **[3]**.

4.1.1. Talent seated at a computer workstation launching the Fiji application.

4.1.2. SCREEN: 69470_4.1.2.

4.1.3. SCREEN: 69470_4.1.3.

4.2. If desired, adjust the image display by selecting **Image**, choosing **Adjust**, and clicking **Brightness Contrast [1]**. Modify the brightness and contrast of individual fluorescence channels to achieve an optimal view of the cells **[2]**.

4.2.1. SCREEN: 69470_4.2.1.

4.2.2. SCREEN: 69470_4.2.2.

4.3. To ease cell tracing, merge channels by selecting **Image**, choosing **Color**, and clicking **Merge Channels [1]**. In the pop-up window, assign the appropriate files to each individual channel and confirm the selection **[2]**.

4.3.1. SCREEN: 69470_4.3.1.

4.3.2. SCREEN: 69470_4.3.2 00:00-00:16.

4.4. For tracing, select **Analyze**, choose **Tools**, and open **ROI Manager [1]**. In the resulting pop-up window, enable both **Show All** and **Labels** options **[2]**.

4.4.1. SCREEN: 69470_4.4.1.

4.4.2. SCREEN: 69470_4.4.2.

4.5. Using the freehand selections tool, carefully trace individual cells based on cell boundaries and the nuclear stain **[1]**. Then, press the t key to add each traced cell to the ROI list **[2]**.

4.5.1. SCREEN: 69470_4.5.1.

4.5.2. SCREEN: 69470_4.5.2.

4.6. Ensure that cells are traced in all 4 corners of the field of view and in the center of the video **[1]**.

4.6.1. SCREEN: 69470_4.6.1.

4.7. Once all cells have been accurately traced, within the ROI Manager window, click **More**

and select **Multi Measure [1]**. In the resulting pop-up window, press **OK** to begin the measurement **[2-TXT]**.

4.7.1. SCREEN: 69470_4.7.1.

4.7.2. SCREEN: 69470_4.7.2. **TXT: Copy the results and plot the values**

5. Calcium Imaging Analysis Using the Calcium Suite

Demonstrator: Abdelkader Daoud

5.1. Open the video file in Fiji **[1]**. Optimize the nuclear and green fluorescence channels for viewing using the image adjustment tools **[2]**.

5.1.1. SCREEN: 69470_5.1.1

5.1.2. SCREEN: 69470_5.1.2.

5.2. Once the channels are optimized, duplicate a single frame by selecting **Image** and choosing **Duplicate [1]**. In the resulting pop-up window, deselect **Duplicate Stack [2]** and save the duplicated image as a PNG image file **[3]**.

5.2.1. SCREEN: 69470_5.2.1.

5.2.2. SCREEN: 69470_5.2.2.

5.2.3. SCREEN: 69470_5.2.3.

5.3. Repeat the frame duplication by selecting **Image** and choosing **Duplicate [1]**. In the pop-up window, press **OK [2]** and save the image as a PNG file **[3]**.

5.3.1. SCREEN: 69470_5.3.1.

5.3.2. SCREEN: 69470_5.3.2.

5.3.3. SCREEN: 69470_5.3.3.

5.4. Merge the two saved images by selecting **Image**, choosing **Color**, and clicking **Merge Channels [1]**. Then, assign each PNG file to the appropriate channel and confirm the merge **[2]**.

5.4.1. SCREEN: 69470_5.4.1.

5.4.2. SCREEN: 69470_5.4.2.

5.5. Upload the merged image into the Cell-pose graphical user interface to generate a cell mask **[1]**. Segment the image using the nuclear stain as Channel 1 and the cytoplasmic stain as Channel 2 by selecting the **Cyto3** model **[2]**.

5.5.1. SCREEN: 69470_5.5.1.

5.5.2. SCREEN: 69470_5.5.2.

5.6. Now, save the generated mask image as a PNG file **[1],** open it in Fiji and convert it to a TIFF *(tiff)* file **[2]**.

5.6.1. SCREEN: 69470_5.6.1.

5.6.2. SCREEN: 69470_5.6.2. 00:10-00:20

5.7. Finally, open both the mask file and the original video file in the Calcium Suite by dragging and dropping them into the software interface. Click **Analyze Data [1]** to generate a downloadable plot and a downloadable CSV file containing normalized and raw intensity values for all analyzed cells **[2]**.

5.7.1. SCREEN: 69470_5.7.1.

5.7.2. SCREEN: 69470_5.7.2.

Results

6. Results

6.1. Fluorescence intensity normalized to baseline increased over time in individual Calu-3 cells following thapsigargin stimulation when measured using both Fluo-4 AM *(Fluo 4-A-M)* and Cal-520 AM *(Cal -five twenty-A-M)* **[1]**.

6.1.1. LAB MEDIA: Figure 1B and C. *Video editor: Show the line graph and highlight the upward traces under thapsigargin addition phase in both B and C*.

6.2. The maximal calcium-dependent fluorescence activation after thapsigargin was significantly higher in Calu-3 cells measured with Cal-520 AM compared to Fluo-4 AM **[1]**.

6.2.1. LAB MEDIA: Figure 1D. *Video editor: Highlight the Cal-520 AM data points*.

6.3. Similarly, individual primary airway and bronchial cells showed increased normalised fluorescence over time after thapsigargin stimulation using either of the dyes **[1]** and maximal calcium-dependent fluorescence activation was significantly higher in cells added with Cal-520 AM **[2]**.

6.3.1. LAB MEDIA: Figure 2C and D. *Video editor: Show the increase line graph signals under "thapsigargin" addition segment*.

6.3.2. LAB MEDIA: Figure 2E. *Video editor: Highlight the Cal-520 AM data points*.

6.4. Machine learning-based analysis identified and quantified calcium-dependent fluorescence changes in 1441 individual cells from a single field of view **[1]**.

6.4.1. LAB MEDIA: Figure 3B.

6.5. Normalized fluorescence traces generated by the software showed calcium responses for all 1441 identified cells following thapsigargin stimulation **[1]**.

6.5.1. LAB MEDIA: Figure 3C. *Video editor: Show the dense set traces under thapsigargin segment*.

1. Calu-3
   Pronunciation link: No confirmed link found

IPA: /ˈkælu: θri:/
Phonetic Spelling: ka·loo·three

2. Confluence
Pronunciation link: https://www.merriam-webster.com/dictionary/confluence
IPA: /ˈkɑ:n.flu:.əns/
Phonetic Spelling: kon·floo·uhns
3. Penicillin-streptomycin
Pronunciation link: No confirmed link found
IPA: /ˌpɛnɪˈsɪlɪn ˌstrɛptəˈmaɪsɪn/
Phonetic Spelling: pen·uh·sil·in strep·tuh·my·sin
4. Phosphate-buffered saline (PBS)
Pronunciation link: https://www.merriam-webster.com/dictionary/phosphate
IPA: /ˈfɑ:s.feɪt ˈbʌf.ərd səˈli:n/
Phonetic Spelling: fahs·fayt buf·erd suh·leen
5. TrypLE
Pronunciation link: No confirmed link found
IPA: /ˈtrɪp.li:/
Phonetic Spelling: trip·lee
6. Celsius
Pronunciation link: https://www.merriam-webster.com/dictionary/Celsius
IPA: /ˈsɛl.si.əs/
Phonetic Spelling: sel·see·uhs
7. Carbon dioxide
Pronunciation link: https://www.merriam-webster.com/dictionary/carbon%20dioxide
IPA: /ˌkɑ:r.bən daɪˈɑ:k.saɪd/
Phonetic Spelling: kar·buhn dye·ok·side
8. Probenecid
Pronunciation link: https://www.merriam-webster.com/dictionary/probenecid
IPA: /proʊˈbɛn.ə.sɪd/
Phonetic Spelling: proh·ben·uh·sid
9. Thapsigargin
Pronunciation link: https://www.merriam-webster.com/dictionary/thapsigargin
IPA: /ˌθæp.sɪˈgɑ:r.dʒɪn/
Phonetic Spelling: thap·sih·gar·jin
10. Transwell
Pronunciation link: No confirmed link found
IPA: /ˈtrænz.wɛl/
Phonetic Spelling: tranz·wel
11. Basolateral
Pronunciation link: https://www.merriam-webster.com/dictionary/basolateral
IPA: /ˌbeɪ.soʊˈlæt̬.ər.əl/
Phonetic Spelling: bay·soh·lat·uh·rul
12. Apical
Pronunciation link: https://www.merriam-webster.com/dictionary/apical

IPA: /ˈeɪ.pɪ.kəl/
Phonetic Spelling: ay·pih·kuhl

13. Microliter
Pronunciation link: https://www.merriam-webster.com/dictionary/microliter
IPA: /ˈmaɪ.kroʊˌliː.ɾər/
Phonetic Spelling: my·kroh·lee·ter
14. Micromolar
Pronunciation link: https://www.merriam-webster.com/dictionary/micromolar
IPA: /ˌmaɪ.kroʊˈmoʊ.lɚ/
Phonetic Spelling: my·kroh·moh·ler
15. Fluorescence
Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescence
IPA: /ˌflʊrˈɛs.əns/
Phonetic Spelling: floor·es·uhns
16. Cal-520 AM
Pronunciation link: No confirmed link found
IPA: /kæl faɪv ˈtwɛn.ti eɪ ɛm/
Phonetic Spelling: kal·five·twen·tee ay·em
17. Fluo-4 AM
Pronunciation link: No confirmed link found
IPA: /ˈfluː.oʊ fɔːr eɪ ɛm/
Phonetic Spelling: floo·oh·for ay·em
18. Hoechst
Pronunciation link: https://www.merriam-webster.com/dictionary/Hoechst
IPA: /hɜːrst/
Phonetic Spelling: hurst
19. Cytoplasmic
Pronunciation link: https://www.merriam-webster.com/dictionary/cytoplasmic
IPA: /ˌsaɪ.toʊˈplæz.mɪk/
Phonetic Spelling: sy·toh·plaz·mik
20. Bronchial
Pronunciation link: https://www.merriam-webster.com/dictionary/bronchial
IPA: /ˈbrɑːŋ.ki.əl/
Phonetic Spelling: brong·kee·uhl
21. Fiji
Pronunciation link: https://www.merriam-webster.com/dictionary/Fiji
IPA: /ˈfiː.dʒiː/
Phonetic Spelling: fee·jee
22. Cellpose
Pronunciation link: No confirmed link found
IPA: /ˈsɛl.poʊz/
Phonetic Spelling: sel·pohz