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Title: Measurement of 3-Dimensional cAMP Distributions in Living Cells Using 4-Dimensional (x, y, z, and λ) Hyperspectral FRET Imaging and Analysis

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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 similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Y**

   If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as reasonably possible.

   Videographer: Screen capture files not yet provided, please film for reference only

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **41**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Naga Annamdevula**: This protocol is significant, as it provides a method for measuring and visualizing FRET/cAMP signals in three spatial dimensions in individual cells **[1]**.

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

REQUIRED:

1.2. **Silas Leavesley**: cAMP distributions may vary axially, in addition to laterally, and it is important to acquire FRET/cAMP image data in three dimensions to sample these distributions **[1]**.

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

Protocol

2. Imaging Preparation

2.1. On the day of imaging, warm Tyrode's buffer to 37 degrees Celsius in a water bath **[1-TXT]** and mount a coverslip seeded with the transfected cells of interest into a cell chamber **[2]**.

2.1.1. WIDE: Talent placing buffer into water bath **TEXT: See text for all buffer and solution preparation details**

2.1.2. Talent mounting coverslip of transfected cells into cell chamber

2.2. Secure the top of the chamber with a mounting gasket to prevent leaking **[1]** and use a delicate task wipe to remove any excess medium or adherent cells from the bottom of the coverslip **[2]**.

2.2.1. Talent securing top of chamber

2.2.2. Talent wiping coverslip

2.3. Add 800 microliters of working buffer **[1]** and 4 microliters of 5-millimolar nuclear label to the cell chamber **[2]** and gently rock the chamber for 5-10 seconds **[3]**.

2.3.1. Talent adding buffer to chamber, with buffer container visible in frame

2.3.2. Talent adding label to cell chamber, with label container visible in frame

2.3.3. Talent rocking chamber

2.4. Then cover the cell chamber with aluminum foil for a 10-minute incubation at room temperature **[1]**.

2.4.1. Talent covering chamber with foil

3. Image Acquisition

3.1. To image the cells, select the 60x water immersion objective on a confocal microscope equipped with a spectral detector **[1]** and add a drop of water to the objective **[2]**.

3.1.1. WIDE: Talent selecting objective

3.1.2. Talent adding drop of water to objective

3.2. Place the loaded cell chamber onto the microscope stage **[1]** and tune the filter knob to select the appropriate filter set **[2-TXT]**.

3.2.1. Talent placing chamber onto stage

3.2.2. Talent selecting filter set **TEXT: *e.g.*, DAPI/FITC/TRITC**

3.3. Select the fluorescence widefield mode **[1]** and use the eyepieces to select a field of view containing cells expressing the FRET-cAMP (fret camp) sensor **[2-TXT]**.

3.3.1. Talent selecting eyepiece mode, with microscope visible in frame

3.3.2. Talent at microscope, selecting FOV **TEXT: cAMP: 3'-5'cyclic adenosine monophosphate; FRET: Förster resonance energy transfer**

3.4. Open the imaging software, select the confocal mode, and unlock the laser interlock button **[1]**.

3.4.1. SCREEN: 61720_Leavesley_Script_Step 3.4.1.mp4 00:04-00:20

3.5. Open the **A1 settings** menu, check the boxes corresponding to the 405- and 561-nanometer laser lines, and set the **spectral detections** to SD, the **resolution** to 10, and the **channels** to 32 **[1]**.

3.5.1. SCREEN: 61720_Leavesley_Script_Step 3.5.1.mp4 00:02-00:25

3.6. Select the start and end wavelength values to set the wavelength range. Open the **A1 settings** menu to select the binning-skip icon, select the number 15 box, and click **OK [1]**.

3.6.1. SCREEN: 61720_Leavesley_Script_Step 3.6.1.mp4 00:05-00:20

3.7. Set the laser intensities to 8% for the 405-nanometer laser and to 2% for the 561-nanometer laser. Set the **detector gain** to 149 and the pinhole radius to 2.4 airy disk units **[1]**.

3.7.1. SCREEN: 61720_Leavesley_Script_Step 3.7.1.mp4 00:02-00:24

3.8. Set the **scan speed** to 0.25 spectral frames per second and select the **unidirectional scan direction**. Set the **count** to 4 and the **scan size** to 1024 x 1024 **[1]**.

3.8.1. SCREEN: 61720_Leavesley_Script_Step 3.8.1.mp4 00:02-00:21

3.9. To define the z-stack acquisition parameters, click **View**, **Acquisition control**, and **ND** (N-D) **acquisition** and enter the file destination and name in the pop window to save the ND file **[1]**.

3.9.1. SCREEN: 61720_Leavesley_Script_Step 3.9.1.mp4 00:02-00:42

3.10. Click the **z-series** box and click **Live** in the **A1 Plus Settings** window to open a live viewing window. Adjust the focus knob to focus on the cells while looking in the preview window. **[1]**.

3.10.1. SCREEN: 61720_Leavesley_Script_Step 3.10.1.mp4 00:02-00:29

3.11. Adjust the focus knob on the microscope to select the top of the cell and click **Top** in the ND acquisition window to set the current position as the top **[1]**.

3.11.1. SCREEN: 61720_Leavesley_Script_Step 3.11.1.mp4 00:02-00:24

3.12. Adjust the focus knob on the microscope to select the bottom of the of the cell and click **Bottom** to set the current position as the bottom **[1]**.

3.12.1. SCREEN: 61720_Leavesley_Script_Step 3.12.1.mp4 00:01-00:38

3.13. Enter 1 micrometer for the **Step size**, select top-bottom for the **z-scan direction**, and click **Run** to acquire a z-stack **[1]**.

3.13.1. SCREEN: 61720_Leavesley_Script_Step 3.13.1.mp4 00:01-00:25

3.14. When the z-stack acquisition is complete, use a pipette to gently add the reagent of interest to the chamber without disturbing the cells **[1-TXT]**.

3.14.1. Reagent being added **TEXT: *e.g.*, forskolin or vehicle control**

3.15. After 10 minutes, change the filename and acquire a second z-stack image **[1]**.

3.15.1. SCREEN: 61720_Leavesley_Script_Step 3.15.1.mp4 00:02-00:24

4. Image Analysis

4.1. After imaging, create new folders with the same filenames as the spectral z-stack images **[1]** and open the spectral image file of interest **[2]**.

4.1.1. WIDE: Talent creating folder, with monitor visible in frame

4.1.2. SCREEN: 61720_Leavesley_Steps 4.1.2, 4.2.1, 4.3.1.mp4 00:00-00:08

4.2. Click **File**, **Import-Export**, and **Export ND document**. In the popup window, select the newly created folder, TIF for the **file type**, **Mono image for each channel**, and **Keep bit depth [1]**.

4.2.1. SCREEN: 61720_Leavesley_Steps 4.1.2, 4.2.1, 4.3.1.mp4 00:08-00:20

4.3. Click **Export** to export the individual files to the folder **[1]** and open the linear spectral unmixing software **[2-TXT]**.

4.3.1. SCREEN: 61720_Leavesley_Steps 4.1.2, 4.2.1, 4.3.1.mp4 00:21-00:30

4.3.2. SCREEN: 61720_Leavesley_Step 4.3.2.mp4 0:11-0:16 **TEXT:** https://www.southalabama.edu/centers/bioimaging/resources.html

4.4. Open the **Linear Unmixing.m** file and click **Run**. Browse and select the folder containing the exported .tif file sequence and click **OK** to open the **Wavelength and Z-Slice** window **[1]**.

4.4.1. SCREEN: 61720_Leavesley_Steps 4.4.1, 4.5.1, 4.6.1.mp4 00:00-00:17

4.5. Copy the filename of first file without the z-slice and channel number from the folder and paste it into the first step of the **Enter the Image Name** box. Enter the number of channels in the **Enter the number of wavelength bands** box, and the number of z-slices in the **Enter the number of Z-slices** box, and click **OK [1]**.

4.5.1. SCREEN: 61720_Leavesley_Steps 4.4.1, 4.5.1, 4.6.1.mp4 00:18-00:34

4.6. Then, in the popup window, browse and select the **Wavelength.mat** file and click **Open**. Browse and select the **Library.mat** file in the new popup window and click **Open** again to initiate the slice unmixing **[1]**.

4.6.1. SCREEN: 61720_Leavesley_Steps 4.4.1, 4.5.1, 4.6.1.mp4 00:35-00:48

5. FRET Efficiency Calculation and Mapping

5.1. To calculate the FRET efficiency, open the **multiFRRCF.m** programming script **[1]** and enter the number of experimental trials to analyze in the **how many folders to reslice** box. Click **OK**, browse to select the unmixed folders, and click **OK** again **[2]**.

5.1.1. WIDE: Talent opening script, with monitor visible in frame

5.1.2. SCREEN: 61720_Leavesley_Steps 5.1.2,5.2.1,5.3.1.mp4 00:00-00:17

5.2. In the new pop window, set the **scaling factor** to 12.4, the **Threshold** to 5.6, the **X**, **Y**, **and Z Frequency** to 5, 5, and 1, respectively, and the **smoothing algorithm** to Gaussian **[1]**.

5.2.1. SCREEN: 61720_Leavesley_Steps 5.1.2,5.2.1,5.3.1.mp4 00:18-00:27

5.3. Then click **Run** to perform the FRET measurements and reslicing **[1]**.

5.3.1. SCREEN: 61720_Leavesley_Steps 5.1.2,5.2.1,5.3.1.mp4 00:28-00:38

5.4. To map the FRET efficiency to camp levels, open the **Mapping_FRET Efficiency_to_cAMP_concentration.m** file and click **Run [1]**.

5.4.1. SCREEN: 61720_Leavesley_Steps 5.4.1,5.5.1.mp4 00:00-00:06

5.5. Navigate to and select the first grey scale FRET image and click **OK [1]**.

5.5.1. SCREEN: 61720_Leavesley_Steps 5.4.1,5.5.1.mp4 00:07-00:25

5.6. Then open the FRET-cAMP images to inspect the distribution of the cAMP signals in three dimensions **[1]**.

5.6.1. SCREEN: 61720_Leavesley_Step 5.6.1.mp4 00:02-00:15

Results

6. Results: Representative 3D cAMP Distribution and Hyperspectral FRET Imaging and Analysis

6.1. In these images, 3-dimensional views of false-colored raw hyperspectral image data acquired using confocal microscopy **[1]** at baseline **[2]** and 10 minutes after forskolin treatment can be observed **[3]**.

6.1.1. LAB MEDIA: Figure 2
6.1.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize Figure 2A*
6.1.3. LAB MEDIA: Figure 2 *Video Editor: please emphasize Figure 2B*

6.2. In this analysis, an acceptor fluorophore was completely photo-destructed **[1]**, allowing spectral signatures of the donor and acceptor with a 1:1 stoichiometry to be obtained **[2]**.

6.2.1. LAB MEDIA: Figures 3A, 3C, and 3E
6.2.2. LAB MEDIA: Figures 3A-3F

6.3. Non-transfected cells labeled with a nuclear dye **[1]** were utilized to obtain the pure spectrum of the dye fluorophore **[2]**.

6.3.1. LAB MEDIA: Figure 3H
6.3.2. LAB MEIDA: Figures 3H and 3I

6.4. Combining the spectra of the donor **[1]**, acceptor **[2]**, and nuclear dye fluorophores **[3]** allows the creation of a 3-component library **[4]**.

6.4.1. LAB MEDIA: Figures 3F, 3G, 3I *Video Editor: please emphasize Figure 3F*
6.4.2. LAB MEDIA: Figures 3F, 3G, 3I *Video Editor: please emphasize Figure 3G*
6.4.3. LAB MEDIA: Figures 3F, 3G, 3I *Video Editor: please emphasize Figure 3I*
6.4.4. LAB MEDIA: Figures 3D, 3F, 3I, and 3J *Video Editor: please emphasize Figure 3J*

6.5. Here the sources of three background spectral signatures can be observed **[1]**.

6.5.1. LAB MEDIA: Figures 4A-4C

6.6. Although these signals are distributed non-uniformly within the sample and cannot simply be subtracted out **[1]**, adding the spectral signatures of these signals to the spectral library **[2]** and using linear unmixing to separate the signals provides an

approach for removing these confounding signals from the donor and acceptor signals prior to calculating their FRET efficiencies **[3]**.

6.6.1. LAB MEDIA: Figures 4A-4C
6.6.2. LAB MEDIA: Figure 4A-4D
6.6.3. LAB MEDIA: Figure 4

6.7. The 6-component spectral library **[1]** can then be used to perform linear spectral unmixing for each slice in the axial image **[2]**.

6.7.1. LAB MEDIA: Figures 4A and 4B
6.7.2. LAB MEDIA: Figures 4 *Video Editor: please show Figures 4A and 4B and sequentially add Figures 4C-4H*

6.8. Here the changes in FRET efficiency **[1]** and cAMP levels in different XY plane slices can be observed **[2]**, allowing a comparison between the baseline conditions **[3]** and the conditions 10-minutes after forskolin treatment **[4]**.

6.8.1. LAB MEDIA: Figure 7 images and legends *Video Editor: please emphasize FRET Efficiency columns*
6.8.2. LAB MEDIA: Figure 7 images and legends *Video Editor: please emphasize cAMP concentration columns*
6.8.3. LAB MEDIA: Figure 7 graph *Video Editor: please emphasize black Baseline data lines*
6.8.4. LAB MEDIA: Figure 7 graph *Video Editor: please emphasize green Forskolin data lines*

Conclusion

7. Conclusion Interview Statements

7.1. **Thomas Rich**: This technique could also be used to quantify the FRET efficiency of other reporters in three dimensions in both cells and tissues **[1]**.

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