Submission ID #: 61481
Scriptwriter Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18751753

Title: Quantifying Spontaneous Ca2+ Fluxes and their Downstream Effects in Primary Mouse Midbrain Neurons

Authors and Affiliations:

Eric A. Bancroft[1], Rahul Srinivasan[1,2]

[1]Department of Neuroscience and Experimental Therapeutics, Texas A&M University Health Science Center, College of Medicine, Bryan, TX, USA
[2]Texas A&M Institute for Neuroscience, College Station, TX, USA

Corresponding Authors:

Rahul Srinivasan (rahul@tamu.edu)

Email Addresses for All Authors:

rahul@tamu.edu
bancroft@tamu.edu

Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, SC all set**

3. **Filming location:** Will the filming need to take place in multiple locations? **No, same building, 2 different floors**

Current Protocol Length

Number of Steps: 14
Number of Shots: 25

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Eric Bancroft:** This protocol quantifies calcium fluxes in dopaminergic neurons, which are lost in Parkinson's disease. This is useful for understanding how abnormal calcium causes dopaminergic neuron loss in Parkinson's disease.

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

1.2. **Eric Bancroft:** For the first time, we demonstrate spontaneous calcium fluxes in cultured primary midbrain neurons. This method can be used to dissect specific receptor contributions involved in calcium-mediated apoptosis.

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

OPTIONAL:

1.3. **Rahul Srinivasan:** Our method provides an avenue for high content drug screens to discover specific and effective neuroprotective compounds for Parkinson's disease. These neuroprotective compounds would prevent calcium-mediated apoptosis in dopaminergic neurons.

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

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University.

Protocol

2. Infection of Cell Culture at 14 DIV with Adeno-associated Viral (AAV) Vectors

2.1. Begin by preparing 1 milliliter of serum free DMEM medium with 1 microliter of hSyn-GCaMP6f *(pronounce 'H-sin-G-camp-6-F')* AAV per dish **[1-TXT]**. Aspirate the cell culture medium from each dish and replace it with 1 milliliter of the prepared DMEM with hSyn-GCaMP6f **[2]**. Place the dishes back into the 37 degrees Celsius incubator for 1 hour **[3]**.

2.1.1. WIDE: Establishing shot of talent preparing medium. **TEXT: 1.0 x 10^{13} titer**

2.1.2. Talent aspirating the cell culture medium and adding the prepared medium.

2.1.3. Talent putting the dishes in the incubator and closing the door.

2.2. After the incubation, aspirate the medium with AAVs and replace it with 3 milliliters of cell culture medium **[1]**. Incubate the dishes at 37 degrees Celsius for 5 to 7 days, changing the medium every 2 to 3 days throughout this period of viral infection **[2]**.

2.2.1. Talent aspirating medium then replacing it, with the cell culture medium container in the shot.

2.2.2. Talent putting the dishes in the incubator and closing the door.

3. Live Confocal Ca^{2+} Imaging at 19 – 21 DIV

3.1. Prepare 1 liter of the HEPES *(pronounce "heeps")* recording buffer, 200 milliliters of 20 micromolar glutamate recording buffer, and 200 milliliters of 10 micromolar NBQX recording buffer according to manuscript directions **[1]**. Fill a sterile 35-millimeter Petri dish with 3 milliliters of the recording buffer **[2]**.

3.1.1. Containers with prepared buffers, all labeled.

3.1.2. Talent adding buffer to a Petri dish.

3.2. Take the Petri dish with the infected cultures from the incubator **[1]**, then carefully grab the edge of one coverslip with fine tip forceps and transfer it into the dish with the recording buffer **[2]**.

3.2.1. Talent taking the cells out of the incubator.

3.2.2. Talent transferring the coverslip with fine forceps to the recording buffer.

3.3. Place the remaining coverslip in medium back into the 37-degree Celsius incubator **[1]** and transport the dish with recording buffer to the confocal microscope **[2]**.

3.3.1. Talent placing the remaining coverslip back in the incubator.

3.3.2. Talent walking to the microscope with the dish in hand.

3.4. Start the imaging software **[1]**. While it is initializing, start the peristaltic pump and place the line into the recording buffer **[2]**, then calibrate the flow to 2 milliliters per minute **[3]**. Transfer the infected coverslip from the Petri dish to the recording bath with fine forceps **[4]**.

3.4.1. Talent at the computer starting the imaging software. *Video Editor: Can also use 61481_screenshot_1 0:10 – 0:50.*

3.4.2. Talent starting the pump and putting the line into the recording buffer.

3.4.3. Talent calibrating the flow of the pump.

3.4.4. Talent transferring the coverslip into the recording bath with fine forceps.

3.5. Using the 10 X water immersion objective and bright-field light, find the plane of focus and look for a region with a high density of neuron cell bodies. Then, switch to the 40 X objective and refocus the sample **[1]**.

3.5.1. Plane of focus and region with neuron cell bodies located, then objective switched and focus readjusted. *Videographer: Film the TV screen attached to the scope for this shot.*

3.6. Select and apply AlexaFluor 488 in the **Dyes List** window. In order to prevent overexposure and photobleaching of the fluorophores, start with low HV and laser power settings **[1]**.

3.6.1. SCREEN: 61481_screenshot_2. 0:06 – 0:25. AlexaFluor 488 selected in the dyes list.

3.7. For the AlexaFluor 488 channel, set the HV to 500, the gain to 1 X, and offset to 0. Set the power to 5% for the 488 laser line. Increase the pinhole size to 300 micrometers and use the **focus x2** scanning option to optimally adjust emission signals to sub-saturation levels. Settings can then be adjusted for optimal visibility of each channel **[1]**.

3.7.1. SCREEN: 61481_screenshot_3_take2. 0:04 – 0:48. HV, gain, and offset selected; Laser power adjusted to 5%; Pinhole size set to 300 micrometers and focus x2 scanning selected.

3.8. Once the microscope settings are optimized, move the stage to locate a region with multiple cells displaying spontaneous changes in GCaMP6f fluorescence and focus on the desired plane for imaging **[1]**. *Videographer: This step is difficult!*

3.8.1. Stage moved and image refocused. *Videographer: Film the TV screen attached to the scope here.*

Video Editor: Can also use SCREEN: 61481_screenshot_4, 0:01 – 0:43.

3.9. Use the **Clip rect** tool to clip the imaging frame to a size that can achieve a frame interval of just under 1 second. Set the **Interval** window to a value of 1.0 and the **Num** window to 600 **[1]**. *Videographer: This step is important!*

3.9.1. SCREEN: 61481_screenshot_5. 0:04 – 0:38. Image clipped, Interval window set, and Num window set.

3.10. To capture a t-series movie, select the **Time** option, then use the **XYt** scanning option to begin imaging. Watch the imaging progress bar **[1]** and move the line from the HEPES recording buffer into the 20 micromolar glutamate recording buffer at the appropriate time point **[2]**. *Videographer: This step is important!*

3.10.1. SCREEN: 61481_screenshot_6. 0:03 – 0:20. Time option and XYt option selected and imaging initiated.

3.10.2. Talent moving the line to the glutamate buffer. NOTE: Normally done in the dark

3.11. When imaging is complete, select the **Series Done** button and save the finished t-series movie **[1]**. Continue to perfuse the glutamate for an additional 5 minutes, so that the cultured neurons have been exposed to glutamate for a total of 10 minutes. Repeat this process for each coverslip to be imaged **[2]**. It is possible to view calcium traces in neuronal cell bodies immediately following the experiment. Use the ellipse tool to draw the desired number of ROIs around neuronal soma and use the series analysis button to visualize the traces **[added]**. *Videographer: This step is important!*

NOTE to VO: Long one, please split up.

3.11.1. SCREEN: 61481_screenshot_7. 0:15 – 0:58. Series Done selected and movie saved.

3.11.2. Glutamate perfusing.

3.11.3. Added shot: Talent drawing ROIs on finished video using the ellipse tool and generating graphs of calcium traces using the series analysis button in the video window.

3.12. After the additional 5-minute exposure to glutamate, remove the coverslip from the bath with fine forceps and place it back into the Petri dish with the recording buffer until all imaging is completed **[1]**. *Videographer: This step is important!*

3.12.1. Talent transferring the coverslip back to the Petri dish with fine forceps.

Results

4. Results: Effect of Glutamate Application on Mesencephalic Neurons

4.1. On the day of imaging, the VM cultures were treated with either glutamate **[1]** or a combination of glutamate and NBQX **[2]**. In both conditions, heterogenous and spontaneous changes in GCaMP6f *(pronounce 'G-camp-6-F')* fluorescence were observed, indicating spontaneous calcium fluxes **[3]**.

4.1.1. LAB MEDIA: Figure 1 A and B. *Video Editor: Emphasize A.*

4.1.2. LAB MEDIA: Figure 1 A and B. *Video Editor: Emphasize B.*

4.1.3. LAB MEDIA: Figure 1 A and B.

4.2. Application of glutamate generated a robust and sustained calcium response in both spontaneously active and quiescent neurons **[1]**. Application of NBQX reduced spontaneous activity and partially blocked the glutamate response **[2]**.

4.2.1. LAB MEDIA: Figure 1 A and Supplemental Movie 1.

4.2.2. LAB MEDIA: Figure 1 B and Supplemental Movie 2.

4.3. The extent to which glutamate application stimulated a calcium response in each condition was quantified using the area under the curve, peak amplitude, and latency to respond **[1]**. The latency to response increased under the NBQX and glutamate condition **[2]**.

4.3.1. LAB MEDIA: Figure 1 C and D.

4.3.2. LAB MEDIA: Figure 2 A and B.

4.4. To measure glutamate-mediated apoptosis, the cells were fixed and stained with an anti-caspase-3 antibody **[1]**. Mean caspase-3 intensity was significantly higher in both treatment conditions compared to untreated controls **[2]**.

4.4.1. LAB MEDIA: Figure 3 A.

4.4.2. LAB MEDIA: Figure 3 B, just the Caspase-3 Activation plot. *Video Editor: Emphasize the Glutamate and NBQX + Glut bars.*

Conclusion

5. Conclusion Interview Statements

5.1. **Eric Bancroft:** A more thorough analysis of excitotoxic cell death can be done by counting the number of immunostained tyrosine hydroxylase positive dopaminergic neurons in control and glutamate treated conditions.

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

5.2. **Rahul Srinivasan:** This technique has paved a way for understanding the relative contributions of different receptors and ion channels involved in calcium-mediated apoptosis in dopaminergic neurons.

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