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Title: Use of Primary Cultured Hippocampal Neurons to Study the Assembly of Axon Initial Segments

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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. **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**.

   ☒ Author interview statement opt out. Statements removed completely.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 18
Number of Shots: 38

Introduction

1. Introductory Statements

Videographer: Authors have opted out of interview statements.
NOTE to VO Talent: Please record statement 1.1 below.

1.1. This protocol is used to quantitatively study the assembly and structure of the axon initial segments of hippocampal neurons that lack pre-assembled AIS due to the absence of a giant ankyrin-G **[1]**.

1.1.1. SCREEN: screen record.mov. 0:06 – 0:12.

Protocol

2. Culture Hippocampal Neurons

2.1. To begin, dissect 6 to 8 hippocampi from postnatal 1-day old pups with HBSS medium in a Petri dish **[1]**. Chop the hippocampi with dissection scissors into smaller pieces **[2]** and transfer them from the dish to a 15-milliliter tube **[3]**.

2.1.1. WIDE: Establishing shot of talent in front of a Petri dish with the hippocampi.

2.1.2. Talent chopping the hippocampi.

2.1.3. Talent transferring the chopped tissue to a tube.

2.2. Wash hippocampi twice with 5 milliliters of HBSS **[1]** and leave them in 4.5 milliliters of HBSS after the wash **[2]**.

2.2.1. Talent washing the tissue with HBSS, with the HBSS container in the shot.

2.2.2. Tissue in the HBSS.

2.3. Add 0.5 milliliters of 2.5% trypsin into 4.5 milliliters of HBSS **[1]** and incubate in a 37-degree Celsius water bath for 15 minutes **[2]**, inverting the tube every 5 minutes **[3]**. Well-digested hippocampi should become sticky and form a cluster. If needed, extend the digestion for 5 more minutes **[4]**.

2.3.1. Talent adding trypsin to the tissue.

2.3.2. Talent putting the tube in the water bath.

2.3.3. Talent inverting the tube.

2.3.4. Well-digested hippocampi in the tube.

2.4. Wash hippocampi with HBSS 3 times for 5 minutes per wash **[1-TXT]**. After the wash, add 2 milliliters of HBSS **[2]** and pipette the hippocampi up and down with a Pasteur pipette 15 times **[3]**.

2.4.1. Talent washing the hippocampi with HBSS. **TEXT: Do not use a vacuum to remove the HBSS**

2.4.2. Talent adding HBSS to the tube.

2.4.3. Talent pipetting the tissue.

2.5. Triturate the tissue with a fire-polished Pasteur pipette 10 times **[1-TXT]** and rest the tube for 5 minutes until all chunks set to the bottom **[2]**. *Videographer: This step is difficult and important!*

2.5.1. Talent triturating the tissue. **TEXT: Do not go beyond 10 X**

2.5.2. Tube resting with chunks at the bottom.

2.5.3. Talent transferring the supernatant to the plating dishes. **TEXT: 10^5 cells/60 mm dish** NOTE: **move step 2.5.3 and 2.5.4 to after step 2.6.1**

2.5.4. Talent shaking the plate.

2.6. Repeat the trituration with the remaining chunks until most of them have disappeared **[1]**. Then, use a 1-milliliter pipette tip to gently transfer the supernatant containing the dissociated neurons to plating dishes **[2.5.3-TXT]**. Add it directly to the pre-incubated plating medium and shake the plate gently **[2.5.4]**. *Videographer: This step is important!*

2.6.1. Talent triturating the tissue. NOTE: put 2.5.3 – 2.5.4 after this one. VO changed accordingly.

2.7. Two to four hours after seeding, check the plating dishes with a light microscope **[1]**. The majority of neurons should have attached to the coverslip **[2]**. Using a fine tip forceps, flip the coverslips to the glia cell feeder dishes containing preconditioned neuronal culture medium with the wax dots side facing downwards **[3]**.

2.7.1. Talent using the microscope.

2.7.2. SCOPE: Attached neurons. NOTE: Author will upload picture.

2.7.3. Talent flipping the coverslips to the glia cell feeder dishes.

2.8. Neurons can grow in the glia cell feeder dishes for up to 1 month. Feed neurons every 7 days with 1 milliliter of fresh neuronal culture medium **[1]**.

2.8.1. Talent putting the feeder dishes in the incubator and closing the door.

3. Disruption of AIS by Knockout of Ankyrin-G at an Early Stage of Neuron Development

3.1. On the third day *in vitro*, flip the coverslips with the wax dots side facing up into a glia cell feeder dish with conditioned neuronal culture medium **[1]**.

3.1.1. Talent flipping the coverslip into the glia cell feeder dish.

3.2. Mix 0.25 micrograms of Cre-BFP DNA with 0.5 micrograms of ankyrin-G-GFP DNA in a 1.7-milliliter tube to transfect 4 coverslips **[1-TXT]**. Add 100 microliters of culture medium **[2]**, then mix the tube and rest it on a rack. If only Cre-BFP is transfected, the GFP plasmid backbone is used to match the total amount of DNA **[3]**.

3.2.1. Talent mixing the Cre-BFP DNA with the ankyrin-G-GFP DNA. **TEXT: ~ 2:1 ratio of DNA copy number**

3.2.2. Talent adding media.

3.2.3. Talent mixing the tube and placing it on a rack.

3.3. Mix 3 microliters of transfection reagent with 100 microliters of culture medium in a new 1.7-milliliter tube and incubate the tube for 5 minutes at room temperature **[1]**. Then, mix 100 microliters of the previously mixed DNA with 100 microliters of transfection reagent **[2]** and leave it for 5 to 10 minutes on a rack **[3]**.

3.3.1. Talent mixing the transfection reagent and media.

3.3.2. Talent mixing the DNA with the transfection reagent.

3.3.3. Talent putting the tube on a rack.

3.4. Add 50 microliters of the DNA mix on top of each coverslip by inserting the tip just below the medium without touching the coverslips. Pipette slowly to avoid spreading the DNA mix **[1]**. *Videographer: This step is important!*

3.4.1. Talent adding the DNA mix to a coverslip.

3.5. Slowly bring the dish back to the incubator and leave it there for 30 to 45 minutes **[1]**. Then, flip the coverslips back to the home glia feeder dish with the wax dots side facing down **[2]** and put the dish back in the incubator **[3]**.

3.5.1. Talent putting the dish in the incubator and closing the door.

3.5.2. Talent flipping the coverslips into the home glia feeder dish.

3.5.3. Talent putting the dish in the incubator and closing the door.

4. Quantification of Axon Initial Segment

4.1. **[1]**.

~~4.1.1. Talent at the microscope setting up the Z-series.~~

4.2. **[2]**.

~~4.2.1. Talent adjusting the laser intensity.~~

~~4.2.2. Talent initiating imaging.~~

4.3. For AIS quantification, open the Z-series images in Fiji and generate a maximum projection of images **[1]**.

4.3.1. SCREEN: screen record.mov. 0:05 – 0:30.

4.4. After subtracting the empty coverslip background signal from the image, draw a line along the AIS, making sure that the width of the line fully covers the AIS. Start the line before the AIS signal is raised above the background and stop after it drops to the background **[1]**.

4.4.1. SCREEN: screen record.mov. 0:31 – 1:15. *Video Editor: Speed this up as needed.*

4.5. Measure the mean pixel intensity along the line and export it to a spreadsheet **[1]**. Then, run a MATLAB script to generate the final figure **[2]**.

4.5.1. SCREEN: screen record.mov. 1:16 – 1:35. *Video Editor: Speed this up as needed.*

4.5.2. SCREEN: screen record.mov. 3:35 – end. *Video Editor: Speed this up as needed.*

Results

5. Results: A Full Rescue of AIS by 480kDa AnkG in *ANK3*-E22/23-flox Neurons Transfected with Cre

5.1. The experiment should include Cre-BFP only transfection as negative control, Cre-BFP plus 480 kilodalton ankyrin-G co-transfection as a positive control, and a non-transfected condition as technique control **[1]**.

5.1.1. LAB MEDIA: Figure 4 A.

5.2. In the Cre-BFP only control, transfected neurons lack the accumulation of AIS markers, including ankyrin-G, beta4-spectrin, neurofascin, and voltage gated sodium channels **[1]**.

5.2.1. LAB MEDIA: Figure 4 A. *Video Editor: Emphasize the areas of the images within the dotted lines.*

5.3. In contrast, Cre and 480 kilodalton ankyrin-G co-transfected neurons have fully assembled AIS, which is demonstrated by the presence of AIS markers **[1]**.

5.3.1. LAB MEDIA: Figure 4 B.

5.4. It is important to confirm the quality of culture via comparison with the non-transfected dishes **[1]**. Unhealthy neurons tend to show abnormal AIS structure, like discontinued or ectopic AIS **[2]**.

5.4.1. LAB MEDIA: Figure 4 C.

5.4.2. LAB MEDIA: Figure 4 C. *Video Editor: Emphasize the area within the white circles and where the white arrows are pointing.*

5.5. To evaluate how an ankyrin-G human neurodevelopmental disorder mutation affects AIS assembly, the averaged AIS intensity was plotted from the soma to the distal axon **[1]**. AIS enriched protein normally shows a fast increase of signal from the proximal axon and a slow decrease of signal to the distal axon **[2]**.

5.5.1. LAB MEDIA: Figure 5.

5.5.2. LAB MEDIA: Figure 5, just the intensity curves.

5.6. When aligned with the non-transfected AIS, the mutant curve is wider and the peak of the curve is lower, suggesting a structure change of AIS **[1]**. The wild type ankyrin-G assembled AIS closely aligned with the non-transfected one **[2]**.

5.6.1. LAB MEDIA: Figure 5, just the bottom intensity curve (red and black). *Video Editor: Emphasize the red curve.*

5.6.2. LAB MEDIA: Figure 5, just the top intensity curve (green and black).

Video Editor: Authors opted out of all interview statements, so there is no conclusion.