Journal of Visualized Experiments

Use of primary cultured hippocampal neurons to study assembly of axon initial segments

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61411R1
Full Title:	Use of primary cultured hippocampal neurons to study assembly of axon initial segments
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Additional Information:	
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TITLE:

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

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KEYWORDS:

Giant ankyrin-G; Axon initial segment; Primary cultured neurons; Transfection; Imaging; AIS Intensity quantification

SUMMARY:

Here, we described a protocol to quantitatively study the assembly and structure of the axon initial segments (AIS) of hippocampal neurons that lack pre-assembled AIS due to the absence of a giant ankyrin-G.

ABSTRACT:

Neuronal axon initial segments (AIS) are sites of initiation of action potentials and have been extensively studied for their molecular structure, assembly and activity-dependent plasticity. Giant ankyrin-G, the master organizer of AIS, directly associates with membrane-spanning voltage gated sodium (VSVG) and potassium channels (KCNQ2/3), as well as 186 kDa neurofascin, a L1CAM cell adhesion molecule. Giant ankyrin-G also binds to and recruits cytoplasmic AIS molecules including beta-4-spectrin, and the microtubule-binding proteins, EB1/EB3 and Ndel1. Giant ankyrin-G is sufficient to rescue AIS formation in ankyrin-G deficient neurons. Ankyrin-G also includes a smaller 190 kDa isoform located at dendritic spines instead of the AIS, which is incapable of targeting to the AIS or rescuing the AIS in ankyrin-G-deficient neurons. Here, we described a protocol using cultured hippocampal neurons from *ANK3*-E22/23-flox mice, which, when transfected with Cre-BFP exhibit loss of all isoform of ankyrin-G and impair the formation of AIS. Combined a modified Banker glia/neuron co-culture system, we developed a method to transfect ankyrin-G null neurons with a 480 kDa ankyrin-G-GFP plasmid, which is sufficient to rescue the formation of AIS. We further employ a quantification method, developed by Salzer and colleagues to deal with variation in AIS distance from the neuronal cell bodies that occurs in hippocampal neuron cultures. This protocol allows quantitative studies of the de novo assembly and dynamic behavior of AIS.

INTRODUCTION:

The axon initial segment is located at the proximal axon in most vertebrate neurons. Functionally, AIS is where action potentials are initiated due to the high-density of voltage-gated sodium channels in this region. AIS of some excitatory neurons are also targeted by inhibitory interneurons through forming GABAergic synapses[1-3]. Therefore, AIS is a critical site to integrate cell signaling and modulate the excitability of neurons. AIS is normally 20-60 μm in length and located within 20 μm of the cell body. The length and position of AIS varies in neurons across brain regions, as well as in different developmental stages of the same neuron[4,5]. Accumulated evidence suggested that the composition and position of AIS are dynamic in responding to the change of neuronal activity[4-7].

480 kDa ankyrin-G is the master organizer of AIS. 480 kDa ankyrin-G is a membrane associated adaptor protein that directly binds to voltage gated sodium channels as well as other major AIS proteins including beta4-spectrin, KCNQ2/3 channels that modulate sodium channel activity[8,9], and 186 kDa neurofascin, a L1CAM that directs GABAergic synapses to the AIS[2,10]. 480 kDa ankyrin-G shares canonical ankyrin domains found in the short 190 kDa ankyrin-G isoform (ANK repeats, spectrin binding domain, regulatory domain), but are distinguished by a giant exon that is found only in vertebrates and is specifically expressed in neurons (**Figure 1A**)[11,12]. The 480 kDa ankyrin-G neuron specific domain (NSD) is required for AIS formation[12]. The 190 kDa ankyrin-G does not promote AIS assembly or target AIS in ankyrin-G-null neurons[12]. However, 190 kDa ankyrin-G is concentrated at the AIS containing 480 kDa ankyrin-G[12]. This ability of the 190 kDa ankyrin-G to target pre-assembled AIS of wildtype neurons has been a source of confusion in the literature and has slowed appreciation of the critical specialized functions of the 480 kDa ankyrin-G in AIS assembly. Therefore, it is critical to study AIS assembly in ankyrin-G-null neurons that lack a pre-assembled AIS.

Here, we present a method to study the assembly and structure of the AIS using cultured hippocampal neurons from *ANK3*-E22/23-flox mice that eliminates all isoforms of ankyrin-G[13] (**Figure 1B**). By transfecting neurons with a Cre-BFP construct before AIS is assembled, we generated ankyrin-G-deficient neurons completely lacking an AIS (**Figure 1B, Figure 2**). The assembly of AIS is fully rescued following co-transfection of 480 kDa ankyrin-G-GFP plasmid with a Cre-BFP plasmid. This method provides a way to study the AIS assembly in a non-pre-assembled AIS environment. We also modified the glia-neuron co-culture system from Gary Banker without using antibiotics, previously designed for embryonic day 18 neurons, for application to postnatal mouse neurons and adapted a AIS quantitation method to average AIS measurements from multiple neurons to normalize the variation of AIS[14,15].

PROTOCOL:

NOTE: This culture method of hippocampal neurons from postnatal 0-day *ANK3*-E22/23$^{f/f}$ mice is adapted from Gary Banker's glia/neuron co-culture system Therefore, it is critical to perform all steps after dissection in a clean hood using sterilized tools. This protocol takes up to 1 month. The workflow is displayed in **Figure 3**. The protocol follows the animal guidelines of Duke University.

1. Preparing of coverslips and neuronal plating dishes

1.1 At least one week before culture day, load coverslips on coverslip rack and soak it in nitric acid (70% W/W) overnight (could be extended for days).

1.2 Wash nitric acid treated coverslips with distilled water on a low speed shaker in a glass jar 2 times, 1 hour each.

1.3 Incubate coverslips in saturated KOH dissolved in 100% ethanol overnight. Add KOH into ethanol till it is no longer dissolved.

1.4 Repeat the wash step with distilled water. Rinse coverslips with 100% ethanol once for 10 minutes.

1.5 Transfer coverslips from the rack to a glass beaker. Cover the beaker with aluminum foil. Bake coverslips in a 225 °C oven overnight to sterilize the coverslips. (Coverslips could be stored in the beaker for weeks).

1.6 Place coverslips in a Petri dish and then apply 3-4 wax dots on the coverslip to serve as feet. Use a Pasteur pipette to dip into boiled wax in a glass bottle. Then quickly touch the coverslip to create a dot. A 60 mm Petri dish can hold 4 coverslips. A 10 mm Petri dish can hold ~10 coverslips.

1.7 2 days before culture day, coat coverslips (the side with the wax dots) with filter sterilized 1 mg/mL poly-L-lysine in 0.1 M boric acid (pH 8.5) for a minimum of 6 hours and rinse with water 2 times, 1 hour each time. Coverslips remain in the same Petri dish.

1.8 Add plating medium (MEM supplemented with glucose 0.6% (wt/vol) and 10% (vol/vol) horse serum) to plates slowly without disturbing coverslips. Put plates in the incubator till the culture day to seed neurons.

2. Preparing glia cell feeder dishes (2 weeks before culture day)

2.1 Dissect the cortex from a postnatal 1-day old mice brain and peel off the meninges.

2.2 Chop the cortex tissue as finely as possible with a clean scissors in a clean Petri dish in a clean bench.

2.3 Transfer the chopped tissue into 12 mL of HBSS and add 1.5 mL of 2.5% trypsin and 1% (wt/vol) DNase. Incubate in a 37 °C water bath for 15 min, swing every 5 min. Well digested tissue become sticky and form a big cluster. Triturate 10-15 times with a 10 mL pipette to break the tissue down and get better digestion.

2.4 Triturate the well digested tissue 10-15 times with a 5 mL pipette till most chunks disappear and the medium turns to cloudy. Pass through a cell strainer to remove remaining chunks and add 15 mL of glia medium (Minimal essential medium (MEM) supplemented with glucose (0.6% wt/vol), 10% (vol/vol) horse serum and Penicillin-Streptomycin (1x) to stop the digestion.

2.5 Centrifuge the cells at 120 x *g* for 5 minutes and aspirate the supernatant. Resuspend the cell pellet with fresh glia medium and seed in cell culture dishes (about 10^5 cells/cm^2).

2.6 Replace medium with fresh glia medium the next day to remove unattached cells.

2.7 Feed the glia dishes every 3-4 days with fresh glia medium. Slap the flask 5-10 times with a hand to dislodge loosely attached cells before changing medium.

2.8 After 10 days of culture, glia cells should be nearly confluent. Detach glia cells with 0.25% trypsin-EDTA and seed about 10^5 cells in a new 60 mm cell culture dish. Remaining cells could be frozen for future use.

2.9 3 days before the culture day, change the glia medium to neuronal culture medium (Neurobasal-A Medium with 1x GlutaMAX-I and 1x B27 supplement).

3. Culture hippocampal neurons

NOTE: All steps are performed at room temperature.

3.1 Dissect 6-8 hippocampi from postnatal 1-day old pups from *ANK3*-E22/23$^{f/f}$ mice with HBSS medium in a Petri dish at room temperate. Chop the hippocampi with dissection scissors to smaller pieces. Transfer hippocampi from the Petri dish to a 15 mL tube.

3.2 Wash hippocampi 2x with 5 mL of HBSS in the tube. Leave the hippocampi in 4.5 mL of 1x HBSS after wash.

3.3 Add 0.5 mL of 2.5% trypsin into 4.5 mL of HBSS and incubate in a 37 °C water bath for 15 minutes. Invert the tube every 5 minutes. Well digested hippocampi should become sticky and form a cluster. If needed, extend the digestion for 5 more minutes.

3.4 Wash hippocampi with HBSS 3 times for 5 minutes each. Do **not** use a vacuum to remove the HBSS. It is very easy to remove the hippocampi.

3.5 Add 2 mL of HBSS after the wash and pipette the hippocampi up and down with a Pasteur pipette 15 times.

3.6 Triturate the tissue with a fire-polished Pasteur pipette (the diameter of the open is narrowed by half) 10 times. Do **not** go beyond 10 times even if there are still chunks remaining.

Overshearing kills neurons.

3.7 Rest the tube for 5 minutes till all chunks set to the bottom. Gently use a 1 mL pipette tip to transfer the supernatant containing the dissociated neurons to plating dishes (10^5 cells/60 mm dish). Add it directly to the pre-incubated plating medium and shake the plate gently.

3.8 Repeat step 3.6-3.7 with the remaining chunks till most of the chunks have disappeared.

3.9 2-4 hours after seeding, check the plating dishes with a light microscope. The majority of neurons should have attached to the coverslip. Attached cells are round and bright. Flip coverslips using a fine tip forceps to the glia cell feeder dishes with preconditioned neuronal culture medium with the wax dots side facing downwards.

3.10 Neurons can grow in the glia cell feeder dishes for up to 1 month. Feed neurons every 7 days with 1 mL of fresh neuronal culture medium.

3.11 Optional step: 1 week after seeding, add cytosine arabinoside (1-β-D-arabinofuranosylcytosine) to a final concentration of 5 μM to curb glial proliferation.

4. Disruption of AIS by Knockout of Ankyrin-G at earlier stage of neuron development

4.1 On 3 div (day *in vitro*), flip the coverslips with wax dots side facing up to a glia cell feeder dish with conditioned neuronal culture medium.

4.2 Mix 0.25 μg of Cre-BFP DNA with 0.5 μg of ankyrin-G-GFP (WT/mutant) DNA in a 1.7 mL tube to transfect 4 coverslips (~ 2:1 ratio of DNA copy number). Add 100 μL of culture medium (e.g., Opti-MEM), mix and rest on a rack. If only Cre-BFP is transfected, the GFP plasmid backbone is used to match the total amount of DNA.

4.3 Mix 3 μL of transfection reagent (e.g., Lipofectamine 2000) (~ 3 times of DNA) with 100 μL culture medium in a new 1.7 mL tube. Incubate for 5 minutes at RT.

4.4 Mix 100 μL of DNA solution from step 4.2 with 100 μL of transfection reagent from step 4.3. Rest for 5-10 minutes on a rack.

4.5 Add 50 μL of DNA mix from step 4.4 right on top of each coverslip by inserting the tip just below the medium without touching the coverslips. Pipette slowly to avoid spreading of DNA mix.

4.6 Slowly bring the dish back to the incubator and incubate for 30-45 minutes.

4.7 Flip the coverslips back to the home glia feeder dish with wax dots side facing down and put the plate back to the incubator.

5. Quantification of axon initial segment

5.1 Fix neurons on 7-10 div and stain with AIS marker following the standard immunocytochemistry protocol for the protein of interesting.

5.2 Collect fluorescent pictures with desired microscopy.

5.2.1. Take Z-series sections to collect the signal of the entire AIS. Keep the same Z-depth for all pictures.

5.2.2. Adjust the laser intensity to reach the best pixel intensity dynamic range.

5.2.3. Make sure all AIS pictures are taken on the same microscope setup.

5.2.4. Always check the signal of Cre-BFP.

5.3 AIS quantification

5.3.1. Open picture with Fiji (https://fiji.sc).

5.3.2. Generate maximum projection of Z-series images.

5.3.3. Subtract the empty coverslip background signal from the image.

5.3.4. Draw a line along the AIS. The width of the line should fully cover the AIS. Start the line before the AIS signal is raised above the background and stop after it drops to the background.

5.3.5. Measure the mean pixel intensity alone the line and export to a spreadsheet (~10-15 AISs are needed).

5.3.6. Generate the average intensity curve of AIS using the MATLAB script adapted from Berger et al.[15].

5.3.7. For each experiment, include Cre only and Cre plus wildtype 480 kDa ankyrin-G transfected neurons as negative and positive controls to make sure that the knockout of AIS is efficiency and the rescue is successful.

REPRESENTATIVE RESULTS:

A complete set of experiment should include Cre-BFP only transfection as negative control, Cre-BFP plus 480 kDa ankyrin-G co-transfection as positive control and a non-transfected condition as technique control. In Cre-BFP only control, transfected neurons lack the accumulation of AIS markers, including ankyrin-G (ankG), beta4-spectrin (β4), neurofascin (Nf) and voltage gated sodium channels (VSVG) (**Figure 4A**)[16]. In contrast, Cre and 480 kDa ankyrin-G co-transfected neurons have fully assembled AIS revealed by the present of AIS markers (**Figure 4B**). It is

important to confirm the quality of culture by comparing with the non-transfected dishes. Unhealthy neurons tend to show abnormal AIS structure, like discontinued or ectopic AIS (**Figure 4C**).

Then we showed an example of evaluating how an ankyrin-G human neurodevelopmental disorder mutation (ankG-K2864N) affects AIS assembly (**Figure 5**). 3 div *ANK3*-E22/23$^{f/f}$ neurons were transfected with Cre-BFP and wildtype 480 kDa ankyrin-G (ankG-WT) or 480 kDa ankyrin-G baring human mutation (ankG-K2864). Neurons were fixed at div7 and stained for ankyrin-G. Images were collected from 10-15 transfected neurons and 10-15 control neurons on the same coverslips and processed with maximum intensity projection. Then we draw a line at the AIS as shown and measure the mean intensity across the line. After averaging the AIS intensity, we plot the AIS intensity from the soma to the distal axon. AIS enriched protein normally showed a fast increase of signal from the proximal axon and a slow decrease of signal to the distal axon. AIS assembled by ankyrin-G with human mutant showed an increase and decrease of signal. But when aligned with the non-transfected AIS, the mutant curve is wider, and peak of the curve is lower suggesting a structure change of AIS. The wild type ankyrin-G assembled AIS closely aligned with the non-transfected one.

FIGURE AND TABLE LEGENDS:

Figure 1. The genomic editing of *ANK3*-E22/23-flox. (A) Schematic representation of protein domains for 3 ankyrin-G isoforms. The location of exon 22 and 23 encoded regions in canonical domain is pointed by the dash line. **(B)** The position of LoxP sites in *ANK3*-E22/23-flox mice is indicated by triangle. In the present of Cre recombinase, exon 22 and 23 is deleted and causes loss the expression of all 3 isoforms of ankyrin-G.

Figure 2. Loss of AIS in *ANK3*-E22/23-flox neurons in the present of Cre recombinase. A diagram shows the time frame of ankyrin-G expression and AIS assembly in wild type neurons versus in *ANK3*-E22/23$^{f/f}$ neurons with Cre transfection at 3 div.

Figure 3. Workflow of protocol.

Figure 4. A full rescue of AIS by 480kDa AnkG in *ANK3*-E22/23-flox neurons transfected with Cre. 3 div neurons of *ANK3*-E22/23$^{f/f}$ mice were transfected with Cre-BFP **(A)** or with a Cre-BFP and wild type 480 kDa ankyrin-G-GFP **(B)**. Neurons were fixed at 7 div and stained for ankyrin-G (ankG), β4-spectrin (β4), neurofascin (Nf) and voltage gated sodium channels (VSVG). White arrow head points to the AIS of a transfected neuron. Scale bar is 20 μm. This figure was adapted from Yang et al [16]. **(C)** Two unhealthy neurons transfected with tdTM and 480 kDa ankyrin-G-GFP were shown. The formation of aggregates (circled in white and enlarged) is a sign of unhealthy neurons. Top: 480 kDa ankyrin-G shows up at the non-AIS region (pointed by white arrow heads. Bottom: Neuron formed 3 AIS and ectopic accumulation of ankyrin-G on soma. Scale bar is 20 μm.

Figure 5. Quantification of AIS structural change. div3 neurons of *ANK3*-E22/23$^{f/f}$ mice were transfected with Cre-BFP and wild type 480 kDa ankyrin-G or 480 kDa ankyrin-G-K2864N. At 7

div, neurons were fixed and stained for ankyrin-G. Representative images show the ankyrin-G signal at the AIS. The green line and yellow line indicate where the line for AIS intensity measurement was drew. White dash line circled the cell body of the transfected neuron. Scale bar is 20 μm. Average AIS intensity for both conditions is plotted alone distance and aligned with non-transfected cells (n=10).

DISCUSSION:

The assembly of AIS is organized by 480 kDa ankyrin-G. However, ankyrin-G has shorter isoforms that can target to the AIS of wildtype neurons, which may lead to difficulty in interpretation of structure-function analyses of AIS assembly. Here we present a method using neurons from *ANK3*-E22/23-flox mice that allows study of de novo assembly of the AIS. By transfecting with Cre-BFP at 3 div, we eliminate the all endogenous isoforms of ankyrin-G. We could also co-transfect 480 kDa ankyrin-G to rescue the formation of AIS. This allows study of AIS formation in a clean system. By further adopting the Banker culture system which improves viability without complications of glial cell over-growth, we could reach a high transfection efficiency, which provide us enough neurons for quantitative measurement of AIS dimensions.

There are several critical steps in this protocol. The first critical step is considering the best time window to do the transfection, which needs to be early enough to prevent the assembly of AIS and late enough to reach the highest transfection efficiency. We tried 0 div electroporation transfection, which gave about 10% transfection efficiency with Cre-BFP only but we were never able to transfect 480 kDa Ankyrin-G at 0 div. We suspect it is due to the large size of the plasmid (about 20 kb). Primary cultured hippocampal neurons have a narrow window for transfection, which is between 3-5 days. The accumulation of ankyrin-G at the AIS starts from 3 div. When we transfect Cre-BFP at 3 div, no AIS formation was seen in transfected neurons (**Figure 4A**). We could get 10-20 neurons transfected with 480 kDa ankyrin-G from one 18 mm coverslip. Also, for the co-transfection rescue experiment, all DNA must be generated under the same promoter and the ratio of Cre-BFP and 480 kDa ankyrin-G-GFP must be matched. In this experiment, we used chicken beta-actin promoter.

Another critical step is the modification to the Banker culture. The Banker culture was developed for culturing embryonic rat neurons. To better support the more sensitive mouse postnatal hippocampal neuron, we include a step of chopping hippocampi into smaller pieces to improve the trypsinization efficiency. Adding KOH treatment after the nitric acid treatment further reduced the toxicity from the glass coverslips, which help neurons attach and grow better.

A remaining challenge is how to control the expression level of ankyrin-G. A dosage screen helped to determine the optimal amount of plasmid used for transfection. Going forward, it is better to use a neuron-specific promoter to control the level of expression. The current data analysis did not measure the position of AIS. This function should be included in the future.

ACKNOWLEDGMENTS:

We thank Dr. Gary Banker for suggestion on neuronal culture protocol. This work is supported by the Howard Hughes Medical Institute, a grant from NIH, and a George Barth Geller endowed

professorship (V.B.).

DISCLOSURES:

The authors have nothing to disclose.

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Fig1

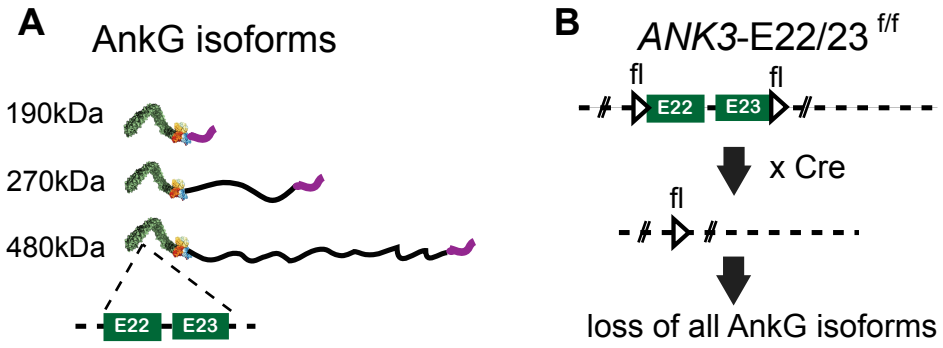


Fig2

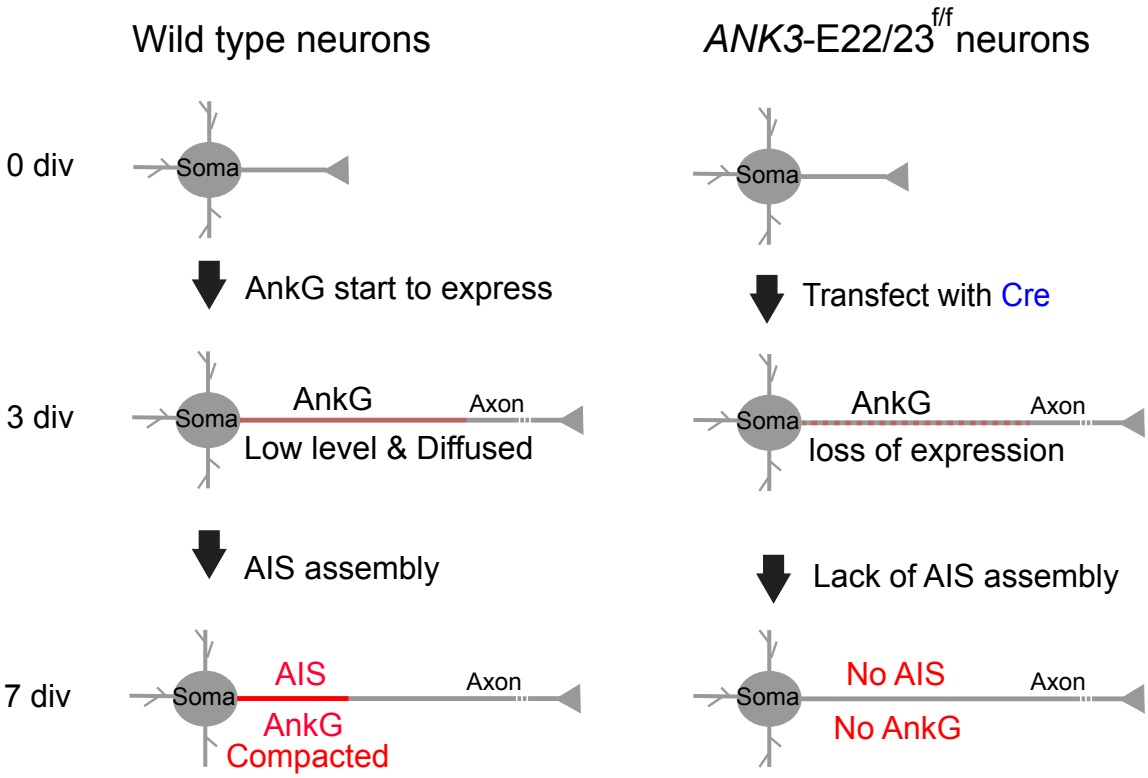


Fig3

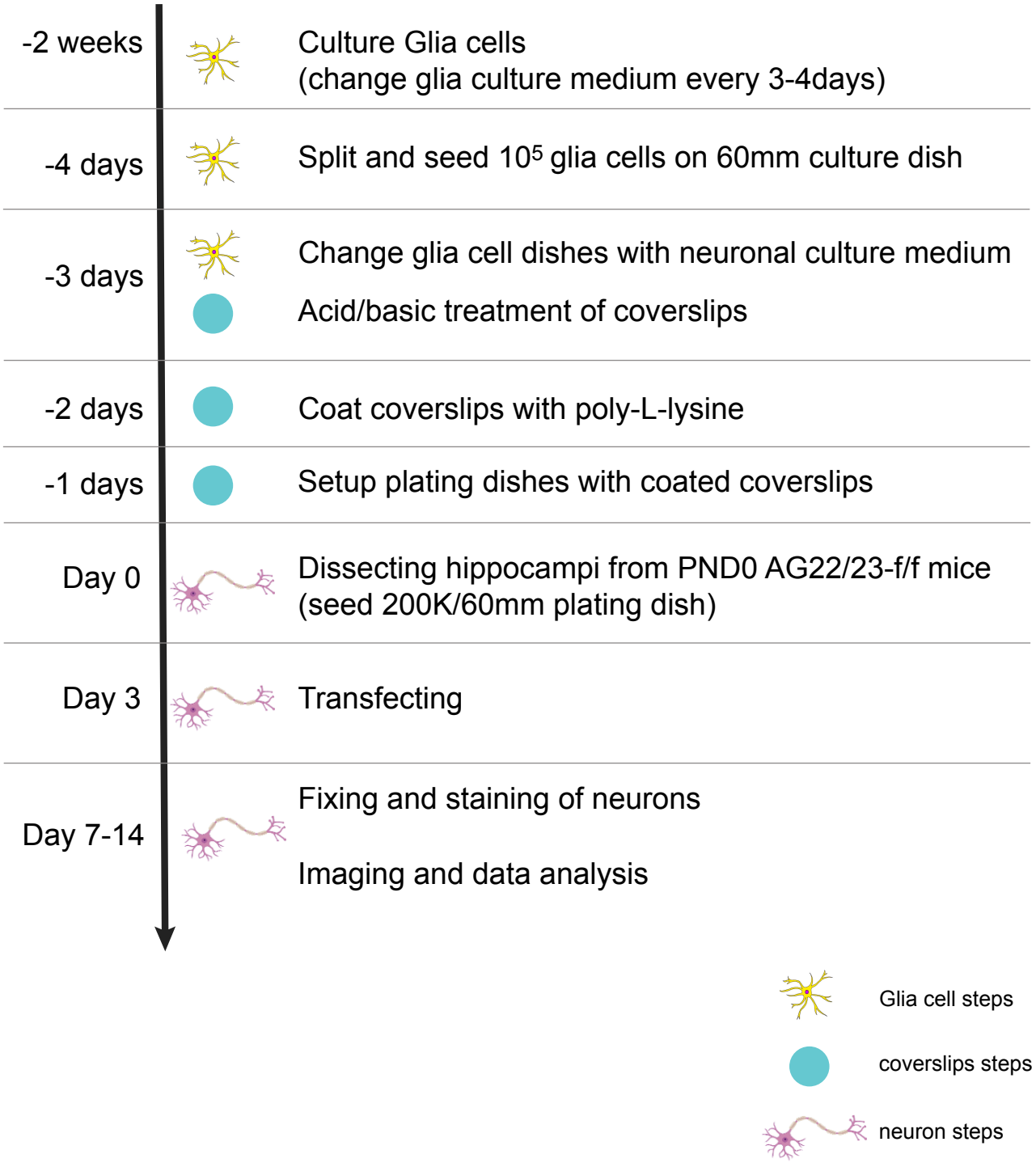


Fig4

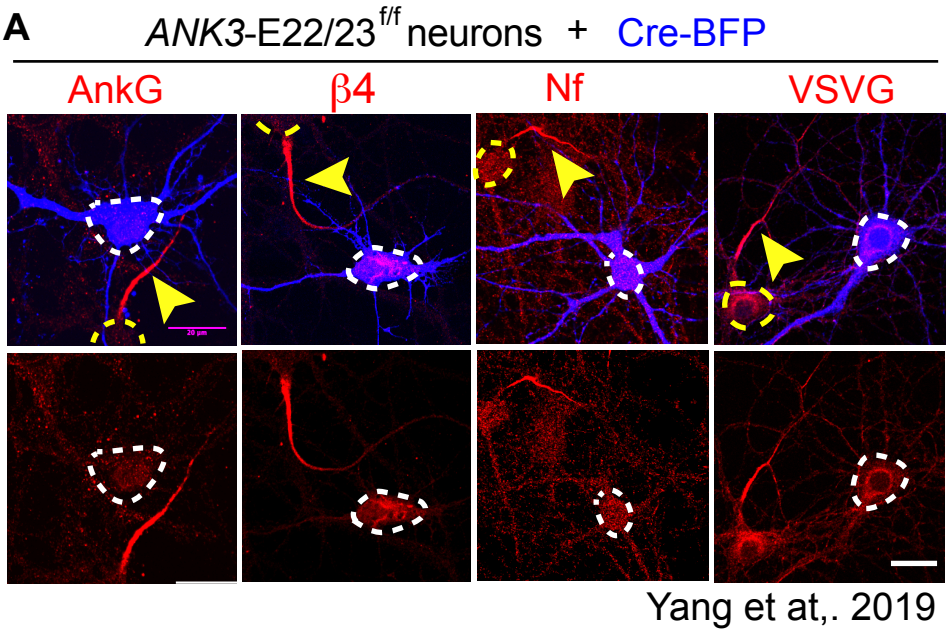
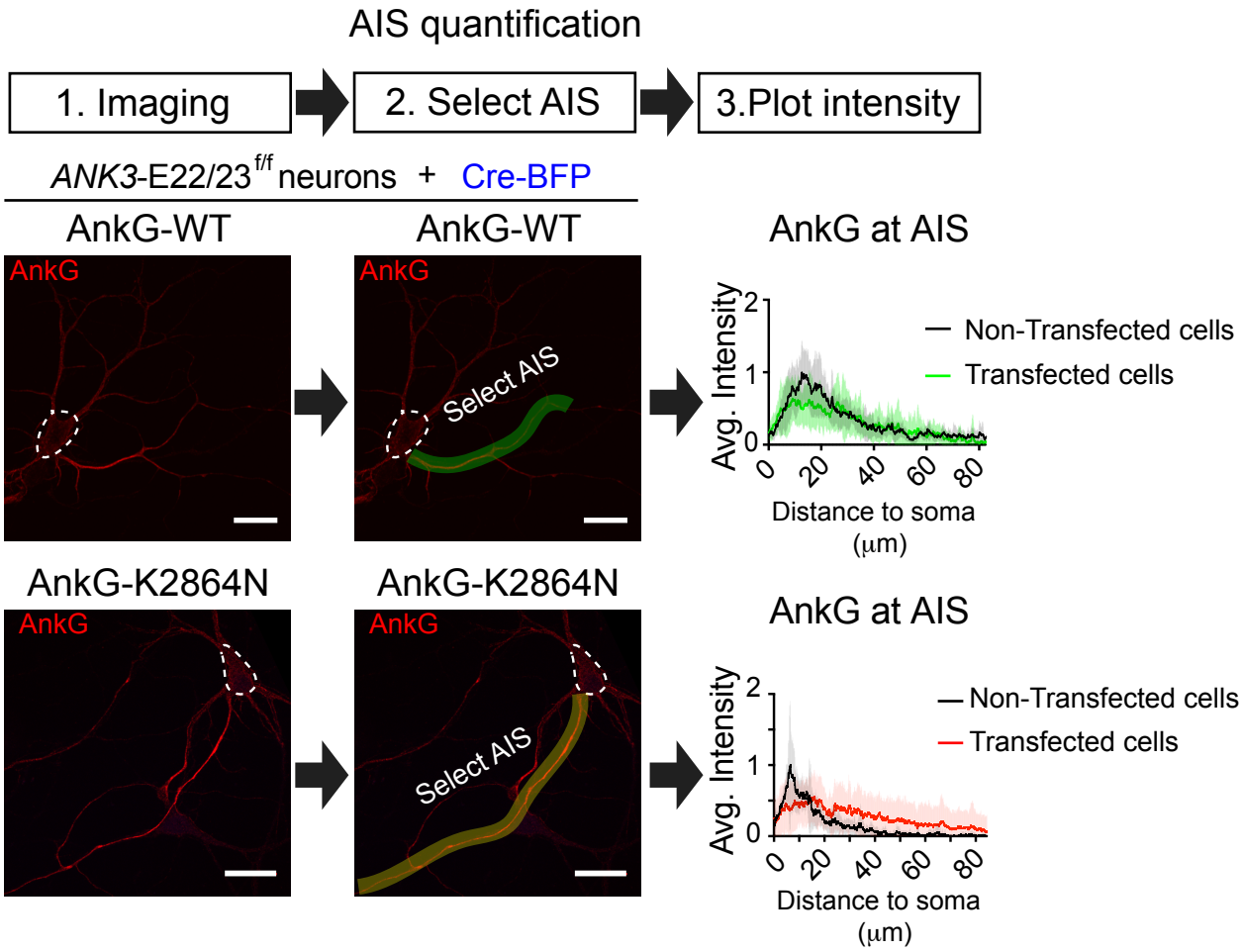


Fig5



Name of Material/Equipment	Company	Catalog Number	Comments/Description
10xHBSS	Thermo Fisher Scientific	14065-056	
18mm coverglass (1.5D)	Fisher Scientific	12-545-84-1D	
190kDa ankyrin-G-GFP	Addgene	#31059	
2.5% Tripsin without phenol red	Thermo Fisher Scientific	14065-056	
480kDa ankyrin-G-GFP	lab made	Provide upon request	
ANK3-E22/23f/f mice	JAX	Stock No: 029797	B6.129-Ank3tm2.1Bnt/J;
B27 serum-free supplement	Thermo Fisher Scientific	A3582801	
Boric acid	Sigma-Aldrich	B6768	
Cell strainer with 70-mm mesh	BD Biosciences	352350	
Ceramic coverslip-staining rack	Thomas Scientific	8542E40	
Cre-BFP	Addgene	#128174	
D-Glucose	Sigma-Aldrich	G7021	
DMEM	Thermo Fisher Scientific	11995073	
GlutaMAX-I supplement	Thermo Fisher Scientific	A1286001	
Lipofectamine 2000	Thermo Fisher Scientific	11668030	
MEM with Earle’s salts and L-glutamine	Thermo Fisher Scientific	11095-080	
Neurobasal Medium	Thermo Fisher Scientific	21103-049	
Nitric acid 70%	Sigma-Aldrich	225711	
Opti-MEM I Reduced Serum Medium	Thermo Fisher Scientific	31985062	
Paraformaldehyde	Sigma-Aldrich	P6148	
Penicillin-streptomycin	Thermo Fisher Scientific	15140122	

Poly-L-lysine hydrochloride	Sigma-Aldrich	26124-78-7
Potassium hydroxide	Sigma-Aldrich	1310-58-3

Dear Dr. Bajaj,

Thank you for sending me the comments. I have gone through all comments and edited the manuscript/figures as required. All of my answers were listed right after each comment and is in blue. Here is one change on the authorship, which was agreed by all listed authors. Rui Yang will be listed as the corresponding author. Vann Bennett will be co-author.

Best regards,
Rui

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The goal of this manuscript is to provide a protocol to culture primary rodent neurons and following immunocytochemistry provide some guidelines on how to quantify the fluorescence intensity of the axon initial segment Ankyrin G.

Major Concerns:
The manuscript does not describe any new protocol that cannot be found in other publications. Both the protocol to culture primary neurons and to quantify immunofluorescence at the axon initial segment have been previously published in books or papers. The primary neuronal culture procedure can be found in much greater details in Culturing Nerve Cells Edited by Gary Banker and Kimberly Goslin, ISBN: 9780262267670 | 678 pp. | August 1998. Likewise, a much more detailed and rigorous analysis of immunofluorescence of markers at the axon initial segment can be found in Imaging of the Axon Initial Segment.
Di Re J, Kayasandik C, Botello-Lins G, Labate D, Laezza F.Curr Protoc Neurosci. 2019 Sep;89(1):e78. doi: 10.1002/cpns.78. PMID: 31532918.

Minor Concerns:
none

Reviewer #2:

Manuscript Summary:
In this manuscript, Yang and Bennett described a highly valuable culture technique, allowing to conditionally knockout a certain gene for cell biological studies. Ankyrin-G plays a key role in organizing the axon initial segment (AIS) of neurons by directly binding to ion channels, cell adhesion molecules and even microtubule-binding proteins. The authors started with primary culture using Ankyrin-G floxed mouse pups based on the Banker culture strategy. When being transfected with Cre-BFP, Ankyrin-G was effectively deleted, resulting in disrupted AIS targeting of other proteins. This disruption can be rescued by co-transfection of Ankyrin-G-GFP constructs. Overall, the manuscript is well written and describes a timely method. This technique and strategy will be very useful to the research community in cell biological study of neurons.

Major Concerns:
None.

Minor Concerns:
1) To further polish the manuscript, it will benefit from some in-house editing.
In line 23, "EB 1 and 3" should be "EB1/EB3".
I have corrected this in the text.
For example, in line 98, "12ml" versus "1.5 ml". Make it consistent using a space between the number and unit.

I have corrected this through the manuscript.
In line 119, "Jackson's Lab" should be "The Jackson Laboratory" or "JAX".
I have corrected this in the text.
In line 158, to "slowly bring the dish back to the incubator and incubate for 30-45 minutes".
The last paragraph, lines 275-278, needs more attention.
I have edited this paragraph.

2) A minor suggestion regarding line drawing along the AIS for measurement. In lines 204-205 and Fig. 5, I would suggest to use the maximal intensity instead of the average intensity. This is because the average intensity includes the intensity outside of AIS in the background, which is dependent upon the line width of each measurement and likely leads to inconsistent results between experiments.
I agree. I have tried both maximal or average intensity to do the quantification. Although average have the potential to dilute the thin part of the AIS with the background outside of AIS. It generates a much smoother curve compare to the maximal intensity curve, which actually reduced the variations between AISs. More importantly, the shape of curves is very similar and represent the intensity of AIS very well.

3) In Fig. 4A, it would be clearer to have arrows pointing to the axons from Cre-BFP-transfected neurons.
In this particular experiment, I didn't stain the neurons with any axonal/dendritic marker. Therefore, it is hard to tell where the axon is. But I agree that this will be a clearer way to present the result in the future.

Reviewer #3:
Manuscript Summary:
The authors describe here a method to assess the role of AnkyrinG in the formation and maintenance of the axon initial segment.
The transfection of Cre in ANK3-E22/23fl/fl mice allows to get rid of all AnkyrinG isoforms in neurons which induce a loss of AIS components. The co-expression of 480kDa-AnkyrinG is used to rescue the AIS formation, whereas the use of AnkyrinG mutants makes it possible to assess the precise role of AnkyrinG in the assembly of the AIS.

This is an interesting approach since AnkyrinG plays such a central role in the assembly of the AIS. It is hard to target all AnkyrinG isoforms with a good efficiency and considering the size of AnkG, the over-expression is also difficult. The method described here allows to overcome these technical challenges.
In general, the writing/annotations should be more consistent throughout the protocol and some technical steps could be better described, see my detailed comments below.

Major Concerns:
One aspect raised by the authors is the variability of AIS length and position across neurons. I do agree with this statement, and it is not clear to me how the proposed approach efficiently allows "to distinguish the real change of AIS structure from normal variations" as stated line 243. Could the authors make it more clear?
I guess what I wanted to say is the variation of AIS need to be normalized before making any conclusion. I have changed the statement to avoid the confusing. Our protocol averaged AIS intensity of multiple neurons to reduce the variation. In addition, we proposed to always include Cre only and a wildtype giant ankyrin-G rescue as technique control to minimize the technique variation.

Another important point raised by the authors is the use of over-expression for rescue experiments. In this respect, they indicate that they screened for the optimal amount of DNA to transfect. Indeed, I think that this is a very important point. Could the authors show some examples in which expression was too high, to help the readers picking the right amount of DNA for their own experiments? Maybe some internal controls could be used as well.

I did a gradient dilution of wild type 480kDa-ankyrinG DNA used in an AIS rescue experiment. I will quantify the AIS of transfected neurons and compare it with the AIS of non-transfected neurons on the same coverslip. The good amount of DNA gives an AIS curve that aligned with the AIS curve from neighboring non-transfected neurons.

The authors show a graph in Figure 4 with intensity profiles of AnkG in case of over-expression and in non-transfected cells. Are the levels different? Could this measure be a reliable way to check for good expression level and integrated as a control into the protocol they are describing?
Yes, what we shown in Figure 4 is how we monitor the expression level. Always compare it with the non-transfected neurons on the same coverslip. I described this in method clearer.

Along the same line, the plasmids used here should be better described (and annotated more consistently), since as mentioned by the authors, the choice of the promoter is crucial.
I have added the promoter information in line 277-279.

In general, it is not clear in which type of cell culture wells/dishes/flasks/coverslips neurons and glial cells are seeded. It should be better described in the protocol and maybe could be added on the workflow in the Figure3.
I have added the information in the table of materials.

Minor Concerns:
In general, the authors should keep their writing consistent, in the tense used to describe the steps, but also in the way to write concentration, amount, time, constructs… and throughout the figures.
I have corrected the format.

The consequences of AnkG WT or mutants on the AIS formation is assessed in neurons co-transfected with Cre-BFP and AnkG-GFP. Is the co-transfection efficient?
This is a very important point. To make sure Cre is co-transfected with AnkG-GFP, I used 2 times of Cre-GFP in transfection. Plus Cre-BFP is much smaller than ankyrin-G, which may be easier for the cell to uptake. In our experience, Cre-BFP is always expressed in the ankyrin-G transfected cells. In addition, we always take pictures from blue channel to be sure Cre-BFP is present.

It is stated in the abstract that the 190kDa AnkG isoforms cannot rescue the AIS. For the clarity of the approach I would also state that the 480kDa is sufficient to rescue AIS formation.
I have added this statement in the abstract (Line30). Thanks for the suggestion!

The neurons are cultured in antibiotic-free medium, which is very burdensome. Is there evidence that it increases the efficacy of the method described here?
I didn't compare the transfection efficiency side. This decision is made based on the experience. With antibiotics, the neuron health condition is worse than the antibiotics free one, which leads to a lower transfection efficiency.

Line 77: "di water" stands for deionized or distilled water?
It is for distill water. I have clarified it in the text.

Line 79: the concentration of KOH is not stated, is it important?
It is saturated KOH. We normally just add KOH to ethanol till it is no longer dissolving. (updated this in Line 80)

Step 1.8: Where are the coverslips + plating medium stored until the culture day?
I have updated the storage place in Line 94.

Step 2.6: How long is the centrifugation step?

I have updated the time in line 108.

Steps 2.6 and 2.8: Where are the glial cells seeded? In cell culture dishes? In flasks? What is the procedure used to passage glia?
I have updated the information in line 113.

Step 2.9: what is the concentration of GlutaMAX-I and B27 supplements in the neuron culture medium?
I have updated the information in line 117.

Step 3.8: The plating is done in the 60mm plating dishes? Or on the coverslips? Or are the coverslips in the dish? It is directly to the plating dishes.
I assume that 'Repeating the 3.6-3.7' is only with the bottom part still containing chunks of tissue? Yes. I have adjusted the text to clarify this.

Step 4.2: Cre DNA is the Cre-BFP construct? What if no AnkG construct is added, then only 0.25ug of DNA is transfected?
Yes. In a parallel experiment, if Cre-BFP is used as control, I will match the copy of AnkG with an empty backbone to composite. I have added a sentence in Line 157.

Step 4.5: 50uL of transfection mix is added PER coverslip? Yes.

Line 182: the authors write that they use a MATLAB script adapted from Berger et al, if there are modifications done, it is important to describe which one and provide the script. I didn't modify the MATLAB script except the number of neurons and channels, which varied from one experiment to another.

Line 184: It is not clear what is important to compare.
I have made it clearer in the text. Line 195.

Line 197: Is it a very interesting observation. Could the authors provide a picture to help identifying unhealthy neurons with damaged AIS structure?
This is a good suggestion. I included two pictures of AIS form unhealthy neurons in Fig4C.

Figure1: What is the color code? I strongly recommend changing the colors for color-blind friendly ones. The way Exons 22-23 are shown is misleading since it seems that they are only found in the 480kDa isoform. I do not think that indicating Exon 37 is necessary.
I have modified Fig 1 based on your suggestion.

Figure2: The label "AG22/23-fl/fl" is not consistent with the other figures and text. Would it be possible to add a third column showing the rescue condition to match with what is shown in Figure 4 ?
I have changed it. But I didn't see how adding a third column can improve the figure. Because the rescue experiment could be designed in different ways depends on the question.

Figure3: it seems that there is a typo in 'palting dishes'
Corrected! Thank you!

Figure4: the legend for Figure4A is missing. Writing 'AG-GFP' is not consistent with the text.
It is on line 233.

The material/equipment list should be more detailed. I could not find for example the type of coverslips used, the reference to the mouse line, the origin of plasmids,… Penicillin/streptomycin is in the list but not mentioned in the text.

Reviewer #4:
Manuscript Summary:
The manuscript summarizes different protocols used to study hippocampal neurons and axon initial segment, and establishes some modifications to get better cultures from postnatal hippocampal neurons. In summary, the manuscript describes in a simple way all the steps to manage the analysis of AIS proteins density and AIS plasticity, and supplies an example knocking down ankyrinG.

Major Concerns:
There are no major concerns.

Minor Concerns:
1) There are a couple of typographic errors. alone instead of along, ...
Thank you for the commands. I have look through and correct the miss spellings.

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