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Measuring Properties of the Membrane Periodic Skeleton of the Axon Initial Segment using 3D-Structured Illumination Microscopy (3D-SIM)

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TITLE:

Measuring Properties of the Membrane Periodic Skeleton of the Axon Initial Segment using 3D-Structured Illumination Microscopy (3D-SIM)

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SUMMARY:

The present protocol describes a method to visualize and measure actin rings and other components of the membrane periodic skeleton of the axon initial segment using cultured rat hippocampal neurons and 3D-structured illumination microscopy (3D-SIM).

ABSTRACT:

The axon initial segment (AIS) is the site at which action potentials initiate and constitutes a transport filter and diffusion barrier that contribute to the maintenance of neuronal polarity by sorting somato-dendritic cargo. A membrane periodic skeleton (MPS) comprising periodic actin rings provides a scaffold for anchoring various AIS proteins, including structural proteins and different ion channels. Although recent proteomic approaches have identified a considerable number of novel AIS components, details of the structure of the MPS and the roles of its individual components are lacking. The distance between individual actin rings in the MPS (~190 nm) necessitates the employment of super-resolution microscopy techniques to resolve the structural details of the MPS. This protocol describes a method for cultured rat hippocampal neurons to examine the precise localization of an AIS protein in the MPS relative to sub-membranous actin rings using 3D-structured illumination microscopy (3D-SIM). In addition, an analytical approach to quantitively assess the periodicity of individual components and their position relative to actin rings is also described.

INTRODUCTION:

The axon initial segment (AIS) is a short, uniquely specialized region of the proximal axon of vertebrate neurons[1]. The AIS comprises a transport filter and diffusion barrier essential in maintaining neuronal polarity by sorting somato-dendritic cargo[2-7]. In addition, the unique

structure of the AIS allows it to accommodate clusters of voltage-gated ion channels that facilitate its function as the site of action potential initiation[8]. A highly stable structural complex underlies the unique functions of the AIS. Research within the last decade has revealed the presence of a membrane periodic skeleton (MPS) containing actin rings connected by spectrin and providing a scaffold for anchoring various AIS proteins[9,10].

The distance between actin rings in the MPS (~190 nm)[9,10] is under the resolution limit of conventional light microscopy. Early attempts to use electron microscopy to visualize the MPS were not successful, as the harsh preparation procedures involved failed to preserve the structure of the MPS. Thus, super-resolution microscopy techniques have proven invaluable in elucidating some of the structural details of the MPS[11]. However, the understanding of the AIS structural complex, the identity and functions of its components, and its spatiotemporal regulation are still incomplete. Recent proteomic studies succeeded in creating a sizeable list of proteins that localize to the AIS close to structural components of the AIS[12,13]. Still, details of their function and precise place in the AIS complex are lacking. Thus, super-resolution microscopy techniques serve as an essential tool to examine the accurate positions of these proteins relative to other MPS components and investigate their functions. Several light microscopy techniques can achieve resolutions higher than the diffraction limit of light, some even capable of localizing single molecules. However, many of these techniques typically require specialized fluorophores or imaging buffers, and image acquisition is often time-intensive[14].

3D structured illumination microscopy (3D-SIM), owing to its ease of use and simple sample preparation requirements, requires no special reagents for imaging or sample preparation, works well with a wide array of fluorophores and samples, can be readily implemented in multiple colors, and is capable of live-cell imaging[15]. While the best possible resolution SIM offers (~120 nm) is low compared to many other super-resolution techniques, it is sufficient for many applications (for example, for resolving the components of the MPS in neurons). Thus, it is crucial to consider the requirement for specific applications to determine if SIM is a suitable choice or if an even higher resolution is necessary. Here, a protocol is described for using cultured hippocampal neurons and 3D-structured illumination microscopy (3D-SIM) to examine the position and organization of putative AIS proteins relative to actin rings in the MPS, as implemented in Abouelezz et al.[16]

PROTOCOL:

Primary hippocampal neurons used in these experiments were harvested[16] from embryonic day 17 Wistar rat embryos of either sex under the ethical guidelines of the University of Helsinki and Finnish law.

1. Sample preparation

1.1. On high-fidelity glass coverslips, allow rat hippocampal neurons to grow in sparse culture conditions (~5,000-10,000 cells/cm^2) for 14 days (14 days-in-vitro).

NOTE: Using sparse cultures reduces the chances of overlapping neurites, which helps quantify

the MPS.

1.2. Fix neurons using 4% paraformaldehyde for 12 min at room temperature. Wash the coverslip once in 0.2% BSA in PBS (BSA-PBS), then incubate for 10 min in a 1% solution of Triton-X in PBS at room temperature. Wash once in BSA-PBS.

1.3. Dilute anti-ankyrin G antibodies (1:200) in BSA-PBS. Add antibody solution to the coverslip and incubate overnight at 4 °C. Optionally, add chicken anti-MAP2 antibodies (see **Table of Materials**) to the antibody solution to demarcate the somato-dendritic domain.

1.4. Wash cells once in BSA-PBS, followed by 0.1% Triton-X in PBS, then do a final wash in BSA-PBS.

1.5. Dilute fluorophore-tagged anti-mouse secondary antibodies (1:200) (see **Table of Materials**) in BSA-PBS, add to the coverslip, and incubate for 1 h at room temperature. Wash the cells once in BSA-PBS, once in 0.1% Triton-X in PBS, then once in PBS.

1.6. Prepare a 1 µM solution of tagged phalloidin in PBS (see **Table of Materials**) and add it to the cells. Incubate for 2 h at room temperature. Wash cells once in PBS, once in 0.1% Triton-X in PBS, then once in PBS.

NOTE: AlexaFluor488-tagged phalloidin was used here, but other tags will also work.

1.7. For mounting the coverslip on a glass slide, apply a drop of mounting media on a glass slide, dip the coverslip in deionized water, and dab using a soft paper towel (to remove excess water).

1.7.1. Place the coverslip on the glass slide. Incubate at room temperature for 24 h.

NOTE: No adverse effects on the sample were observed using hard-setting mounting media (refractive index 1.47 after curing).

2. Imaging

2.1. If possible, consult with the microscopy facility's personnel before imaging. Use an immersion oil calculator (see **Table of Materials**) to select the immersion oil suitable for the sample.

2.2. Once the samples are ready, ensure the coverslips are clean and clear of any residue or excess mounting media. If necessary, use a cotton tip dipped in water or ethanol to clean. Place the sample in a 3D SIM-capable microscope (see **Table of Materials**) and find a cell to image.

2.3. Adjust the power of the relevant laser lines and the exposure times to maximize the signal-to-noise ratio without significantly bleaching the sample.

NOTE: A good signal-to-noise ratio will show a clear grid-like appearance focused on the sample and lead to accurate high-resolution reconstructions. 488 nm and 640 nm laser lines were used to visualize F-actin and ankyrin G, respectively.

2.4. Set the upper and lower limits of the sample in the z-dimension and proceed to acquire a stack.

NOTE: z-step size of 125 nm was used here.

2.5. For successful SIM reconstruction, ensure that the microscopy software (see **Table of Materials**) has an optical transfer file (OTF) suitable for the used dyes.

NOTE: These are typically created and maintained by specialized personnel. In the absence of a functional OTF, open-source tools are also available to perform reconstructions based on estimates[17]. In this work, up-to-date OTF files were used controlled by specialized personnel.

2.6. Run the reconstruction algorithm on the stack to obtain a super-resolved reconstruction. Check the reconstructions for known artifacts and, if necessary, adjust the parameters to correct them.

NOTE: The default algorithm parameters typically give accurate results. Many of these artifacts involve some repeating patterns: striped lines along with multiple directions on one z-plane, 'ghosting' (repeated features appearing in various z-planes), or a hexagonal 'honeycomb' pattern emerging in some areas. These artifacts can often be fixed with better sample preparation, a better signal-to-noise ratio, adjusting the parameters of the reconstruction algorithm, or ensuring the refractive index of the chosen immersion oil is suitable for the mounting medium and sample. For a more detailed discussion of SIM artifacts and a freely-available tool to check for the presence of artifacts, see Ball et al.[18]

2.7. Compare the SIM reconstruction to a wide-field image to observe improvements in resolution.

2.8. When performing multi-color SIM, use the alignment algorithm to align the different channels correctly once a satisfactory reconstruction is ready.

NOTE: The alignment algorithm uses an alignment reference file generated using a microsphere bead preparation as per the instruction of the microscope manufacturer.

3. Image analysis

3.1. Actin rings in the MPS have a distinctive periodic appearance. Using image analysis software (see **Table of Materials**), create a maximum intensity projection image using all the focal planes where actin rings are visible.

3.2. On the maximum intensity projection image, draw a perpendicular line across visible adjacent rings and record the fluorescence intensity along with the software's line 'Plot Profile' function.

3.3. To calculate the mean inter-peak distance, note the local maxima in the line profile and measure the distance between individual adjacent fluorescent intensity peaks.

NOTE: This can be easily calculated, for example, using the 'findpeaks' function in MATLAB or the (open-source) Octave platform.

3.4. To evaluate the colocalization of different proteins with actin rings in the MPS, run a colocalization analysis procedure[19-21] on SIM reconstructions of actin and the candidate protein. Manually draw a selection to define the AIS as a region of interest for the EzColocalization plugin in the software platform[19] and run the analysis to calculate the Pearson's coefficient of correlation (PCC) co-localization[19-21]. A PCC value close to 1 indicates strong colocalization.

NOTE: This protocol is summarized in **Figure 1**.

REPRESENTATIVE RESULTS:

Using cultured rat hippocampal neurons and 3D-SIM, a protocol is described to visualize and measure actin rings and other components of the MPS in the AIS. Reconstructions of image stacks showed clear periodicity of actin rings (**Figure 2A**). In our hands, the mean inter-peak distance of actin rings in the MPS, visualized using Alexa 488-tagged phalloidin, was 190.36 ± 1.7 nm (mean ± SEM). This is in line with the previously reported average distance of ~190 nm between actin rings in the MPS. Similarly, an anti-ankyrin G antibody was used to visualize ankyrin G (**Figure 2B**). The colocalization of ankyrin G and actin rings was tested in the AIS using a colocalization analysis procedure to calculate the PCC of co-localization[19-21]. The PCC of colocalization of ankyrin G and actin rings fluorescence was 0.36 ± 0.03 (mean ± SEM, **Figure 2C**). As ankyrin G and actin rings bind βIV-spectrin at different domains, they do not show significant colocalization. These data were adapted from Abouelezz et al.[16]

FIGURE LEGENDS:

Figure 1: Schematic representation of the protocol for sample preparation for 3D-SIM imaging. Hippocampal neurons are harvested from rat pups, dissociated, and allowed to grow on glass coverslips in culture for 14 days. Cells are then fixed and stained using tagged phalloidin and appropriate antibodies, then mounted on glass slides for 3D-SIM imaging.

Figure 2: 3D-SIM reconstruction of the membrane periodic skeleton in the AIS. (**A**) F-actin (green) and ankyrin G (magenta) show a regular distribution in the AIS, visualized by 3D-SIM. Scale bar = 1 µm. (**B**) Pearson's coefficients of correlation (PCC) of colocalization of ankyrin G with F-actin in the MPS in the AIS. The mean PCC of ankyrin G was 0.36 (black circle). Gray diamonds represent individual cells (n = 16), mid-line represents the median, error bars represent 25th and

75th percentiles. The data is adapted from Abouelezz et al.[16]

DISCUSSION:

The protocol described here provides a method for visualizing and measuring MPS proteins using the super-resolution technique. As actin rings and other MPS components display a periodicity of ~190 nm[9,10], conventional diffraction-limited imaging approaches cannot reveal the details of the MPS. Several microscopy setups may resolve diffraction-limited structures in super-resolution, and SIM represents a robust and uncomplicated option. Importantly, SIM is compatible with the most widely-used fluorophores, providing significant flexibility. Furthermore, SIM is effective in visualizing potentially dim structures in the MPS, such as actin rings in latrunculin-treated neurons[22], as well as live neurons[15].

An essential aspect for the success of this protocol is preserving the integrity of the MPS in the first place. The most critical steps to successful SIM imaging occur during sample preparation. For example, moderate fixation (4% PFA for 12 min at room temperature) and strong permeabilization (1% Triton-X for 10 min) provide the best results. It is crucial to keep in mind that harsh treatments that may be required for specific preparations may have a negative effect on the structural integrity of the MPS. Therefore, it is perhaps best to consider modifying such treatments to maximize the preservation of the MPS.

The other crucial factor to consider when preparing samples for imaging is to maximize the labeling density. Ideally, every molecule would be tagged and detected. For example, the anti-ankyrin G antibody used in this experiment is commonly used to label the AIS. It provides outstanding performance in conventional fluorescence microscopy, even when used at a dilution of 1:1000 and incubated for just 1 h at room temperature. However, to obtain good labeling density for super-resolution microscopy, it is highly effective to use 5-fold that concentration (1:200) and incubate the antibody overnight at 4 °C. While the specific requirements for each antibody or labeling technique will vary and need to be determined experimentally, it is perhaps helpful to keep this in mind.

In addition, it is essential to note that achieving a high signal-to-noise ratio lends itself well to accurate and successful SIM reconstructions. A good rule of thumb is that the grid pattern should be visible in the individual images once the SIM modality is engaged. However, this is not always possible.

Finally, it is essential to note that SIM is among the weakest super-resolution techniques in resolving power[14]. Thus, while it is generally sufficient for revealing periodicity and overall organization of MPS proteins, it is significantly less capable of providing details about their interactions than stochastic optical reconstruction microscopy (STORM)[10]. Furthermore, the technique's utility described here is limited to the study of proteins for which a specific, well-performing antibody is available. However, this can partly be circumvented through exogenous expression of tagged proteins[15].

ACKNOWLEDGMENTS:

Dr. Pirta Hotulainen is acknowledged for her critical comments, invaluable for preparing this manuscript. Dr. Rimante Minkeviciene is acknowledged for her help in preparing the neuronal cultures used for the original experiments. All imaging was performed in the Biomedicum Imaging Unit. This work was supported by the Academy of Finland (D.M., SA 266351) and Doctoral Programme Brain & Mind (A.A.)

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

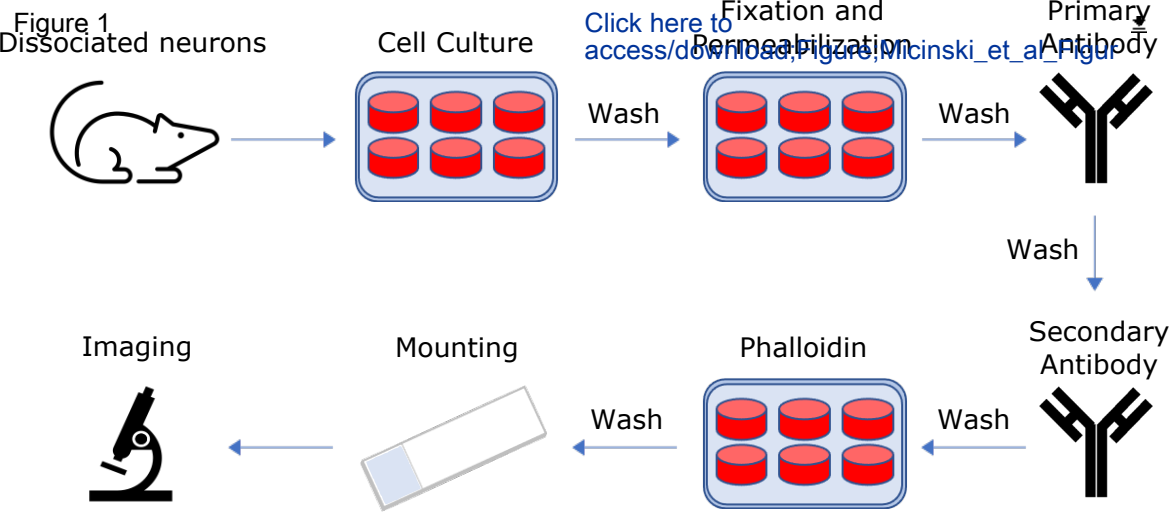
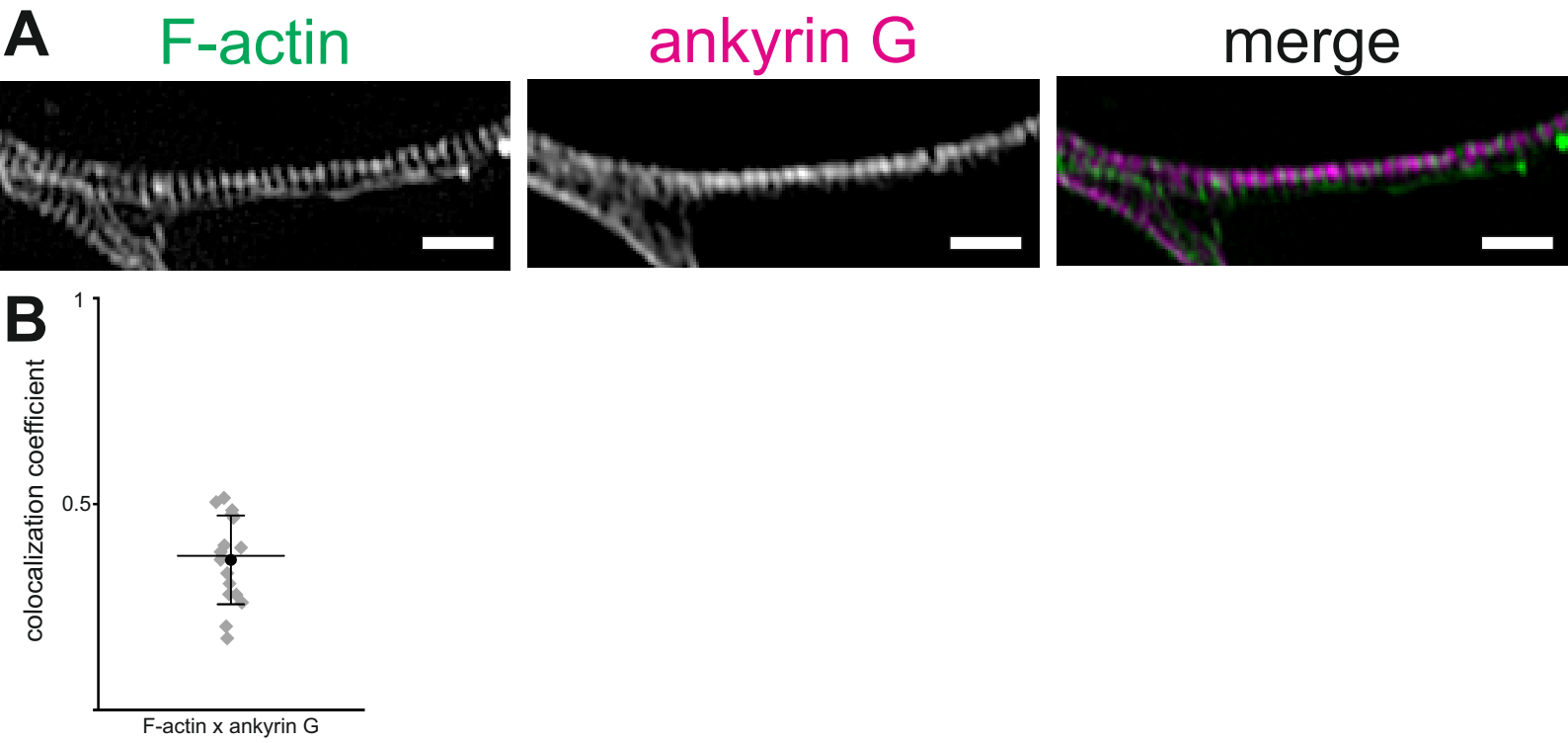


Figure 2

Click here to access/download;Figure;Micinski_et_al_fig2_R1.pdf



Dear Dr. Krishnan,

We are grateful for the constructive comments we received on our manuscript titled “Measuring properties of the membrane periodic skeleton of the axon initial segment using cultured rat hippocampal neurons and 3D-structured illumination microscopy (3D-SIM)”. We sincerely thank both reviewers for their time and thorough examination.

Please find below our point-by-point response to each comment.

Sincerely,

Amr Abouelezz

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Amends in lines 47 and 247

2. Please provide an institutional email address for each author.

These are listed along with author names and affiliations, lines 7, 9, and 12.

3. Please reword the lines to avoid the issue of plagiarism:35-36. Please refer to the ithenticate report attached.

Rephrased.

4. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

Rewrote the introduction to address these points.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example:

We do not use the symbols mentioned, and have avoided all commercial language.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Reformatted the protocol to contain numbered steps.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Added “Note” in line 147.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Rewritten.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Added detailed instructions.

10. Line 58: Are the rat hippocampal cells isolated or purchased commercially. If isolated, please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Added ethics statement (line 70).

11. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Added space and highlighted essential steps for the video.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

The figure is not a reprint from the original publication listed, but is from the same data. The publication is open-access and does not require a license.

13. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Edited the Discussion section.

14. Please do not use the &-sign or the word "and" when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

We use the journal's own EndNote style file.

15. Figure 2: Please include scale bars in all the images of the panel and define the magnification in the figure legends.

Added.

16. Please ensure that the Table of Materials includes all the supplies (reagents, chemicals, instruments, equipment, software, etc.) used in the study. Please sort the table in alphabetical order.

Sorted alphabetically.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors present a protocol to visualize and measure the localization and periodicity of MPS/AIS components in 3D-SIM images. 3D-SIM provides sufficient resolution and due to its robustness, it is an ideal tool to map the localization of the previously identified MPS/AIS components.

Major Concerns:

At the current form, the protocol is aiming more experienced researchers, who already know how to culture neurons, use a SIM setup and do basic image analysis. In order to be more useful for a less experienced researcher, the imaging and image analysis part should be more detailed. The authors should elaborate on potential pitfalls.

We now added more detail about the imaging and image analysis to make the protocol more accessible and elaborated on potential pitfalls.

Minor Concerns:
-Line 84: The authors state that it is important to check the SIM reconstructions for known artifacts. The authors can add a few sentences here to discuss these artifacts and how to deal with them.

We added a few lines about artefacts and included a reference to a freely available tool that can be used to check for their presence and evaluate the quality of the data (SIMcheck).

-Line 87: The authors mention an alignment algorithm. Is that a system specific tool? It should be clarified.

Clarified.

-Line 92: Elaborate on how to measure the distance between individual peaks (using a peak finder script or fitting multiple Gaussians…). It is not obvious to a beginner.

Explained in more detail.

-Line 95: The authors use pearson's correlation to test colocalization. The authors should discuss how to interpret the results of these measurements.

A brief explanation included.

Reviewer #2:
Manuscript Summary:
In their manuscript, Micinski et al. described a method to visualize and analyze the relative localization of MPS proteins with the periodic actin rings in the AIS region of the axon. This work is addressing an important technical question in neuroscience and cell biology. However, the protocol is too general in its current shape and doesn't provide sufficient practical information to guide the experiment. I provide my several major concerns below.

Major Concerns:
1. Fluorophores and phalloidin concentration is critical for the success of the actin ring staining. So could the author specify What kind of tags are used for the phalloidin? 647? 647N?

Specified.

2. Hard-setting mounting media are generally believed to require more laser power to get the similar fluorophore density (Wegel et al, 2016, scientific report). Have you ever tried vector shield liquid mounting medium?

We have only tried vector shield liquid media in different contexts. We find that ProLong Gold gave a good balance of ease of use and storage, preserving the sample integrity, high refractive index, and cost. In some of our experiments using ProLong Gold we get excellent signal-to-noise ratio with low laser power (<1%). It is possible that we make up for the increased laser power requirement by stronger staining.

3. Line 77-78: Essential setting parameters of the microscope should be listed.

Added more details about the microscope settings and ways to adjust them. Also added a reference that offers a tool for reconstructions in the absence of a proper OTF.

4. line 76: Channel alignment parameters should be derived prior to the experiment using the 100-200 nm fluorescent spheres (TetraSpeck beads, Invitrogen).

Indeed. Now clarified in text.

5. Line81-82 As the periodic pattern is very specific, which is the 190 nm lattices, Grid size, rotation number, and z-stack step size should all be given for the optimization of different wave-length fluorophores. you can check Wegel et al, 2016, scientific report for some information and Wang et al, Journal of Cell Biology, 2020 for more information.

Added information about the z-stack step size. As the grid size and rotation number are parameters that are out of our control and instrument-specific, we opted for not specifying these. We are not aware of how changes in these parameters would affect the capabilities of the instrument, and how much this is relevant in the context of commercial 3D-SIM setups.

6. line 91: can you specify the software you used?

Specified.

7. Line 95: more detailed steps for the colocalization analysis should also be provided.

More details now included.

Minor Concerns:
Figure 1: I guess you should also describe this step in more detail as well.
Line 134: SIM is also capable of imaging actin ring dynamics in live axons (Wang et al, 2020 Journal Cell Biology).

Added.