ISTITUTO DI RICERCHE
FARMACOLOGICHE
MARIO NEGRI · IRCCS

August 07, 2020

Nam Nguyen, Ph.D.
Manager of Review
JoVE
1 Alewife Center Suite 200,
Cambridge, MA, 02140

Dear Nam,

It is with great pleasure that I am writing to submit a revised version of the manuscript entitled: “Super-resolution imaging to study co-localization of proteins and synaptic markers in primary neurons”.

We have addressed all comments and concerns of the reviewers and the manuscript is now much more detailed and helpful. As per suggestion, we have also modified the title, to better reflect the content of the protocol.

Please find below a description of the changes that have been made (in black our responses).

Thank you for receiving our revised manuscript and considering it for publication. We appreciate your time and look forward to your response.

Best,

Luca Colnaghi, PhD
Department of Molecular Biochemistry and Pharmacology
Istituto di Ricerche Farmacologiche Mario Negri IRCCS
Via Mario Negri, 2. 20156 Milano, Italy
Phone: +390239014447

Editor's comments:
Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

We have not addressed the comments on the novelty of the findings.

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes

that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

We would like to thank the reviewers for their helpful comments. We have addressed all concerns and we have described the changes below. Thanks to the suggestions and critics of the reviewers, the manuscript is now much more helpful and detailed.

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

A native English-speaking person has proofread the manuscript to ensure that there are no spelling or grammar issues.

2. 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 and Reagents.

We have removed all commercial language but the brand of the microscope. This information is crucial for the protocol. Alternatively, we could just list the microscope system in the materials list.

3. 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.
4. 1.1: What are the culture conditions? Cell density?

We have added the required details (lanes 92-130 and lane123).

5. 1.4: Please provide a citation here.

We have now added specific culturing instructions and substitute step 1.4 (lanes 92-130).

6. Please provide specific experimental parameters instead of a generalized one. Please provide an example protocol of choice.

We have now added specific culturing instructions (lanes 92-130).

7. Please discuss limitations of the protocol in the Discussion.

We have discussed the limitations of the present protocol (lanes 422-438).

Changes to be made by the Author(s) regarding the video:

We have edited the video as requested. The intro section is much shorter, as well as the preparation of primary cultures and the staining of neurons. A results section has been added and, in general, more emphasis has been given to the calibration of the scope and to the acquisition of 3D-SIM images.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The protocol describes the use of a commercially available Nikon N-SIM microscope and its stock NIS Elements software for acquiring and processing images from fixed, immunostained cell cultures. This protocol could be potentially useful for users who have access to an N-SIM system, but have no access to training from core facility staff or other experts. However, the presentation of some of the more important steps, such as

calibration, lack necessary detail. The parts of the protocol on cell culturing and immunostaining aren't very well suited for the video format, as these consist of series of washes.

Major Concerns:
1. Despite the statements made in the abstract, the protocol does not describe the use of controls, or anything about the interpretation of the data or statistics (other than recommending an arbitrary number of images to be taken). This is the biggest issue of this protocol. Figure 2 summarizes the problems with the analysis. How are the included synapses selected? Why do we care about line profiles if the colocalization analysis is image based? The analysis should be done in a way that the profiles from many lines are aligned on the peak of the PSD signal. Which areas of the image are included in the colocalization analysis? How the manual thresholds are set? Are the MAP2 and DAPI channels used to create masks for dendritic areas? Is 3D information from the z stacks used in any way? What do the numbers that are derived from this analysis mean biologically? What are the numbers for a known presynaptic, a known postsynaptic, a known extrasynaptic protein, and for negative control? Unless these points are properly addressed in the video, the analysis section isn't useful, and the time could be better spent on showing how to actually obtain and process the images.

We would like to thank the reviewer for the helpful comments and insights. We have edited the manuscript to reflect his suggestion of adding more controls, or anything about the interpretation of the data or statistics. (lanes 166-175; lanes 199-225; lanes 263-279; lanes 422-430)

2. The protocol on primary neuronal cultures takes up a good chunk of the video but has limited usefulness in its current form, because users either have their protocols or need other resources to successfully culture cells. I recommend to omit these steps and instead list the type of substrates that work well with this protocol and discuss their pros and cons (such as chambered coverslips and round coverglass).

We have edited the video to just list the type of substrates that work well with this protocol and discuss their pros and cons.

3. Similarly, the protocol on immunostaining has limited usefulness due to an unfortunate combination of lack of specificity in critical details while over-specifying non-critical steps. For example, the method of fixation and permeabilization (which can vary greatly depending on the protein target) is specified, but the fluorescent dye combinations recommended for SIM are not discussed. What wavelength combinations of DyLight and Alexa dyes work well? Which combinations are optimal for good spectral separation, and which offer the best resolution? Considerations for the choice of mounting medium or potential alternatives (which I assume is critical) are not described. Finally, this would be a good place to recommend a positive control (primary and secondary antibodies with catalog numbers) and describe the negative controls. It is odd to devote time on explaining why we use blocking solution.

We have edited the text and video to give more information on DyLight and Alexa dyes and on the mounting medium. We now give specific information on a positive control (an antibody against PSD95). We also now describe negative controls (lanes 405-408; 397-403). Wavelength combinations depend on the system in use, although with proper calibration of the single channels most combinations can be used for SIM.

4. While the protocol is claimed to be applicable for any SIM system, it is specific to the Nikon system. "Follow the guided steps for calibration" is not useful for a more general audience.

We have edited the protocol to make it more general (lanes 178-254).

5. The steps that would be the most valuable for readers are the calibration, and the optimizing of acquisition and image reconstruction steps (3.3-5.5). The video should emphasize these, and appropriate description of the possible artefacts, as well as possible quality control criteria.

We have added new steps and paragraphs to discuss the calibration of the system, the optimizing of acquisition and image reconstruction steps (lanes 178-254).

Minor Concerns:
1. The introduction could do more in discussing the limitations of the method, as well as the strengths and weaknesses in comparison to other methods. For example, for what type of biological questions would one pick SIM over confocal microscopy or electron microscopy or other super-resolution methods? There are many

reviews in this topic that could be referenced here and summarized in a few sentences. For example, the concept of diffraction limit deserves an introduction, while the current first paragraph on our knowledge about synapses is vague and not very useful, given that the imaging method isn't really specific for the study of synapses. A particularly confusing issue is the multiple mention of dynamics in the abstract and introduction, while the protocol takes static images of fixed samples.

We added the concept of diffraction limit and we have edited the paragraph on synapses(lanes 63-73). We have edited the manuscript to only focus on static images of fixed samples.

2. Some of the useful advice and considerations are in the discussion. Consider moving these to the relevant places in the protocol.

We did not move the parts as requested by JoVE guidelines.

Reviewer #2:
Manuscript Summary:
The manuscript by Colnaghi et al describes a simple method to perform super-resolution SIM of synaptic molecules in neuronal cultures. The authors give a short introduction and provide a detailed protocol of the method.

Major Concerns:
none

Minor Concerns:
Please include information on the secondary antibodies used rather than saying you use Alexa and DyLight. Maybe add a line in the text saying that you can use more or less any fluorophore with SIM. Also add the word antibody behind MAP2 etc. in the table.

We have added more information on the secondary antibodies used (lanes 405-408).

Reviewer #3:
Major Concerns:
In its current form, the work "Super-resolution imaging of synaptic proteins in primary neurons" by Colnaghi et al. is very preliminary and limited in scope. The Protocol lacks clarity (see specific examples of unclear instructions below, where line of occurrence is indicated).
The title promises far more than is delivered in the Protocol; two synaptic marker proteins and the protein SUMO1 with a DAPI nuclear staining is far from being a generic lineout of how to apply SIM to the study of synaptic proteins. And the reader can find the specifics on SUMO1 imaging in synapses in the Frontiers paper by the same authors.

We would like to thank the reviewer for the comment. We have edited the manuscript to add more useful details on the protocol and analysis. We have also edited the title to better reflect the content of the manuscript.

But the most worrying aspect of the protocol is that it fails to arguably demonstrate the need to use superresolution optical microscopy for the illustrated purpose. What is the gain in spatial resolution resulting from application of structured illumination microscopy as compared with wide-field microscopy? The single example in Fig. 2 is an asymmetric spot well above the diffraction limit. The reader would wonder why an expensive superresolution microscope is needed to do that.

We have edited the manuscript to add more information on the reasons why super-resolution microscopy is helpful to study co-localization of neuronal proteins (lanes 57-73).

Although the authors claim that the Protocol is valid for other hardware systems, the specifics of the commercial instrument they use (one of the best available) becomes apparent. This reviewer understands that it is extremely difficult to produce a completely "aseptic", brand-free protocol applicable to any hardware configuration, but efforts should be devoted either to attempt such a task or otherwise refer to the instrument's manual when applicable.

We have edited the protocol to include more general instructions (lanes 178-254).

Nothing is said about the preparation of calibration beads.

We have added a section about calibration beads (lanes 178-225).

Nothing is said about artifacts and how to prevent and/or correct them.

We have added sections about how to detect and prevent artifacts (lanes 221-279)

Spatial resolution is not dealt with.

We have added a section on spatial resolution (lanes 57-73).

The densitometric profile shown in Fig. 2 does not convey seem to correspond to a superresolution image.

We have replaced the image, now image 7.

Minor Concerns:
Examples of missing aspects of the Protocol.

Line 133 3.3. Calibrate the system to achieve spatial resolution as close as possible to 100 nm.
How?

We have added details (lanes 178-225).

Lines 157-158. The step size/acquisition time should be related to the area to be imaged.

We have eliminated the statement.

Line 165 5.1. Process the raw images to obtain restructured SIM images. "Restructured"?

We have corrected the mistake.

Line 237-238. "diffraction index of the microscope"

We have corrected the mistake.

Reviewer #4:
This protocol by Colnaghi and colleagues, Super-resolution imaging of synaptic proteins, provides a mechanism to meet the need for high resolution imaging of synapses in vitro using Structured Illumination Microscopy (SIM). In both the video and the manuscript, the authors make a compelling case for the need for such techniques, as understanding synapses in nervous system function and synaptopathies in disease often requires resolution greater than traditional light microscopy techniques can offer. There is not, however, a lot that is truly new here, and the authors should do a little more to place their protocol in the larger context of colocalization and synaptic imaging approaches. There are also a couple technical issues that need to be addressed. The video is quite well done and overall the report has merit for JoVE readers.

Major concerns
1. This technique relies on identifying synapses based on a single marker, rather than any more specific method (eg, co-localization of a pre- and a postsynaptic marker). As Fig. 1A makes clear, the postsynaptic marker at least is present throughout the cell. Much of this signal is clearly non-synaptic (proteins in transport or degradation, background or other artifact). A technique that relies on co-localization of an experimental signal with all PSD95, particularly if the ROI is not carefully chosen, could be misleading vis a vis co-localization at synapses specifically. This should be carefully discussed as a limitation.

We have added the details suggested by the reviewers. This is a crucial limitation and we would like to thank him/her for the helpful suggestion (lanes 432-438).

2. Both the Pearson's and Mander's coefficients are relatively low in the examples chosen, implying that the majority of the time these signals do not overlap. Perhaps this is expected for these markers, but it seems an odd choice to demonstrate the utility of the method. This deserves discussion.

We did not discuss the finding since it is not the scope of this JoVE article. It is nevertheless a good scientific point, that we describe in more details in a recent publication (https://doi.org/10.3389/fncel.2019.00486).

3. What efforts are made here to measure and eliminate chromatic aberration? Especially at high-resolution, chromatic aberration could result in overlapping signals appearing separate. Multiply fluorescent microbeads offer a logical option.

We have added the requested details (lanes 178-279).

4. It would be very helpful in the discussion to generally discuss what would be necessary to translate this technique to brain tissue. Where would the difficulties lie (eg, challenges with out of focus light in thicker specimens for SIM)? Would the authors expect the approach to work with minimal modification, or should readers know that this approach is only likely to work on cultured neurons? Can the authors recommend alternatives for imaging synapses in tissue using super-resolution?

Although extremely interesting, we do not have experience with SIM in tissue. While the sections on controls and calibrations are general, the sample preparation instructions are likely to only work for cultured neurons.

5. Prolong diamond glass? I believe these are separate products: prolong diamond, and prolong glass. The catalog number in the table of reagents (P36970 Thermo Fisher Scientific) specifically references Prolong Diamond. Prolong glass would be more appropriate here, as it is better matched to the RI of the coverglass. Using prolong diamond for SR imaging would prohibit imaging more than a few microns deep due to spherical aberrations. It would be worth noting either way the great importance of adequate refractive index matching for SR applications.

We apologize for the mistake. We have fixed it.

6. It is furthermore important to understand this technique as one application of colocalization in microscopy. Colocalization is heavily used in the analysis of fluorescence micrographs, both diffraction limited and SR. It would be useful for the authors to cite some of the prior reports describing these approaches, and clearly lay out what sets this method apart—what it brings that is new or different—so that the reader can decide whether it is appropriate for his/her problem.

We have added details to better describe why SIM can be used for co-localization studies (lanes 57-73).

7. It only becomes clear what the readouts of this method are upon close inspection of the images provided. To make utility more apparent, the authors could describe what quantification parameters this method will achieve (e.g. quantification of synapses, measurement of synaptic distances, etc.) in the abstract and introduction

We have added details to better describe what the readouts of the methods are (lanes 81-88; lanes 432-438).

8. Little information is provided about SIM and SUMO. To further explain the utility of this protocol, the authors could use the intro to very briefly describe the mechanism by which SIM improves resolution of light microscopy and explain the function of SUMO.

Details about SIM and SUMO are beyond the scope of this manuscript. More info could be found here https://doi.org/10.3389/fncel.2019.00486.

9. Qualifiers should be added to the last para of the discussion re live cells. If the authors do not have data to support the translation to live cells, then this language is too strong.

We have taken that part out.

Minor concerns
1) Abstract: specify that SIM achieves 100 nm resolution in the XY plane, specifically. Resolution is generally substantially poorer in Z, which has implications for accurate colocalization.

We have added details about Z dimension (lanes 71-73).

2) Introduction, para 1: the last sentence is confusedly worded, and the point is lost. Suggest revising.

We have revised the last part of the introduction.

3) Is triton not included in the blocking step (2.9)? Is there a reason cells are incubated for a short period in triton, then in a blocking solution without triton, then in a staining solution that again contains triton?

We have found that blocking the cells in a solution without triton helps reducing the staining background. We have added the details in the text (lanes 142-143).

4) Step 4.8 provides a range of settings that seems so broad as to be unhelpful

We edited the manuscript to reflect better the meaning of 4.8. We try to keep laser power below 50% to prevent photobleaching (lanes lanes 242-249).

5) Step 4.11: this guidance will vary greatly depending on the nature of the experiment and the quality of the sample. This guidance is probably more misleading than helpful. At least it should be qualified as referring only to the specific stains and analyses reported here as an example. The same can be said for step 8.2.

We agree with the reviewer that the number of images to be analyzed may vary between experiments. As guidance, we suggest the minimum numbers of images to analyze and we are not suggesting these numbers to be absolute.

6) While the video provides number of cells to be plated, the protocol does not (line 86). This information is necessary to properly follow the protocol.

We added this info in the text (lane 123).

7) Poly-L-lysine is not included in necessary reagents table.

We have added it.

Reviewer #5:
Manuscript Summary:
This protocol by Colnaghi et al. describes how to image synapses with SIM, and is based on their publication from 2019 where they used this method to image SUMO proteins at synapses (Colnaghi et al., 2019, Frontiers in Cellular Neuroscience). Here, the authors go through the immunostaining protocol and how they image with the SIM. Overall, the protocol misses many of the key details and intricacies of using a super-resolution imaging method such as SIM. There are numerous challenges associated with using SIM to image synapses, including poor signal-to-noise ratio, the large number of artifacts found in images and the limited increase in resolution provided if not utilized correctly. These issues are highlighted in two Nature protocol papers: Demmerle et al., 2017 doi:10.1038/nprot.2017.019- See Box 2 for publishing guidelines, and Kraus et al., 2017, doi:10.1038/nprot.2017.020
Here, this protocol presents a 'super-easy' way of obtaining images from a SIM microscope, but due to the under-appreciation of many of the critical nuances and technicalities of SIM imaging and image reconstruction, which is likely to produce low-quality images, I cannot support its publication in JoVE.

Some general points:
-There is no quality control to assess the quality the images produced, and how the reconstruction parameters were chosen. There needs to be some independent assessment of the resulting image to determine the correct parameters. Furthermore, at least 2 quality control checks should be used to assess the quality of every image acquired. First, the FFT image needs to be checked in each channel to assure that reconstruction worked well (a

large 'flower', defined 'petals', sharp image). Second, Sim Check (Ball et al., 2015, Scientific Reports) should always be used to check resulting raw and reconstructed SIM images. It is not clear that any quality control measures are in place.

We would like to thank the reviewer for suggesting the use of unbiased quality controls. We have added information on Fourier transform and SIMCheck (lanes 255-279; lanes 300-317; lanes 422-430).

-Nothing is mentioned about channel registration:
If you are doing any type of co-localization using this technique you HAVE to do some type of channel registration (drift correction). Image Registration should be performed within the Nikon software and the system has to be checked before each imaging session to make sure the registration is still valid with a Bead Alignment:
Take a SIM image of 0.1uM beads (TetraSpeck) - reconstruct, Check bead alignment in the XY,Check bead alignment in the Z

We added the required information (lanes 178-225).

-For co-localization experiments you should always take an image in all selected wavelengths before moving z-position.

We edited the text to better describe this step.

-Is 2D or 3D SIM used?

We now specify 3D-SIM throughout the text.

-Ln 271: 'The current protocol can also be applied to the analysis of live cells. In this case, cells expressing fluorescent-tagged proteins should be used. To achieve this, the cells should express fluorescent pre- or post-synaptic protein markers and target proteins.' This is an incredibly simplistic comment, and demonstrates how little the authors appreciate the technicalities and complexities of this method. To perform live imaging with SIM and use the correct fluorophores (let alone have one expressed post synaptically and one presynaptically), environmental chamber set-up, perfect focus settings etc. i.e. takes months/years of optimization.

We took this part out.

Some specific points:
3. 1: 'Let the system warm up (15-30 minutes). Per NIKON's Instructions - The system needs at least 3hrs to warm to reach thermal equilibrium. It is also recommended that the slides are at the same temperature as the microscope, so it is good to put the slides near the microscope to warm.

We edited the text to include this (lanes 196-197.

3.3: 'Calibrate the system to achieve spatial resolution as close as possible to 100 nm.'
- What does this mean? Instructions for this? This process is essential for the production of high-quality, high-resolution imaging.

We added instruction for this (lanes 177-225).

3.6 'Carry out grating focus....'
-They authors use a one color bead for (FluoSpheres carboxylate-modified microspheres, 0.1 µm, yellow-green fluorescent) for the grating focus. You would need to run the grating focus at each wavelength that will be used, and therefore beads should be used that fluoresce at all wavelengths required (e.g. TetraSpeck beads).

We added the required instructions (lanes 206-210).

4.7. 'Using histogram windows, set laser power and exposure time to reach a target intensity of 16,000 to keep the linear response of the camera or 30,000-45,000 to maximize the dynamic range of the acquisition.' The values quoted here are dependent on the microscope used, and are not universal.

We edited the text to reflect this (lanes 242-246).

-A lot of time in the video is devoted to the staining the neurons, which could be skipped (as everyone can do that). The unique points specific to SIM imaging could be described (using Alexa fluor dyes, #1.5 coverslips, mountant). More time could be used to address the details of SIM and how to produce high-quality super-resolution images.

We edited the video to reflect this comment.

-Before using SIM to image anything, the quality of all immunostaining should be verified by confocal imaging. If the staining doesn't work for confocal, it will never work for SIM.

We edited the text to add this information (lanes 230-232).

Sede Legale
Mario Negri Milano

Via Mario Negri, 2 - 20156 Milano
Tel. +39 02 390141
mnegri@marionegri.it

Centro di Ricerche Cliniche
per le Malattie Rare "Aldo e Cele Daccò"
Villa Camozzi

Via G.B. Camozzi, 3 -24020 Ranica (BG)
Tel. +39 035 45351
villacamozzi@marionegri.it

Centro Anna Maria Astori
Parco Scientifico Tecnologico
Kilometro Rosso

Via Stezzano, 87 - 24126 Bergamo
Tel. +39 035 42131
bergamo@marionegri.it

marionegri.it

Fondazione per ricerche eretta in ente morale, D.P.R. 361 Del 5/4/1961 - Registro Persone Giuridiche Prefettura Milano N.227
Cod. Fisc. E Partita Iva 03254210150 - Anagrafe Nazionale Ricerche Cod.G1690099