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TITLE:

Super-Resolution Imaging to Study Co-Localization of Proteins and Synaptic Markers in Primary Neurons

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SUMMARY:

This protocol shows how to employ super-resolution microscopy to study protein co-localization in primary neuronal cultures.

ABSTRACT:

Synapses are the functional elements of neurons and their defects or losses are at the basis of several neurodegenerative and neurological disorders. Imaging studies are widely used to investigate their function and plasticity in physiological and pathological conditions. Because of their size and structure, localization studies of proteins require high-resolution imaging techniques. In this protocol, we describe a procedure to study in primary neurons the co-localization of target proteins with synaptic markers at a super-resolution level using structured illumination microscopy (SIM). SIM is a patterned-light illumination technique that doubles the spatial resolution of wide-field microscopy, reaching a detail of around 100 nm. The protocol indicates the required controls and settings for robust co-localization studies and an overview of

the statistical methods to analyze the imaging data properly.

INTRODUCTION:

The understanding and view of the synapse has changed enormously since its first description by Foster and Sherrington in 1897[1]. Since then, our knowledge of neuronal communication and the molecular processes behind it has grown exponentially[2]. It has become clear that synapses can be thought of as a two-compartment system: a pre-synaptic compartment containing vesicles for the release of neurotransmitters and a post-synaptic compartment with receptors[3]. This simplistic view, in the past twenty years, has evolved into a complex network of the proteins required to transduce transmitter binding into signaling[4].

The gains in the understanding are partially due to super-resolution techniques that overcame the diffraction limit of conventional light microscopy to suit the dimension of synapses better[5–10]. Due to the diffraction limit, an optical microscope cannot reach a resolution above 200 nm laterally[11,12]. To bypass this limit, super-resolution techniques were created, using different approaches and reaching different sub-diffraction limit resolutions: SIM, STED (Stimulated Emission Depletion Microscopy), PALM (PhotoActivated Localization Microscopy) and STORM (Stochastic Optical Reconstruction Microscopy)[13,14]. SIM doubles the spatial resolution of laser-based wide-field microscopy systems by inserting a diffraction grating into the excitation beam path[15]. The movable grating diffracts the laser beams to create a known illumination pattern, usually stripes. This purposely structured light pattern is superimposed to the unknown spatial distribution of the fluorescent dye (of the sample). The interference fringes formed by the two patterns encode for otherwise indistinguishable fine details with normal wide-field microscopy. The final super-resolved image is obtained by combining and decoding with mathematical methods several raw images of the same sample obtained by the translations and rotations of the diffraction grating. The resolution of the super-resolved images reaches 100 nm in the lateral and 500 nm in the axial directions for 2D-SIM[15] or 100 nm in the lateral and 250 nm in the axial directions for 3D-SIM[16].

The new understanding of the synapse is even more important in the light of the many neurological disorders where synaptic dysfunction plays a major role in onset and progression[17,18]. Alzheimer's disease, Down syndrome, Parkinson's disease, prion diseases, epilepsy, autism spectrum disorders and fragile X syndrome among others have been linked to abnormalities in synaptic composition, morphology and function[19–22].

Recently, using a set of SUMO-specific antibodies, we used SIM to show co-localization in primary hippocampal neurons of the SUMO proteins with the pre- and post-synaptic markers synaptophysin and PSD95 at super-resolution level[23]. This enabled us to confirm biochemical and confocal microscopy evidence of SUMO localization in neurons.

Here, we describe a protocol to study the localization of proteins in mouse hippocampal primary neurons. At the same time, this protocol may be adapted to different types of primary neuronal cultures.

PROTOCOL:

1 Primary cultures

1.1. Culture mouse hippocampal primary neurons in chambered coverslips (such as Ibidi µ-Slide 8 Well or Nunc Lab-Tek Chambered Coverglass) that match the objective requirement for #1.5 (0.17 mm) coverslip thickness.

1.2. Coat chambered coverslips with 100 µL of poly-L-lysine (100 µg/mL).

1.3. The next day, wash the chambered coverslips twice with sterile phosphate-buffered saline (PBS).

1.4. To obtain mouse primary neurons, isolate hippocampi from P1-P4 pups[23].

1.5. Place dissected hippocampi in 10 mL of Dissection Media (**Table 1**) and let them deposit at the bottom of the tube.

1.6. Using a sterile pipette, carefully remove the Dissection Media, leaving the hippocampi undisturbed at the bottom of the tube.

1.7. Add 10 mL of Media 1 (**Table 1**) to the hippocampi and incubate for 30 minutes at 37 °C.

1.8. Using a sterile pipette, carefully remove Media 1, leaving the hippocampi undisturbed at the bottom of the tube.

1.9. Add 10 mL of Media 2 (**Table 1**) and leave the (capped) centrifuge tube under the hood horizontally for 45 minutes.

1.10. Let the centrifuge tube stand vertically to allow the tissue to settle at the bottom of the tube.

1.11. Using a sterile pipette, carefully remove Media 2, leaving the hippocampi undisturbed at the bottom of the centrifuge tube.

1.12. Add 2 mL of Media 3 (**Table 1**).

1.13. Using a p1000 pipette with a filtered tip, mechanically dissociate cells from the tissue.

1.14. Transfer the supernatant, in which are located the isolated neurons, to a 15 mL centrifuge tube.

1.15. Centrifuge the cell suspension for 2 min at 300 x *g* at room temperature (RT).

1.16. After centrifugation, cells are located at the bottom of the centrifuge tube. Using a sterile pipette discard the supernatant.

1.17. Resuspend cells in 1 mL of Media 4.

1.18. Use a 70 µm filter to eliminate undissociated cells.

1.19. Count viable cells in a Bürker chamber by adding 1 µL of 0.4 % Trypan blue solution to 19 µL of the cell suspension.

1.20. Plate cells at 70,000 cells/well in a volume of 200 µL per well.

1.21. Allow the cells to attach for 2 h in a humidified incubator at 37 °C and 5% CO_2.

1.22. Take out the chambered coverslips from the incubator and carefully replace the medium with 200 µL of Culture Media.

1.23. Leave the chambered coverslips in a humidified incubator at 37 °C and 5% CO_2.

1.24. Replace one third of the medium with fresh culture media every 5-7 days.

1.25. Wait until hippocampal primary neurons are fully matured (12-14 days after plating) to perform co-localization studies.

2 Immunofluorescence staining

2.1. Take the chambered coverslips from the incubator.

2.2. Remove the medium.

2.3. Quickly wash the wells with 200 µL of PBS.

2.4. Add 4% paraformaldehyde (PFA) in PBS (200 µL/well) to neurons to fix them quickly.

2.5. Incubate the cells for 15 min at RT.

2.6. Remove the PFA solution.

2.7. Permeabilize the cells by adding PBS with 0.2% Triton X-100 (200 µL/well).

2.8. Incubate for 1 min at RT.

2.9. Remove the solution and incubate the samples with 1% bovine serum albumin (BSA) in PBS (200 µL/well) for 1 h at RT to passively cover all free binding surfaces of the plate with an

irrelevant protein for the analysis. A BSA-based blocking buffer without Triton X-100 reduces the antibody background more efficiently than the same buffer with 0.2% Triton X-100.

2.10. Remove the solution.

2.11. Add the primary antibody of choice diluted in a PBS solution containing 1% BSA and 0.2% Triton X-100 (120-200 µL/well, depending on the antibody dilution and availability). Incubate the samples for 2 h.

2.11.1. As a negative control, do not add any primary antibody to one of the wells. Multiple antibodies of different species against different targets may be used at the same time. Use an antibody against MAP2 (a neuronal marker) raised in chicken, an antibody against either PSD95 or synaptophysin raised in mouse, and an antibody against a target protein raised in rabbit. This allows three-color SIM analyses.

2.12. Quickly wash the wells three times with PBS (200 µL/well).

2.13. Add secondary antibodies (dyLight and Alexa secondary antibodies can both be used) diluted in a PBS solution containing 1% BSA and 0.2% Triton X-100 (200 µL/well). Incubate the samples for 1 h at RT.

2.14. Quickly wash the wells three times with PBS (200 µL/well).

2.15. Add Hoechst dye at a concentration of 1 µg/mL diluted in PBS (200 µL/well) to stain nuclei. Incubate the samples for 10 minutes at RT.

2.16. Quickly wash the wells twice with PBS.

2.17. Mount cells using a SIM-compatible mountant. Use 10 µL/well of ProLong Glass Antifade Mountant.

2.18. Cover and protect the cells with a coverglass (e.g., a round coverglass with a diameter of 8 mm). Square ones can also be used.

2.19. Store the chambered coverslips at RT and wait at least 48 h before acquiring images. Diamond Glass requires at least two days of curing before super-resolution acquisitions.

3 Antibody specificity control

NOTE: Use two strategies to assure antibody specificity. The first strategy is to use at least two different antibodies targeting the same substrate. The second strategy is antibody neutralization by incubation with the purified protein target or the epitope used to raise the antibody.

3.1. Incubate the antibody of choice with five times excess of the recombinant target or epitope for 1 h at RT in 1% BSA in PBS.

3.2. After the incubation, use the neutralized antibody at the usual concentration for staining as described above from 2.11.

4 Microscope calibration

NOTE: We routinely use an N-SIM Super-Resolution Microscope System manufactured by Nikon for the super-resolution studies. However, several other companies also offer super-resolution microscopes in their catalogues. Although specific indications for Nikon's N-SIM system are described, the instructions that follow can be generalized to other systems. Before the acquisition of SIM images, the system requires a proper calibration with specific sub-resolution size fluorescent beads. An example is the TetraSpeck microspheres. These beads are stained with different fluorescent dyes to allow the calibration of different lasers with one sample.

4.1 In a water bath sonicate around 1.8 x 10^8 fluorescent microspheres for 10 minutes. Nikon's N-SIM system requires a sparsely populated multicolor beads sample for the calibration. This could differ for other systems that require a dense single layer of sub-resolution size fluorescent beads. Adjust the number of fluorescent particles accordingly.

4.2 Dilute the fluorescent microspheres 1:500 in double distilled water.

4.3 Sonicate a second time for an additional 10 minutes.

4.4 Pipet 15 µL of the diluted beads into a well of a chambered coverslip.

4.5 Let the solution dry for 5 minutes at RT.

4.6 Add 10 µL of the mounting solution and place an 8 mm coverslip on top.

4.7 Wait at least 48 hours to allow proper curing.

4.8 Turn on the microscope and lasers.

4.9 Let the system warm up to reach the thermal equilibrium of all microscope components. N-SIM Super-Resolution Microscope System requires at least 3 hours.

4.10 Select the 100x objective.

4.11 Start the calibration by aligning the lasers to the center of the diffraction grating block. In the N-SIM system, a micrometer knob and a dedicated camera allow centering of the light beams to the target.

4.12 Insert the chambered coverslip in the microscope for viewing. Set the system to the chambered coverslip thickness by adjusting the objective correction collar. NIS software, the proprietary software provided with N-SIM Super-Resolution Microscope systems, has an automatic function to regulate correction collars.

4.13 Adjust grating block focus for each channel to ensure focused structured pattern illumination on the sample. NIS software provides an automatic function for this task.

4.14 Next, acquire raw 3D-SIM images of the multicolor microspheres. Reconstruct the raw images to obtain a super-resolved image using the microscope software or the open-source software platform for the analysis of biological images ImageJ[24] and the plugin fairSIM[25].

4.15 Calculate, for each separated wavelength, the Fourier transform of the super-resolved image obtained in 4.14. If the transformed image fails to obtain a correct flower-like pattern, restart calibration from 4.11 since super-resolution has not been achieved.

4.16 In the super-resolved image, select a single microsphere and calculate its intensity profile for each channel to measure the resolution achieved. It should now be close to 100 nm laterally.

4.17 Next, perform channel registration by overlaying a multichannel acquisition of the microspheres. The goal is to collimate all channel signals laterally and axially. This will eliminate chromatic aberrations due to the misalignment of the different channels and help the co-localization analysis.

4.18 Confirm the quality of calibration by using the functions “Illumination Phase Steps” and “Illumination Pattern Focus” of SIMcheck[26], a suite of plugins for the open-source application ImageJ. To this end, prepare a chambered coverslip to obtain a dense single layer of TetraSpeck microspheres and acquire a 3D-SIM image of the sample. Analyze the image in ImageJ and, if aberrations are detected, restart microscope calibration from step 4.11.

5. Acquisition

5.1. Start analyzing the sample using a 40x objective in confocal or widefield mode. This allows navigation to the sample, maintaining good details and a large field of view.

5.2. Use MAP2 antibody signal to identify an area representing neuronal processes.

5.3. Acquire images of the sample in confocal mode to determine the quality of the staining. Poor confocal quality will reflect in poor SIM quality, therefore requiring the samples to be discarded.

5.4. If the area and the quality of the images are satisfactory, switch the objective to 100x.

5.5. Apply oil to the 100x objective.

5.6. Acquire a widefield or confocal image that will be used later to assess the quality of the super-resolved image (**Figure 1A,B**).

5.7. Switch to 3D-SIM mode.

5.8. Using dialog windows to set parameters for acquisition, select the highest bit-depth setting available to maximize color information. Typically, 16-bit is the standard choice. Moreover, to improve signal-to-noise ratio, select a low frequency value for acquisition, such as 1 MHz.

5.9. Using histogram windows, set lasers power to obtain a linear response of signal. To avoid loss of information, limit saturated pixels in the images. The N-SIM system uses an Andor iXon3 camera. When working at 16-bit, choose a target intensity of 16,000 to ensure the linear response of the camera. Alternatively, choose a range between 30,000-45,000 to maximize the dynamic range of the acquisition.

5.10. Set laser power between 0.1% and 50% when imaging the samples and exposure times between 50 ms and 2 s. Laser powers above 50% may cause rapid photobleaching of the fluorophores in use.

5.11. Start acquiring the images in 3D-SIM mode.

5.12. Use SIMcheck, a suite of free plugins for ImageJ, to assess the quality of acquisition of the raw images.

5.13. If SIMCheck does not detect any artifacts or quality issues, acquire a minimum of 10 images from 4 technical replicates to allow statistical analysis.

6 Post-production: image reconstruction

NOTE: 3D-SIM acquired images are raw images that need to be processed to obtain reconstructed super-resolved images. Incorrect reconstruction of raw images can lead to artifacts that would affect the analysis of the samples. Great attention should therefore be paid to properly choosing reconstruction parameters.

6.1. Process the raw images using the microscope reconstruction analysis software to obtain a super-resolved image (**Figure 1C**). Alternatively, use the freely available ImageJ plugin fairSIM to reconstruct raw images.

6.2. Calculate the Fourier transform of the super-resolved images using the microscope reconstruction software or ImageJ plugin SIMCheck. A good reconstructed image should return, for each channel, a flower-like image. If the reconstructed images fail to recreate a flower-like shape, restart from the raw images and reconstruct them by modifying the reconstruction

parameters such as Wiener filtering, apodization and zero-order suppression[27]. In NIS software, using the preview to monitor how changing the parameters affects the final resolved image, modify the parameters i) Illumination Modulation Contrast, ii) High Resolution Noise Suppression and iii) Out of Focus Suppression.

6.3. Next, analyze the reconstructed image to unbiasedly detect artefacts by using NanoJ-SQUIRREL[28], an ImageJ-based plugin to assess the quality of super-resolved images.

6.4. If NanoJ-SQUIRREL detects artifacts, restart from the raw images and reconstruct them by modifying the reconstruction parameters such as Wiener filtering, apodization and zero-order suppression. In NIS software, using the preview to monitor how changing the parameters affects the final resolved image, modify the parameters Illumination Modulation Contrast, High Resolution Noise Suppression and Out of Focus Suppression.

6.5. Use the super-resolved images to calculate the co-localization profile and/or Pearson's and Mander's coefficients.

7 Co-localization with profile analysis

NOTE: As a first step to study co-localization between synaptic markers and a protein of interest, take a super-resolved image and analyze a single locus to determine signal overlap.

7.1. Identify a single locus on the super-resolved image.

7.2. Obtain the intensity profiles of the fluorescent signals of the locus of interest.

7.3. Export the data.

7.4. Use GraphPad Prism, or a similar analysis software, to normalize all signal peaks and obtain comparable signal intensities for each channel with the final goal of determining locus specific co-localization.

8 Quantification of Pearson's and Mander's coefficients

NOTE: If profile analysis has suggested single locus co-localization, a more general analysis of the whole image can be carried out by calculating Pearson's and Mander's coefficients[29,30].

8.1. Use JACoP[31], an ImageJ plug-in, to determine the two parameters of co-localization: Pearson's and Mander's.

9 Statistical analysis

9.1. Use GraphPad Prism, or a similar analysis and graphing software, to process data collected with JACoP.

9.2. Use at least 40 SIM images for each condition analyzed to obtain graphs and for statistical relevance.

REPRESENTATIVE RESULTS:

We present here the standard workflow to study neuronal proteins co-localization. We first calibrated the microscope and next we performed SIM analysis of the samples. To calibrate the system, we used fluorescent microspheres of 0.1 µm diameter. Upon obtaining super-resolved 3D-SIM images of the beads, the underlying image data are Fourier-transformed to re-convert them to a spatial frequency representation. In **Figure 2A**, the distinct flower pattern is presented as an indication of super-resolution detail levels. We next measured the resolution achieved by calculating the full width at half maximum (FWHM) of the peak of a single bead's intensity profile (**Figure 2B,C**). Finally, we corrected chromatic aberration by channel registration, again using fluorescent microspheres (**Figure 3A,B**). Next, we started analyzing the sample with a 100x objective and we acquired 3D-SIM images. We used SIMCheck to assess evenness of field-illumination or movement during acquisition (**Figure 4A**). We checked differences in intensity between illumination pattern angles (**Figure 4B**) and we calculated the ratio of the modulation contrast to noise to measure the local stripe contrast (**Figure 4C**). Finally, we estimated the effective resolution of the reconstruction (**Figure 4D**).

We next confirmed the quality of the super-resolved images by using NanoJ-SQUIRREL. In the first reconstructed image (**Figure 5A**), NanoJ-SQUIRREL detected the presence of artefacts (**Figure 5C**). We changed the reconstruction parameters to obtain a new super-resolved image (**Figure 5B**) and NanoJ-SQUIRREL confirmed the lack of artifacts (**Figure 5D**). After having calibrated the system and assessed the quality of the reconstructed images, we next started analyzing the primary neuronal cultures stained with an antibody against MAP2, a neuronal marker, PSD95, a post-synaptic marker and the target protein SUMO1. We first analyzed the sample performing four-channel confocal microscopy with a 40x objective (**Figure 6A**). Upon selecting an area representing neuronal processes, we switched to a 100x objective. We acquired both confocal and SIM images of the same area to assess quality of reconstruction with NanoJ-SQUIRREL and perform co-localization analysis. In **Figure 6B**, we show the super-resolved 3D-SIM image of neurons stained for SENP1 and drebrin. Co-localization in super-resolved images can be analyzed with profile analysis (**Figure 7A**) and quantification of Pearson's and Mander's coefficients (**Figure 7B**).

FIGURE LEGENDS:

Figure 1: Comparison of widefield, confocal and SIM acquisitions. (**A**) Widefield image of primary hippocampal neurons immunostained for SENP1 (green), drebrin (red) and MAP2 (mauve). DAPI was used to stain nuclei. Scale bar 5 µm. (**B**) Confocal image of the same sample of panel **A**. (**C**) SIM image of the same sample of panel **A** and **B**.

Figure 2: Analysis of a 3D-SIM image of microspheres for microscope calibration. (**A**) Fast Fourier transform of an acquisition of microspheres with its flower-like shape. (**B**) Selection of a single microsphere to determine lateral spatial resolution. (**C**) Intensity profile of the single

microsphere in **B** with the measurement of its FWHM. The values represent the resolution achieved by the instrument.

Figure 3: Three channel registration. (**A**) Acquisition of multicolor (wavelengths 488nm, 555nm and 647nm) TetraSpeck microspheres before registration. (**B**) Acquisition of the same sample after calibration.

Figure 4: Quality assessment of raw and reconstructed images using SIMcheck. (**A**) *Motion and Illumination Variation* analysis using SIMCheck. Signal grey to white represents homogeneous illumination and absence of movement during acquisition. (**B**) *Channel Intensity Profile* obtained by analyzing the raw image. In this example there intensity variation is minimal, to suggest lack or bleaching or fluctuations. (**C**) *Raw Modulation Contrast* to calculate the ratio of the modulation contrast-to-noise within the image. The heatmap shows modulation contrast variations. (**D**) *Reconstructed Fourier Plot* to analyze the amplitude Fourier spectrum to determine the effective resolution of the reconstruction.

Figure 5: Assessment of super-resolution image quality using NanoJ-SQUIRREL. (**A**) Reference super-resolution image with artefacts. (**B**) Reference super-resolution image of good quality. (**C**) Image representing NanoJ-SQUIRREL error map of **A**. Lighter areas represent large scale artifacts, while darker ones represent correct reconstruction. (**D**) NanoJ-SQUIRREL error map of **B**.

Figure 6: SIM image sample. (**A**) Confocal microscopy of primary neurons. A 40X-objective was chosen to obtain an overview of the sample while maintaining good resolution. Cells were immunostained for SUMO1 (green), PSD95 (red) and MAP2 (mauve). DAPI was used to stain nuclei. Scale bar 50 µm. Images were displayed as Z projection. (**B**) SIM images for SUMO1 and PSD95 on the area highlighted in the green box in panel A using a 100X objective. Red arrowheads indicate the position of the inset shown in **A** used to calculate the intensity profile. Scale bar 5 µm.

Figure 7: Co-localization analysis. (**A**) Super-resolved image of primary neurons stained with an antibody against SENP1 (in green) and drebrin (in red), scale bar 0.5 µm, and its intensity profile. The values of the graph were normalized for each channel to 100 (arbitrary unit) and correspond to the pixel intensity shown by the blue arrow. (**B**) Analysis using JACoP to calculate Pearson's Correlation Coefficient and Mander's coefficient between SENP1 and drebrin. Windows of the plug-in set up and visual threshold are shown. Mander's coefficient is expressed by two values – SENP1 fraction that co-localizes with drebrin (M1) and the drebrin fraction that co-localizes with SENP1 (M2).

DISCUSSION:

Elucidating the structure and composition of the synapse is crucial for understanding the physiological and pathological processes that regulate memory and cognition. While in the normal state, synapses are the building blocks of memory, they also underlie complex neurological disorders such as Alzheimer's disease[32]. The protocol described here serves to study the co-localization of neuronal proteins with a super-resolution microscopy technique called SIM.

Using a particular pattern of illumination, SIM can reach a resolution of about 0.1 µm, which is suited for the study of synapses, which normally measure between 0.03 and 0.15 µm. For even greater detail, other super-resolution techniques such as STED (Stimulated Emission Depletion Microscopy), PALM (PhotoActivated Localization Microscopy) or STORM (Stochastic Optical Reconstruction Microscopy), that can reach a resolution of 10-20 nm, may be applied[33].

Here, we describe the analysis of co-localization of target proteins with synaptic markers in primary neurons. The protocol can be applied to any primary culture of neuronal cells, such as hippocampal, cerebellar or cortical neurons and even to cultures of primary neurons that do not belong to the central nervous system, such as enteric nervous system neurons. The key to the analysis at super-resolution level, however, is the reagents used during acquisition, such as chambered coverslips and mounting solutions compatible with the diffraction index of the objective. We used chambered coverslips for their ease of use, but the classical, cheaper method of growing primary neurons on coated coverglass is nevertheless valid, particularly with a high precision #1.5H (0.17 mm) coverglass. In addition, a mounting media that can reach a refraction index as close as possible to the refraction index of glass (1.52) and an immersion oil for the 100x objective with a refraction index of 1.515 should be used. Constant room temperature and stabilized tables are also mandatory to guarantee the accuracy of the acquisitions.

We use both dyLight and Alexa secondary antibodies in the SIM studies. Due to their narrow peaks of excitation and emission and good quantum yield, they are indicated for super-resolution techniques that require the best signal to noise ratio. Dempsey et al. compared Alexa, dyLight and other fluorophores for super-resolution imaging[34].

During acquisition, we routinely set the camera at 1 MHz over 10 MHz. 1 MHz, thanks to a slower acquisition speed, gives the images more accuracy and less noise than 10 MHz. 1 MHz read-out mode can also record with a bit depth of 16 bit (compared to the maximum 14 bit of 10 MHz), giving more color information and a more precise color gradient to the images. However, 10 MHz, with its speed, is useful for live images. To avoid bleaching and preserve fluorophore, we also set laser power as low as possible. To improve signal intensity, gain values can be increased. It is worth noting that lower gain guarantees cleaner images without enhancing noise. In general, best results are obtained while imaging within 7 µm from the bottom of the chambered coverslip. This is especially important when using a 100x objective with oil immersion. If deeper acquisition across the cells/tissues is required, a better choice may be the use of a 60x objective with water immersion.

One of the main challenges in performing SIM studies is image reconstruction[35]. Obtaining super-resolved images without artefacts and aberration requires not only the use of ad-hoc experimental conditions, but also careful calibration of the system and parameter optimization to obtain the final images. In the protocol, we describe how to avoid some of the most common mistakes by assessing calibration of the system and quality analysis of raw and reconstructed images. Specifically, we describe the use of the ImageJ plugins SIMCheck and NanoJ-SQUIRREL to assure correct instrument settings to prevent common artifacts of super-resolved images. The

applications allow for an unbiased quality assessment of the final images that is not based on subjective benchmarking the results against prior knowledge of the structures of study.

We suggest using synaptophysin and PSD95 or drebrin as pre- and post-synaptic markers, though other markers are valid as well. A huge body of literature describes proteins such as bassoon as synaptic markers[36,37]. It is worth noting that pre- and postsynaptic markers are however present throughout the cell, excluding the nucleus. Much of their signal is non-synaptic but represents proteins in transport or degradation, background or other artifacts. It is therefore important to carefully choose the area of the analysis. We use MAP2 antibody signal to choose axon and dendritic terminals.

In the analysis of co-localization we use two approaches. The first is a visual approach, based on profile analysis that shows single events of co-localization and identifies the contribution of each channel. A caveat of this approach, however, is the poor statistical power. For this reason, we decided also to use a second method based on analysis of a larger number of events representative of the entire field of each image. This method is based on calculation of the Pearson's correlation coefficient and Mander's M1 and M2 coefficients. We use the Pearson's correlation coefficient to describe the overlap of signals in the image and Mander's M1 and M2 to describe reciprocal co-localization between signals of interest[38]. For the calculation, we employ the ImageJ plugin JACoP, since it has a feature that allows you to set a manual threshold to discard any background contribution to the analysis, especially critical for Mander's analysis.

DISCLOSURES:

The authors have nothing to disclose.

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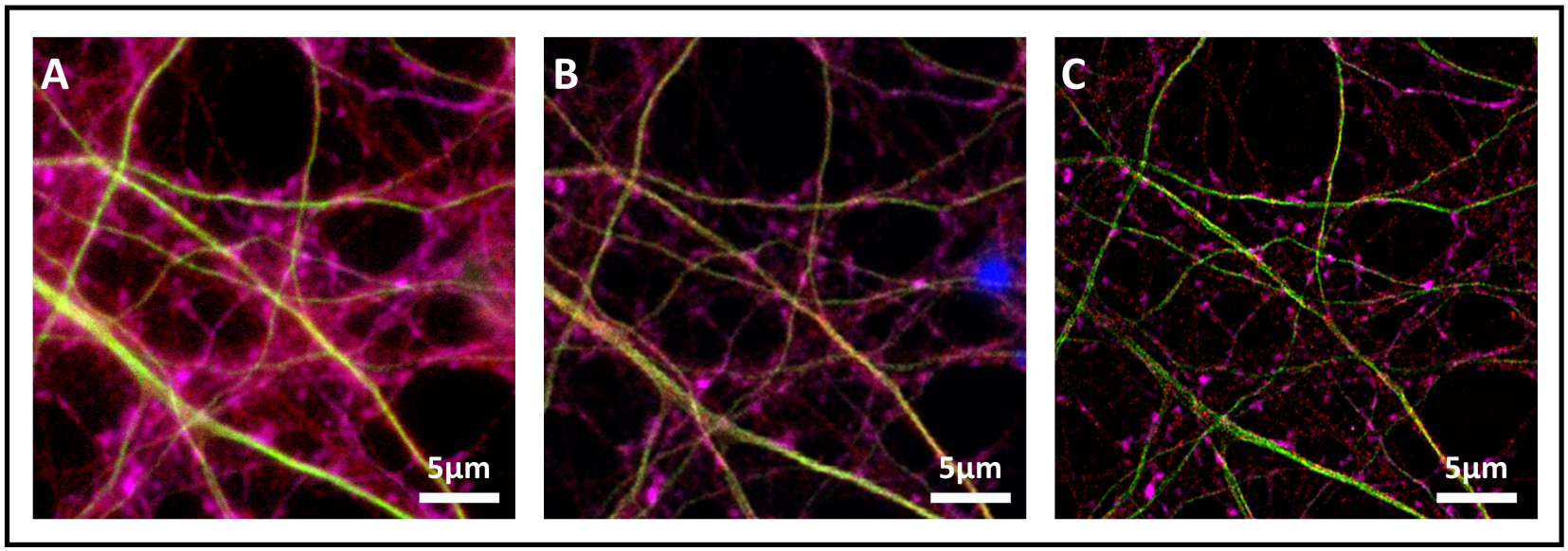


Figure 1

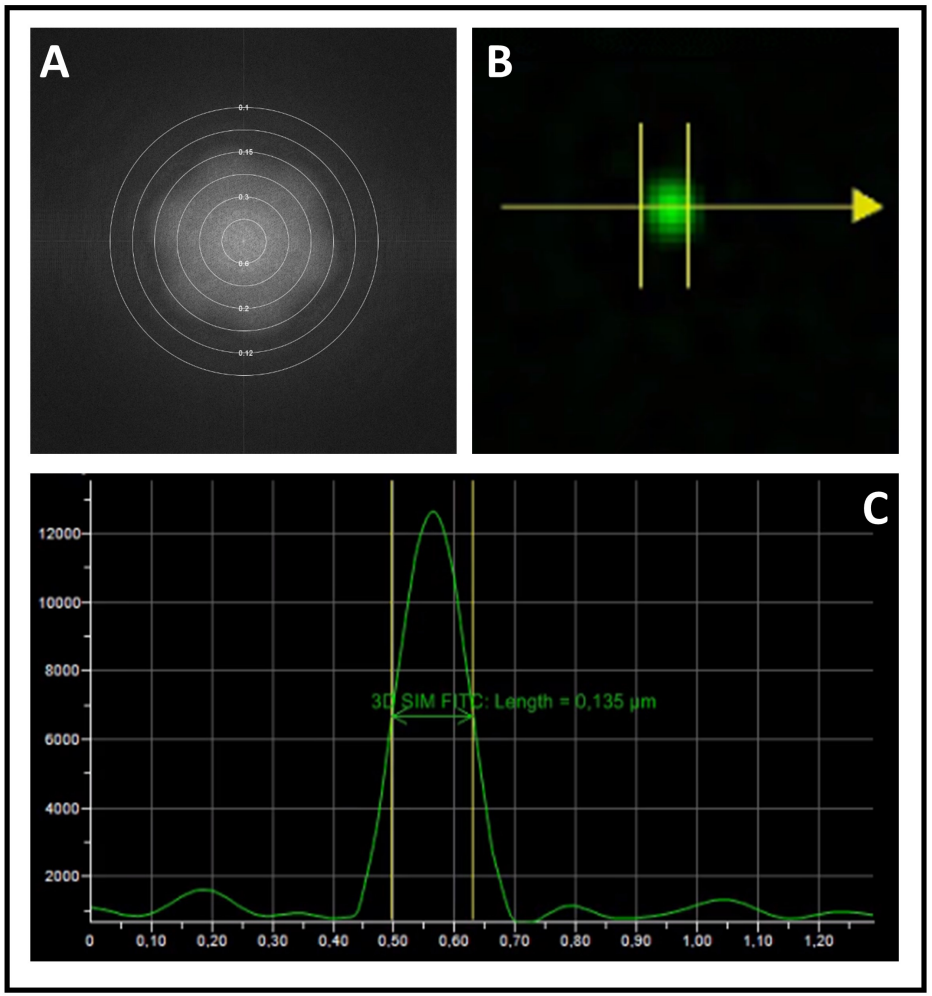


Figure 2

Figure 3

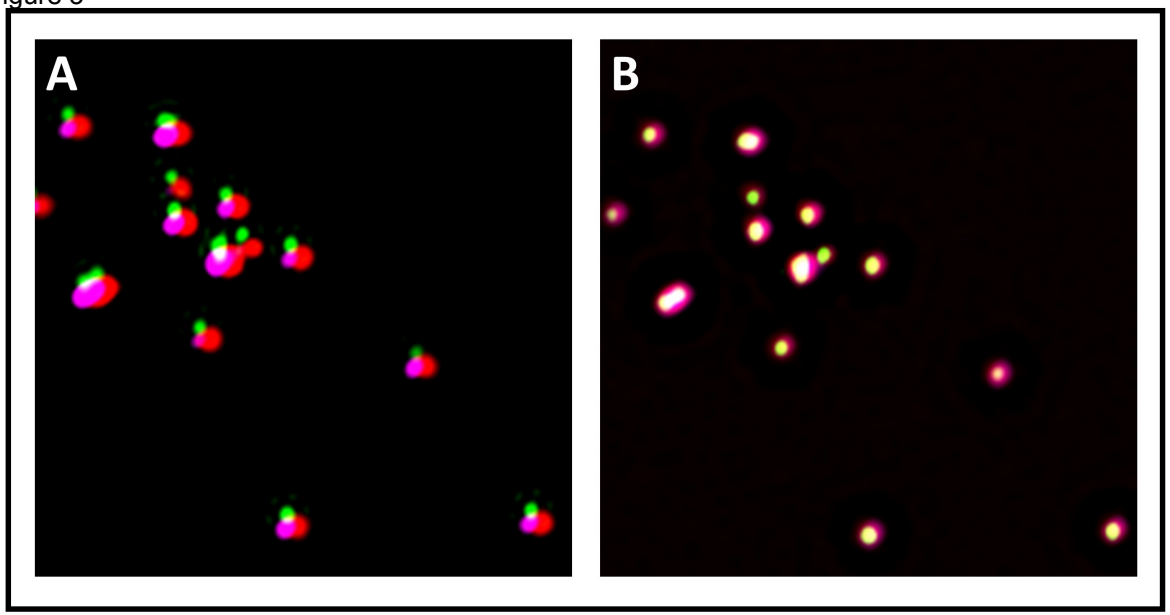


Figure 3

Figure 4

Click here to access/download;Figure;FIG4-converted.pdf

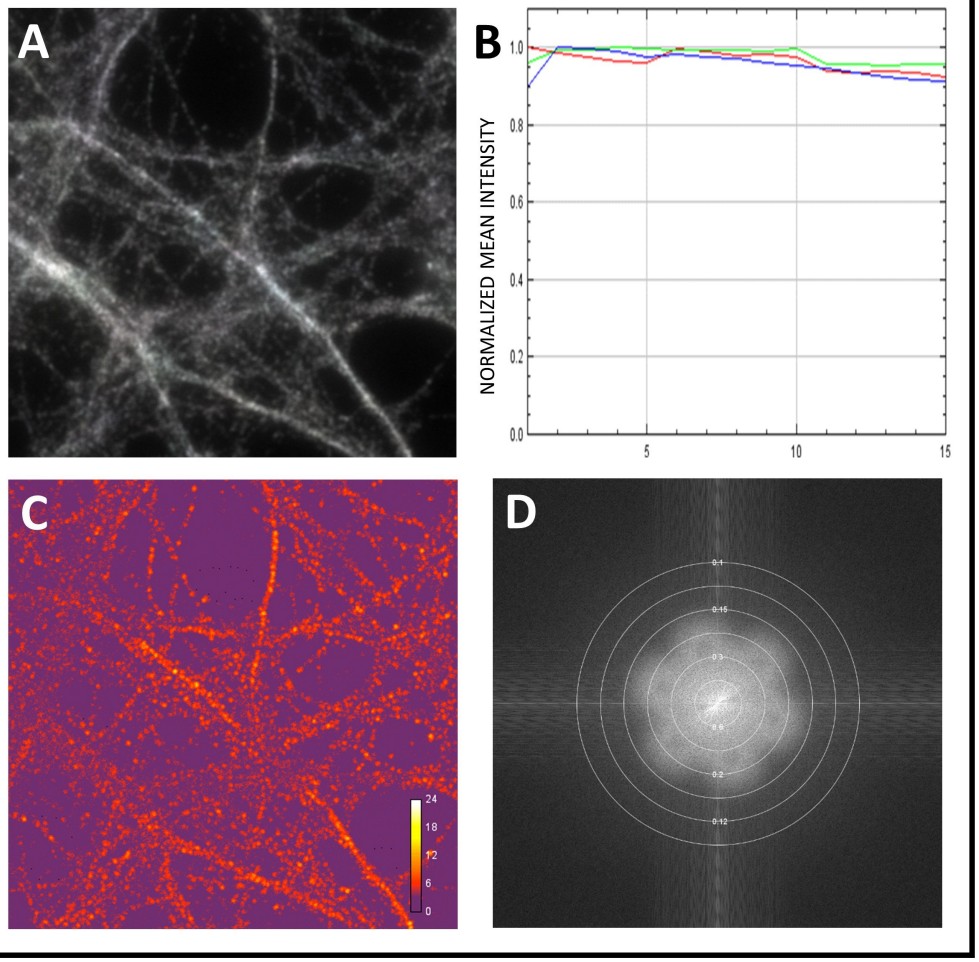


Figure 4

Figure 5

Click here to access/download;Figure;FIG5-converted.pdf

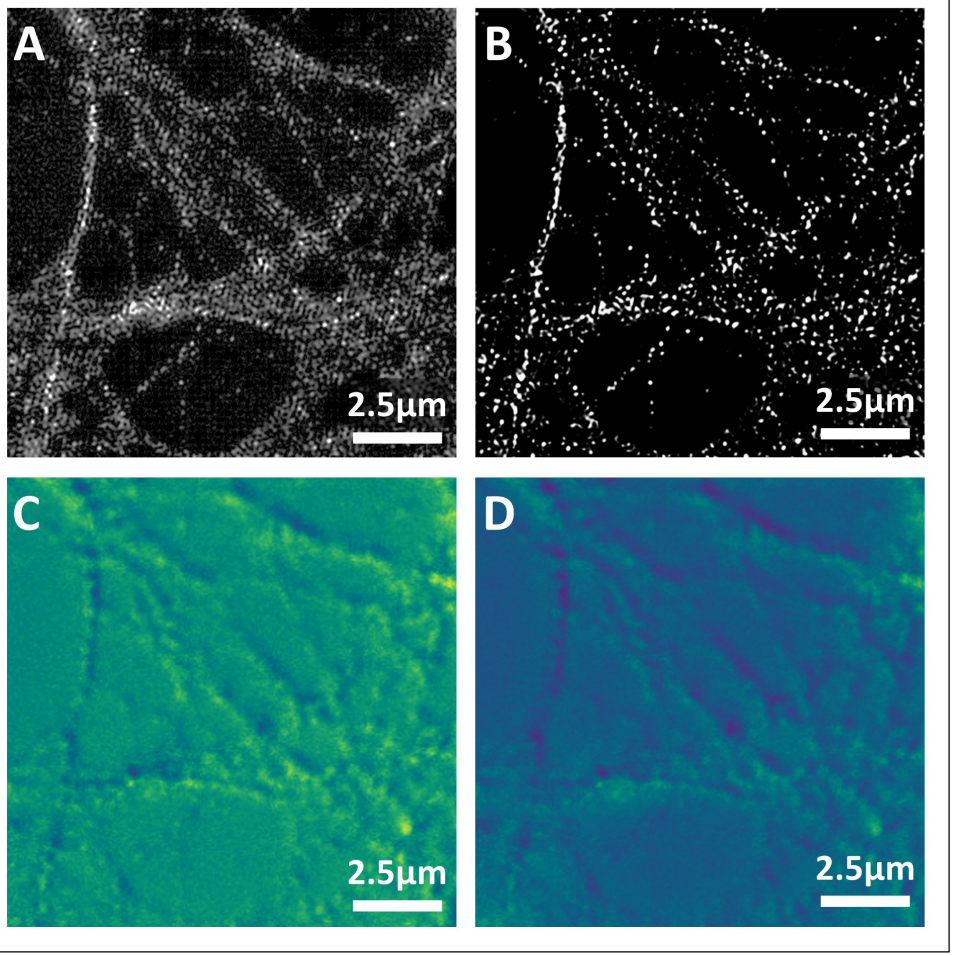


Figure 5

Figure 6

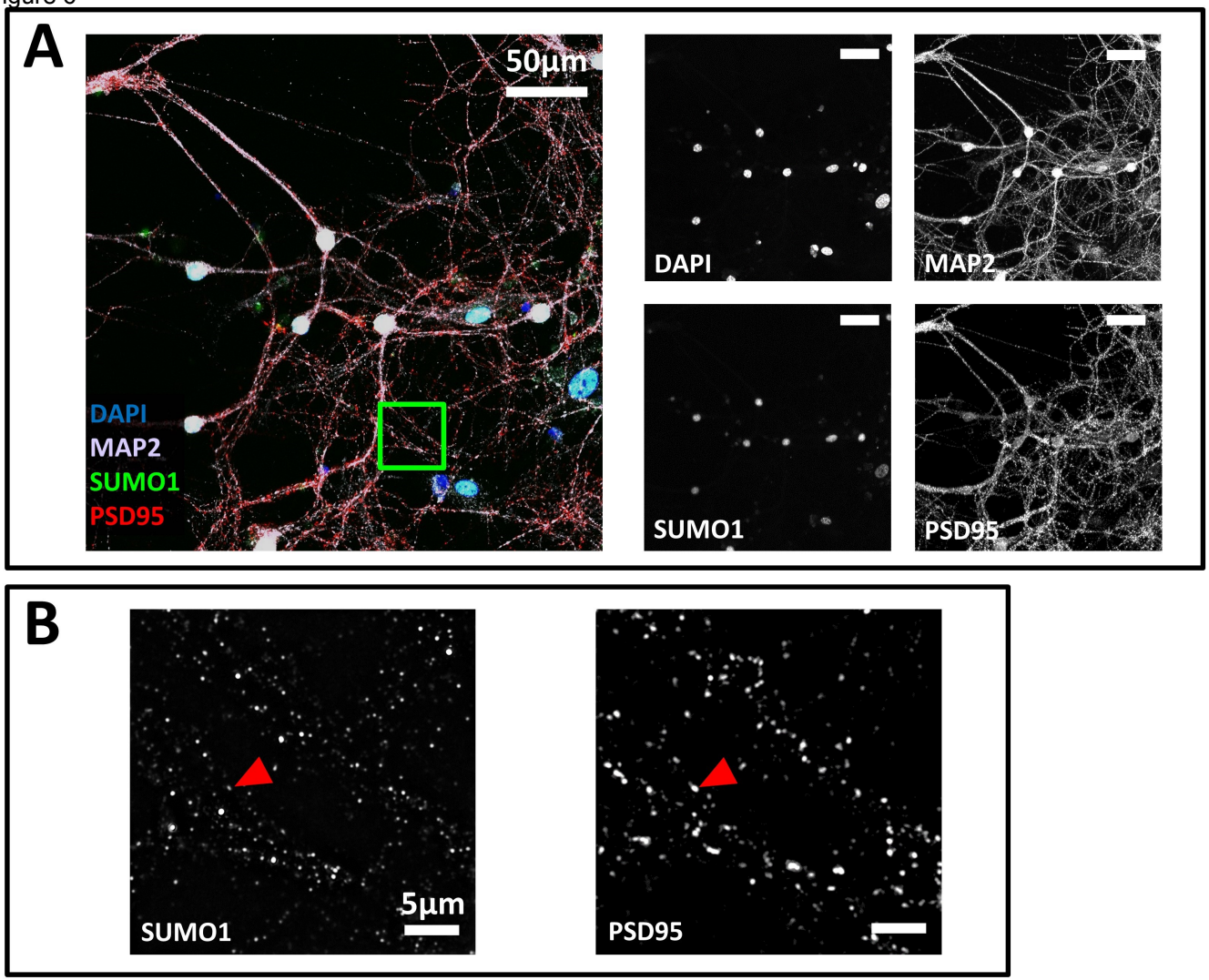
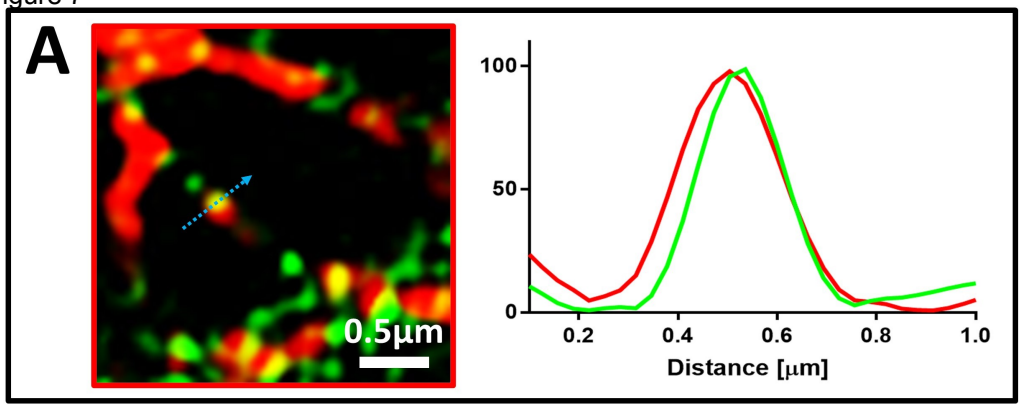


Figure 6

Figure 7

Click here to access/download;Figure;FIG7-converted.pdf



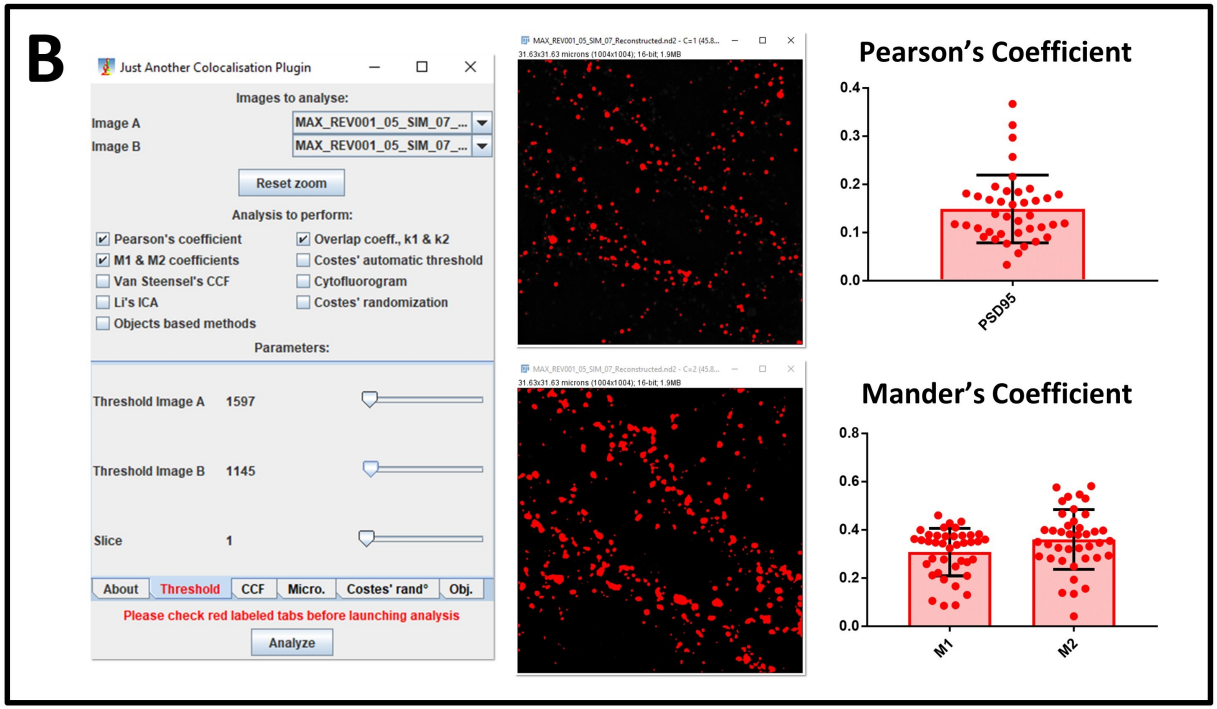


Figure 7

Table

	CNDM	Glucose	Pen/Strep 100X	L-Cystein	Papain	Trypsin inhibitor	MEM with 50mM Glutamine	FBS	Neurobasal A
Dissociation media	9.7 mL	20 mM	0.5 mg/mL						
Media 1	9.64 mL	20 mM		5.7 µM	20 U/mL				
Media 2	9.64 mL	20 mM				130 nM			
Media 3		20 mM					8.7 mL	1 mL	
Media 4 (for 50 mL use)		20 mM	0.5 mg/mL				43.5 mL	5 mL	
Culture Media			0.5 mg/mL						9.6 mL

Glutamax 50mM	B27 Supplement (50X)	MgCl2	CaCl2	HEPES	NaOH
		0.58 mM	0.05 mM	0.32 mM	0.02 mM
		0.58 mM	0.05 mM	0.32 mM	0.02 mM
		0.58 mM	0.05 mM	0.32 mM	0.02 mM
125 µM	200 µL				

Table of Materials

Item	Catalogue #	Manufacturer	Comment
0.4% Trypan blue solution	15250061	Thermo Fisher Scientific	Chemical
70 µm filter	352350	Corning	Equiment
Alexa	-	Thermo Fisher Scientific	Antibody
Antibody SENP1	sc-271360	Santa Cruz	Antibody
B27 Supplement	17504044	Life Technologies	Chemical
Bovine serum albumin	5470	Merck	Chemical
$CaCl_2$	21115	Merck Life Science	Chemical
Chambered coverslips	80826	Ibidi	Equiment
DyLight	-	Thermo Fisher Scientific	Antibody
FBS (Hyclone)	SH3007002 (CHA1111L)	GIBCO	Serum
FluoSpheres carboxylate-modified microspheres, 0.1 µm, yellow–green fluorescent	F8803	Thermo Fisher Scientific	Equiment
Glucose	G8769	Merck Life Science	Chemical
Glutamax	35050061	GIBCO	Chemical
HEPES	H3537	Merck Life Science	Chemical
L-Cystein	C6852-25g	Merck Life Science	Chemical
MAP2	AB15452	Merck	Antibody
MEM	21575022	Life Technologies	Medium
MgCl	M8266	Merck Life Science	Chemical
NaOH	1,091,371,000	VWR International	Chemical
Neurobasal A	10888022	Life Technologies	Medium
N-SIM Super Resolution Microscope	-	Nikon	Instrument
Papain	P-3125	Merck Life Science	Chemical
paraformaldehyde	28908	Thermo Fisher Scientific	Chemical
Pen/Strep 10x	15140122	Life Technologies	Chemical
phosphate-buffered saline	10010023	Gibco	Chemical
Poly-L lysine	P2636	Sigma	Chemical
ProLong Diamond Glass Antifade Mountant	P36970	Thermo Fisher Scientific	Chemical
PSD95	K28/43	NeuroMab	Antibody
Round coverglass	12052712	Thermo	Equiment
SUMO1	ab32058	Abcam	Antibody
Synaptophysin	S5768	Merck	Antibody
Triton X-100	T8787	Merck	Chemical

Trypsin inhibitor	T9003-500MG	Merck Life Science	Chemical

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
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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).

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