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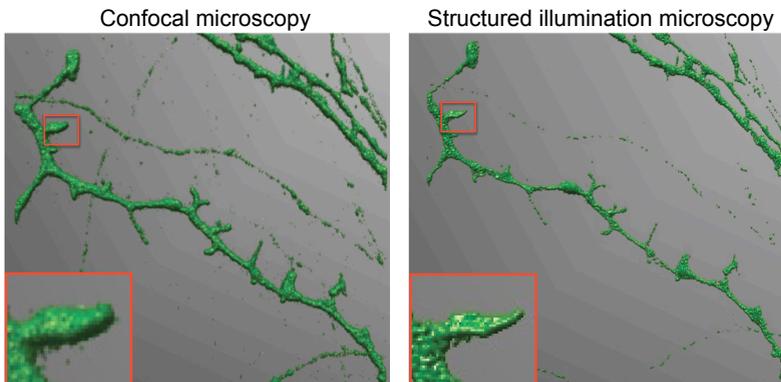
Imaging Dendritic Spines of Rat Primary Hippocampal Neurons Using Structured Illumination Microscopy

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Graphical abstract



3 dimensional reconstructions of a dendritic spine obtained by conventional confocal microscopy (left) and structured illumination microscopy (SIM, right). In this chapter we provide a working protocol for obtaining dendritic spine images in primary hippocampal cultures using SIM. We further show that, compared to confocal microscopy, a significantly higher resolution can be obtained using this protocol.

Abstract

Dendritic spines are protrusions emerging from the dendrite of a neuron and represent the primary postsynaptic targets of excitatory inputs in the brain. Technological advances have identified these structures as key elements in neuron connectivity and synaptic plasticity. Despite their relevance for many physiological and pathological processes in neurons, the quantitative analysis of spine morphology using light microscopy remains an essential problem due to technical limitations associated with light's intrinsic refraction limit. Dendritic spines can be readily identified by confocal laser-scanning fluorescence microscopy. However, measuring subtle changes in the shape and size of spines is difficult because spine dimensions other than length are usually smaller than conventional optical systems resolution fixed by light microscopy's theoretical resolution limit of 200nm.

Several recently developed super resolution techniques have been used to image cellular structures smaller than the 200nm limit, including dendritic spines. These techniques are based on classical far-field operations and therefore allow the use of existing sample preparation methods and to image beyond the surface of a specimen. Described here is a working protocol to apply super resolution structured illumination microscopy (SIM) to the imaging of dendritic spines in primary hippocampal neuron cultures. Possible applications of SIM overlap with those of confocal microscopy. However, the two techniques present different applicability. SIM offers higher effective lateral resolution, while confocal microscopes, due to the usage of a physical pinhole, achieve resolution improvement at the expense of removal of out of focus light¹.

In this protocol, primary neurons are cultured on glass coverslips using a standard protocol, transfected with DNA plasmids encoding fluorescent proteins and imaged using SIM. The whole protocol described herein takes approximately 2 weeks, because dendritic spines are imaged after 16-17 days *in vitro*, when dendritic development is optimal. After completion of the protocol, dendritic spines can be reconstructed in 3D from series of SIM image stacks using specialized software.

Introduction

A dendritic spine is a small protrusion of the neuron membrane. This characteristic structure is specialized to typically receive input from a single synapse and represents the physical contact area between two neurons. Most functionally mature dendritic spines consist of a globular tip, termed head, and a thin neck that connects the head to the dendritic shaft. However, spines are not static and actively move and change their morphology continuously even in the adult brain². Within a 2 week period of time, rat primary hippocampal neuron cultures derived from late embryonic or early postnatal time develop complex dendritic arbors with numerous membrane protrusions that evolve from early filipodia to spine-like structures³. Based on this dynamic behavior and other characteristics, dendritic spines are thought to provide an anatomical substrate for memory storage and synaptic transmission^{4,5}.

Given the critical role that dendritic spine size and shape have in synaptic function, it is important to measure their dimensions accurately. Spines vary from around 200 to 2000 nanometers in length and can be readily identified by confocal laser-scanning fluorescence microscopy. However, spine dimensions other than length are usually below the conventional optical systems' resolution, theoretically fixed by diffraction around 200 nanometers⁶. These resolving powers are insufficient for imaging finer details, such as the width of spine necks and heads. Much work has been dedicated to solve this problem and many relatively new super-resolution microscopy techniques have provided substantial progress. In particular, it is possible to achieve resolution beyond the classical limit without discarding any emission light by using laterally structured illumination microscopy (SIM) in a wide-field, non-confocal microscope⁷⁻¹⁰. Using this technique in combination with non-linear microscopy techniques, it is theoretically possible to improve the lateral resolution of the optical microscope by an unlimited factor¹¹. However, in most experimental circumstances, SIM allows to surpass the resolution limit by a factor of two¹. Other super-resolution optical microscopy techniques such as Stimulated emission depletion (STED) microscopy¹² and photo-activation localization microscopy (PALM)¹² have been applied to imaging of dendritic spines. Localization-based methods such as PALM require very large numbers of raw images to achieve super-resolution and

are therefore limited in speed. On the other hand, STED can achieve high imaging speed, although at relatively low photon counts and small fields of view, which may not be the case for SIM¹³.

In this article the aim is to provide a working protocol to image dendritic spines from rat primary hippocampal neurons cultured in vitro using SIM. The protocol consists of two distinguishable phases: an initial one consisting of establishment, development, transfection and immunohistochemistry of rat primary hippocampal neuron cultures and a late phase dedicated to sample imaging.

Protocol

All experimental procedures involving animals were optimized to reduce animal suffering and were approved by the Commission for Animal Experimentation, University of Amsterdam, DEC protocol # DED204 and DED250.

Section 1 : Coverslip preparation.

1.1) Cut down the coverslips to a size of 15mm x 15mm using a carbide or diamond scribe, so that they fit into the wells of a 12-well plate.

1.2) While working in a fume hood, start coverslip coating by fully submerging coverslips in concentrated nitric acid solution (70% wt/wt) in a glass container. To ensure even distribution shake, then incubate for a minimum of 4hr. It is possible to reuse the concentrated nitric acid solution for approximately 2 times, but it can lose color by exposure to light.

1.3) Remove the concentrated nitric acid solution and wash the coverslips four times in distilled water for 30min, carefully shaking after each wash.

1.4) With caution remove the water.

Note: the next three steps are performed in a sterile lamimar flow cabinet.

1.5) Soak the coverslips in 96% EtOH. Remove the coverslips from the EtOH solution and let them dry.

1.6) Flame the coverslips and put them in a glass container. Use fine forceps to handle the coverslips (Dumont style no.5 forceps for example).

1.7) Cover the container with aluminum foil and bake in a dry-heat oven for 12-16hr at 180°C. Baked coverslips can be stored at room temperature for up to 1 month if covered tightly.

Section 2: Coverslip coating.

The coverslip coating procedure favors neuron attachment to the glass surface and dendritic arborization¹⁴.

2.1) In a sterile laminar hood, use sterile small forceps to place the individual coverslips in single wells of a 12- wells plate.

2.2) Add 500µl of Poly-L-lysine solution (or sufficient amounts to submerge the coverslips).

2.3) Wrap the plate in aluminum foil to prevent evaporation and leave it overnight at room temperature.

2.4) Before starting the culture, aspirate the Poly-L-lysine solution carefully in a sterile laminar hood.

2.5) Wash each well with 1ml of sterile water twice, whilst preventing them to dry out.

2.6) Aspirate water completely, add 1ml of plating medium and leave the coverslips in the tissue culture incubator until you are ready to plate the cells. It is recommended to plate the cells within 24hr.

Section 3: Removal of brains from E16-E19 rat embryos.

3.1) Sterilize the surgical instruments by heating them in a dry sterilizer overnight or washing them with 70% EtOH. Dry thoroughly if EtOH is used.

3.2) Prepare several 30mm dishes with 1xHBSS buffer and keep them on ice.

3.3) Euthanize the rat dam with an intraperitoneal injection of Euthasol (160mg/kg Euthasol in a volume of ±0.4ml).

3.4) Check for the absence of reflexes.

3.5) Spray the dam's abdomen with 70% EtOH.

3.6) Make an incision along the abdomen and remove the uterus.

3.7) Remove the embryos from the uterus and place them in a 100 mm diameter petri dish.

3.8) Remove the heads of the embryos with large scissors and place the heads in a new petri dish containing cold 1xHBSS buffer.

Note: from here to step 7.1 the procedure is performed under sterile conditions in a laminar flow cabinet.

3.9) Hold down the heads along the sides with big forceps.

3.10) With small scissors, make a saggital cut in the skin on top of the head, then laterally peel the skin down with a large forceps.

3.11) Use the same approach as 3.10 to remove the skull. Make a saggital cut starting at the caudal end, gently opening the skull without damaging the brain tissue. Fold the two halves of the skull away laterally exposing the brain.

3.12) With the blunt spatula, scoop out the brain and place it in fresh cold 1xHBSS.

Section 4. Dissection of the hippocampi.

It is very important that the dissection is done as quick and sterile as possible to ensure cell viability. Keep the samples cold on ice.

4.1) Remove and discard the cerebellum with the fine scissors.

4.2) Separate the two hemispheres of the brain by making a sagittal cut along the midline.

4.3) Take each hemisphere and put them in a new 30 mm dish containing fresh cold 1xHBSS.

4.4) Place each hemisphere such that the temporal lobe faces the bottom of the dish.

NOTE: From here on, it is recommended to use a dissecting microscope.

4.5) Gently hold the midbrain using a small forceps and remove the midbrain with another pair of forceps. Leave the remainder of the hemisphere intact containing the cortex and the hippocampus.

4.6) Turn over the tissue, so that the hippocampus is now facing the bottom of the dish.

4.7) Gently hold the hemisphere in place with a fine forceps. By using another fine forceps, carefully and gently remove the meninges. It is easier to start at the olfactory bulb. Use caution so as not to damage the hippocampus.

4.8) Orient the tissue so that the hippocampus is now facing up. You can now see the hippocampus by its characteristic C-shaped structure.

4.9) By using fine forceps, dissect out the hippocampus. Collect it in a new 30mm dish containing fresh cold 1xHBSS.

Section 5: Cell dissociation and plating.

5.1) Count the total amount of hippocampi, then cut them into small pieces.

5.2) Collect the pieces in a 15ml centrifuge tube containing 3ml 1xHBSS.

5.3) Centrifuge at 300xg for 5 min and carefully remove the supernatant.

5.4) Add 6µl Trypsin per hippocampus.

5.5) Incubate for max 20min at 37°C. Swirl after 3min.

5.6) Wash two times with 5ml of fresh cold 1xHBSS and discard the supernatant.

5.7) After the second wash, add 1.5ml of plating medium, pre-warmed to 37°C. The serum in the plating medium will inactivate Trypsin activity.

5.8) Slowly triturate 30x with a fire-polished Pasteur pipette until all pieces of tissue are homogenously dispersed into single cells. Avoid any bubbling.

5.9) Add 5ml of plating medium pre-warmed to 37°C.

5.10) Count cells using a Trypan blue vital staining.

5.11) Seed 50.000 cells per well of a 12-well plate in 1 ml plating medium, prepared in Section 2 (total volume 2ml).

5.12) Gently rock the plate to evenly distribute the cells.

5.13) Incubate at 37°C, 5% CO₂. After 2-3 days, replace half of the plating medium (0.5ml) with culture medium containing 10µM FUDR.

Dendritic spine imaging is performed 16-17 days after plating (16-17 days *in vitro*, DIV).

Section 6: Rat hippocampal primary neuron transfection using Lipofectamine.

On DIV 14-15 neurons are transfected using the following protocol:

6.1) Prepare plasmid DNA expressing GFP and incubation medium (10ml of Neurobasal medium with 100µl of glutamax).

6.2) Pre-warm the incubation medium to 37°C.

6.3) Prepare DNA mix (Tube A). For each coverslip add 1µg of DNA in 100µl of plain Neurobasal medium. Gently mix.

6.4) Prepare Lipofectamine mix (Tube B). For each coverslip add 2µl Lipofectamine in 100µl of plain Neurobasal medium. Gently mix.

6.5) Add the Lipofectamine mix to the DNA mix dropwise.

6.6) Incubate in the laminar flow hood for 30min at room temperature.

6.7) 10min before the end of the incubation, pipette the conditioned medium from the original culture plate (plate 1) to a new 12-well plate (plate 2).

6.8) Add 1ml of pre-warmed incubation medium to plate 1.

6.9) Store plate 2 for 5min at 37°C, 5% CO₂.

6.10) Gently add dropwise 200µl of the DNA/Lipofectamine mix to each well and incubate for 45min at 37°C, 5% CO₂.

6.11) With the use of small forceps lift the coverslips containing the neurons and rinse them by dipping them in a 3 cm dish containing fresh warm Neurobasal medium and move them to plate 2.

6.12). Incubate the transfected neurons at 37°C, 5% CO₂ for 48hr.

6.13). Check for transfection efficiency 24hr after transfection.

Section 7: Immunostaining and mounting of rat hippocampal primary neurons.

To improve fluorescence intensity in transfected cells, perform an immunostaining protocol to enhance GFP detection 48hr after transfection.

7.1) Prepare 4% PFA and 0.05M TBS.

7.2) Warm the 4% PFA solution to 37°C.

7.3) Gently aspirate the medium from the wells containing the coverslips.

7.4) Add 500µl of warm 4% PFA carefully to prevent damaging the dendrites.

7.5) Incubate at room temperature for 15min.

7.6) Wash with 1xHBSS 3 times for 5min.

NOTE: at this point samples could be stored up to 3 weeks at 4°C or start the immunostaining immediately.

7.7) If samples were stored, wash with 0.05M TBS 3 times for 5min.

7.8) Block with TBS-BSA (1%) solution at room temperature for 30min.

7.9) Wash with 0.05M TBS 3 times for 5min.

7.10) Add the primary antibody diluted in incubation mix.

7.11) Incubate the plates for 1hr at room temperature.

7.12) Further, Incubate overnight at 4°C with gentle shaking.

7.13) Remove the primary antibody.

7.14) Wash with 0.05M TBS 3 times for 5min.

NOTE: from this point on, keep the coverslips protected from light.

7.15) Add the secondary fluorescent-conjugated antibody diluted in incubation mix.

7.16) Incubate at room temperature for 2hr.

7.17) Remove the secondary antibody.

7.18) Wash with 0.05M TBS 3 times for 5 minutes.

7.19) Wash with 10xTB 2 times for 5min.

7.20) Use fine tweezers to remove to coverslips from the wells.

7.21) Dry any excess of TB with a tissue and mount the coverslips using mounting medium.

7.22) Seal with nail polish to prevent evaporation of mounting medium.

Section 8: Dendritic spine Imaging using Structure Illumination Microscopy.

Dendritic spine imaging using the SIM system described in the materials has a lateral resolution (XY) value of approximately 85-110nm and an axial (Z) resolution value between 200-250nm, providing a factor of 2 improvement in resolution compared to wide-field microscopy.

NOTE: Dendritic spine imaging using SIM is done typically 2 days after step 7.22, but could be done up to 3 weeks later if samples are kept in the dark and under a controlled temperature of 22-23°C.

8.1) Turn on the 488nm laser, the mercury lamp, the stage controller, the piezo controller, the halogene lamp for transmitted light and the PC and start up the SIM software in the "ANDOR for N-SIM" mode.

8.2) Clean the 100x TIRF objective with 95% ethanol three times, and if necessary with petroleum ether.

8.3) Filter settings used were: 520LP with a 488 dichroic.

8.4) Put a drop of immersion oil on the objective. Check that there are no air-bubbles in the oil-drop. Move the objective upwards until the oil touches the sample.

Note: Place a cover over the stage to protect the sample from ambient light and dim the lights in the room as much as possible.

8.5) Set the correction collar of the objective to 37°C, 200µm, to obtain the best symmetry of PSF. In order to set the correct collar position, the objective ring was first positioned at the optimal nominal position and then a 100nm beads sample was checked. According to the best PSF's symmetry, the collar position can slightly change around the nominal one.

8.6) For illumination 3D-SIM grating (3D 1layer 100x/1.49 all wavelengths) is used. The grating alignment can start after placing the selected grating block into the SIM illuminator, with the 100x/1.49 objective in place. The alignment grating is done by using a 100nm bead sample mounted in media, with a concentration that can allow isolating 10-15 beads for a field of view (FOV). After setting the objective correction collar to the desired position, select the 3D-SIM illumination and start the software-guided alignment procedure. It will run (5 phases) x (1 direction) x (100 Z-planes) images, from which it will reconstruct the FOV with beads. Once selected a single bead via an appropriate ROI, covering the entire bead including the out-of-focus blurred light, the software will start an automatic PSF fitting and it will adjust the grating position according to the result.

8.7) To check the performance of the microscope, the grating alignment has to be repeated every 2 weeks, because of the possible misalignment caused by table movements and/or temperature drifting. Furthermore, the laser intensity and stability are also checked, according to the manufacturer's suggestions.

8.8) Clean the sample surface with 95% ethanol three times.

Note: For the next steps see figure 3 for an overview of the control panels and the correct settings within the SIM software.

8.9) In the SIM software, select the Optical Configuration Eye FITC and select an empty filter block in Turret 1 for visual inspection with white light.

8.10) Set the focusing speed and the travel speed of the XY table to "Fine".

8.11) Open the shutter and quickly focus on the sample.

8.12) Close the shutter again and set the Z-coordinate to zero.

8.13) Select the green filter in Turret 1 for visual inspection using the green channel.

8.14) Set the intensity of the mercury lamp to the lowest setting.

8.15) Move to the border of the sample carefully, making sure that the objective does not touch the sealant.

8.16) Open the shutter and quickly scan through the sample with the lowest possible intensity.

8.17) Upon encountering a sufficiently bright dendrite of interest and centering a segment of interest in the field of view, close the shutter.

8.18) Set the software to Optical Configuration 3D-SIM 488 and the camera settings to read-out mode EM, gain 1MHz 16-bit, exposure time 100ms, laser power 5% and EM gain 200.

NOTE: Check that the green filter in Turret 2 is selected and that Turret 1 is empty.

8.19) Check that the grating is set to 'Moving' and click Live to view the sample with laser light and through the camera.

8.20) Activate the Look Up Table.

8.21) Center the object of interest if necessary and focus with the focusing speed set to Extra Fine.

NOTE: in the read-out mode EM gain 1MHz 16-bit, the target intensity for a good SIM image is between 30,000-45,000.

8.22) Quickly adjust the camera settings to get an intensity value between 30,000-45,000 in the read-out mode EM gain 1MHz 16-bit. Use initially:

1. Laser power: 0%-20% (with samples prepared as described above, 5% or 2.6mW is sufficient)
2. Exposure time: 50ms-2s
3. Read-out mode: EM gain 1MHz 16-bit
4. EM gain: 0-300
5. Conversion gain: 1x-5.1x
6. Format for Live: No binning
7. Format for Capture: No binning

NOTE: with these settings 6.3%±1.3% bleaching is routinely achieved, within a 10% limit of acceptable maximum bleaching, which could significantly affect the image quality¹⁵.

8.23) Click Stop to turn off Live view.

8.24) Configure the settings of the 3D Z-stack in the ND Sequence panel to:

1. Range: 2µm
2. Set size: 120nm
3. 9 images per Z plane
4. Click Home position
5. Select the Optical Configuration 3D-SIM 488 in the Lambda section.

8.25) Select 3D-SIM as the acquisition mode in the N-SIM pad.

8.26) Run the ND Sequence acquisition and save the raw data.

8.27) Select the Optical Configuration Eye FITC again and repeat steps 8.15-8.27 until the entire sample has been imaged

8.28) 3D Image reconstruction: The data acquired in step 8.23 can either be reconstructed right away or later on. Start by reconstructing the Z-stack with the default reconstruction settings in Reconstruct Slice or Reconstruct Stack mode and adjust if necessary.

NOTE: for best results, Z-stacks should be made with the indicated step size and reconstructed in Reconstruct Stack mode. Always check the validity of the reconstructed image by comparing it to the raw data or, preferably, a wide-field image. The parameters that can be adjusted for the reconstruction process are the contrast and high frequency noise suppression. Both of them influence how different raw image properties are taken into account during the reconstruction process. In case of low modulation depth raw data, the contrast parameter plays an important role. If the user has datasets with a low signal-to-noise ratio, then the high frequency noise suppression parameter can influence the reconstruction quality severely.

8.29) When done with imaging, center the XY stage and move the objective all the way down to its resting position.

8.30) Unmount the sample and clean both the sample and objective with 95% ethanol.

8.31) Shutdown the software and the PC and switch off all other devices.

8.32) 3D reconstruction and spine classification of the acquired images can be carried out after converting the files to TIFF as described before using NeuronStudio software (see figure 4)¹⁶.

8.33) NeuronStudio parameters used for reconstruction:

1. Volume: Voxel dimensions: X: 0.03µm; Y: 0.03µm; Z: 0.120µm
2. Dendrite detection: Attach ratio: 1.3; Minimum length: 5µm; Discretization Ratio: 1; Realign junctions: Yes
3. Spine detection: Minimum height: 0.2µm; Maximum height: 5.001µm; Maximum width: 3µm; Minimum stubby size: 10voxels; Minimum non-stubby size: 5voxels
4. Spine Classifier: Neck ratio (head-neck ratio): 1.1; Thin ratio: 2.5; Mushroom size: 0.35µm

8.34) NeuronStudio parameters used for rendering:

1. Neurite vertex shape: solid eclipse
2. Neurite vertex color: by type
3. Neurite edge shape: line
4. Neurite edge color: single color
5. Spine shape: solid eclipse
6. Spine color: by type

After 3D reconstruction it is also possible to set the volume render. The default threshold was set to 20 with the 'Regenerate volume rendering' option active. Opacity was set by 'Automatic Intensity' checked. 'Surface only' and 'Use Point Pre-Rendering' options were also active

Results

Described here is a standardized working protocol for imaging dendritic spines from rat primary hippocampal neurons in vitro using SIM. The protocol workflow and its crucial steps are shown in Figure 1. Overall, the protocol takes approximately 2 weeks of experimental work separated in a first phase of sample preparation, including culture, development and transfection of rat primary hippocampal neurons and immunohistochemistry, and second phase of sample imaging using SIM. The rat primary hippocampal neurons are fixed approximately 2 weeks after start of the culture, when

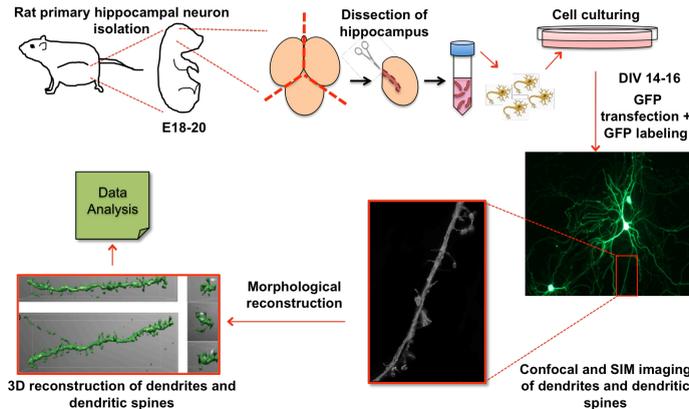


Figure 1 - The scheme shows a protocol workflow, its steps and timing.

neurons have developed complex dendritic arbors bearing numerous dendritic spines^{3,5}. Using the protocol described in detail in sections 1-8, it is possible to systematically image dendritic spines with super resolution. In comparison with a conventional dendritic spine imaging method using confocal fluorescence microscopy that is described before^{16,17}, the protocol described herein using SIM provides significantly better image resolution and 3D reconstruction (Figure 2), allowing the identification and classification of neuron membrane protrusions ranging from early filipodia to spine-like structures.

Discussion

In this article a working protocol to image dendritic spines from rat primary hippocampal neurons cultured *in vitro* using SIM is described. The primary hippocampal neuron culture method is an adaptation of the original method described by Kaech and Banker¹⁸. The main differences are the use of Neurobasal/B27 culture medium, which eliminates the requirement of astroglial feeder cultures, and the addition of the mitotic inhibitor FUDR on day 3 which promotes neuronal survival while suppressing glial proliferation, as described by Brewer *et al.*¹⁹.

The critical steps of the protocol are:

1. Thickness of the coverslips used to plate the cells is crucial for an accurate SIM experiment.
2. Sterility during coverslip preparation and coating.

3. Do not let poly-l-lysine-treated coverslips dry during 2.3 and 2.4.
4. The diameter of the flame-polished pipette used in step 5.8 is crucial. A too narrow a tip will result in low cell viability at later stages.
5. Isolate the hippocampi as quick as possible to ensure high cell viability.
6. Timing of incubation and trypsin concentrations are crucial to ensure high cell viability. Loss of trypsin enzymatic activity may affect cell viability too.
7. The addition of FUDR in step 5.14 is crucial to promote neuron survival and inhibit glial proliferation.

The sample imaging phase is straightforward when performed following strictly the protocol described here and results in the acquisition of super resolution images that can be readily reconstructed in 3D to analyze and classify dendritic spines according to their morphological features. As shown in Figure 2, the quality of the images acquired in the SIM mode is substantially better than images of exactly the same dendritic segments and individual spines acquired using the confocal mode of the same microscope. This result suggests that the use of SIM could provide an excellent opportunity to image more than subtle changes in dendritic spine morphology, as quantified in Figure 2E&F.

So far, mostly (fluorescent) wide-field microscopy has been used to image live cells, due to its low phototoxicity. Similarly, due to its low phototoxic effects and good

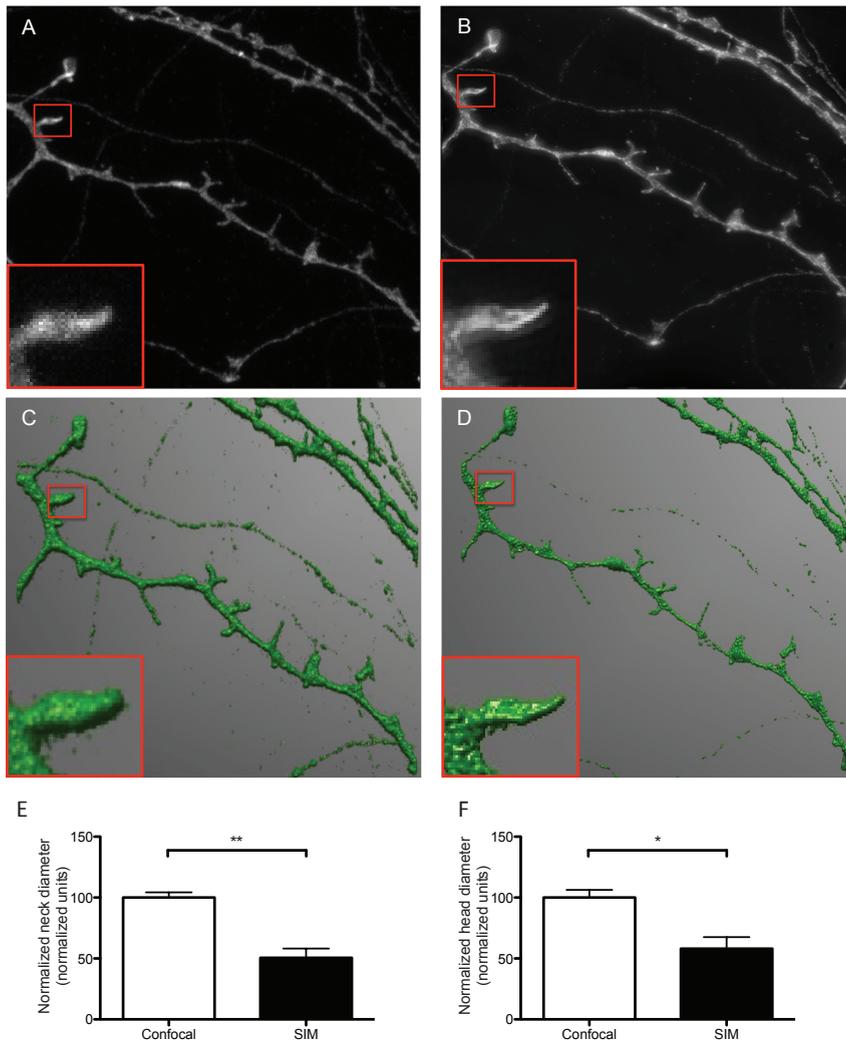


Figure 2 - Representative micrographs of dendrites and dendritic spines imaged with confocal microscopy and SIM

(A) The representative SIM micrograph was acquired as described in section 8. (B) the representative confocal micrograph was acquired using physical pinhole size: 30 μ m laser power 2.6mW, no averaging. Acquisitions were reconstructed from confocal and SIM images (C and D, respectively) using NeuronStudio software as described before¹⁶. Dendrites were traced and spines were classified automatically with the software after adjusting main parameters such as neck length, neck diameter and head diameter. The boxed areas show one individual dendritic spine imaged with confocal (A) and SIM (B) and reconstructed from their corresponding Z-stacks (C and D), depicting differences in resolution and accuracy of the resulting 3D reconstructions. According to SIM's higher resolution, quantitative analysis of both head (E) and neck (F) diameter reveals that SIM measures significantly smaller dimensions than confocal microscopy for the same dendritic spines, indicating that SIM is capable of detecting smaller changes in dendritic spine morphology. Data in E and F are normalized to the reference confocal measurements. Results are presented as mean \pm SD of 3 dendritic spines extracted from 5 dendritic segments imaged in both confocal and SIM microscope modes. For neck diameter there was a significant difference (**p = 0.0049) between confocal (100.0 \pm 4.296 normalized units) and SIM (50.61 \pm 7.642 normalized units) images, as tested with a Students t-test (E). For head diameter there was a significant difference (*p = 0.0209) between confocal (100.0 \pm 6.255 normalized units) and SIM (58.12 \pm 9.451 normalized units) images, as tested with a Students t-test.

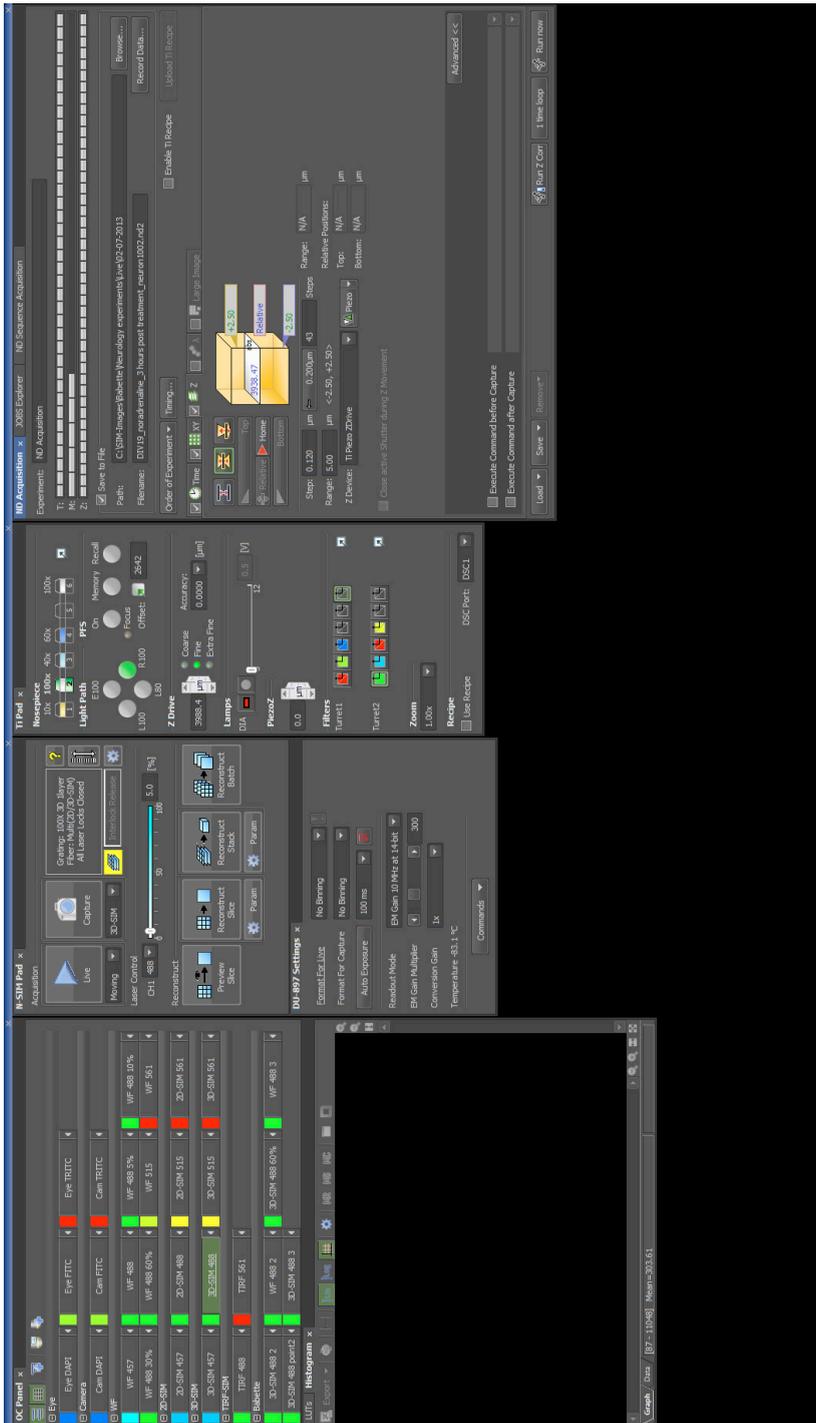


Figure 3 - Screenshot from the Nikon's NIS Elements 6.14 SIM software package with the settings as described in this protocol (rotated 90° counter clock-wise).

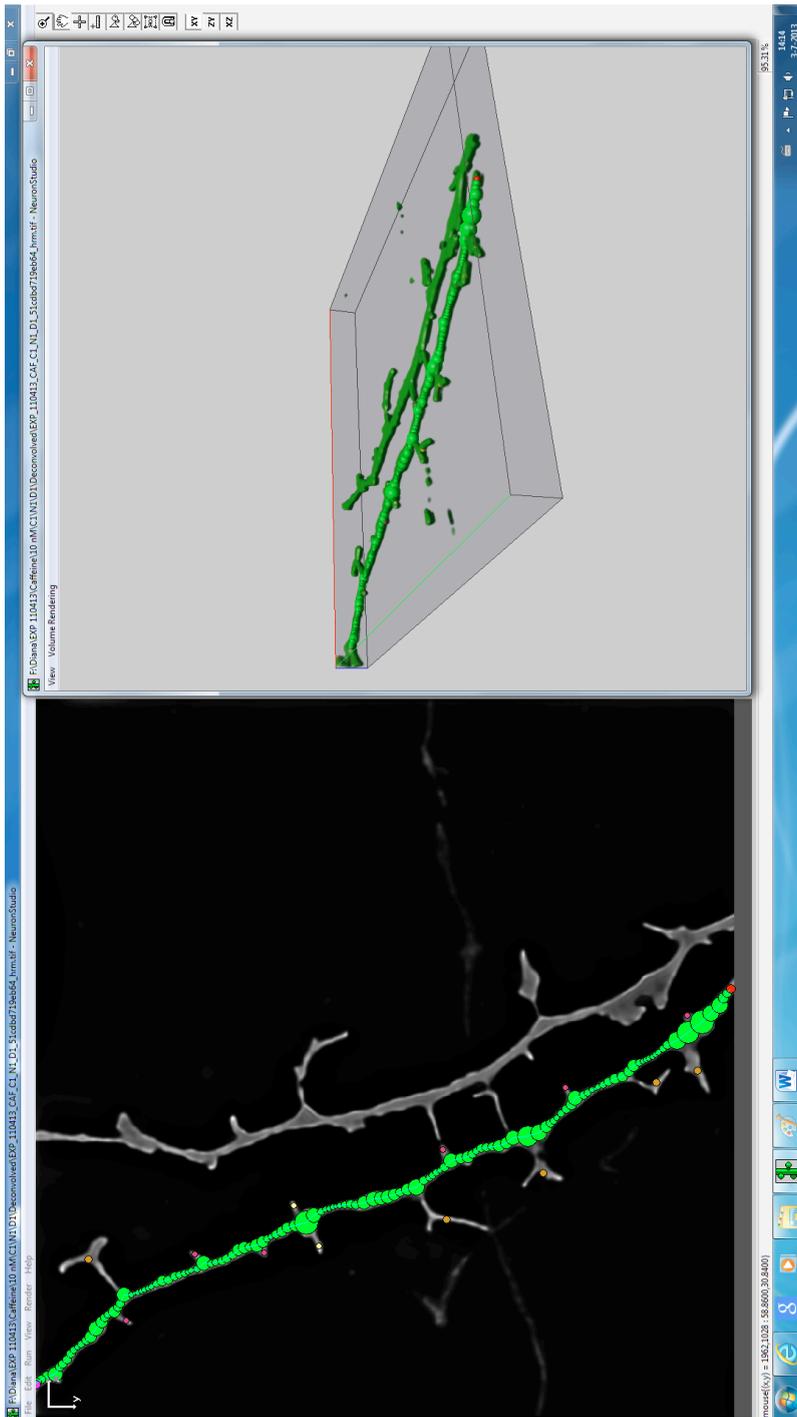


Figure 4 - Screenshot from NeuronStudio 3d reconstruction and spine classification software of a representative image of a dendrite (rotated 90° counter clock-wise).

combination with conventional (genetic) fluophores, SIM allows live cells imaging and identification of dendritic spines in low fluophore expressing cells at super-resolution. In comparison to other super-resolution microscopy methods such as STED or PALM, SIM provides a quick and affordable method for the imaging of dendritic spines from rat primary hippocampal neurons *in vitro*. Although in practice SIM only increases resolution by two fold compared to conventional confocal microscopy.

One example of a limitation of the SIM is that it relies on fluorescence, which in some experimental setups can be difficult to apply. To this end, microscopy techniques which do not rely on fluorescence such as electron microscopy may provide a possible solution. Nevertheless, electron microscopy in particular is a tedious, expensive and slow method. Moreover, electron microscopy can only be carried out on fixed samples. Therefore, SIM is more suitable for super-resolution imaging of live cells. The rationale for applying a fluorescent protein encoding plasmid transfection is that it results in a scarce, yet reproducible cytoplasmic labeling of isolated cells, preventing overlap of dendrites from different cells and the identification of individual dendritic spines. Combination of transfection with immunostaining has been shown previously to enhance fluorescence¹⁷. Nevertheless, other fluorescent techniques could also be applicable to the imaging of dendritic spines with SIM, for example sufficient fluorescent staining could be acquired using recently developed actin binding probes²⁰.

Since recent technical developments have allowed the application of SIM to dynamic cell imaging, demonstrating that high-speed structured-illumination microscope is capable of 100nm resolution at frame rates up to 11Hz¹³, a very logical future application of the protocol described herein could be its application to time-lapse SIM of live rat primary hippocampal neurons and the analysis of fast dynamic changes in dendritic spine morphology. A next challenge for this application could be the expected cumulative phototoxicity associated with long time intervals of live microscopy.

Table 1 Reagents, media and materials can be found on: https://www.jove.com/files/ftp_upload/51276/51276table1.jpg

Video Link The video component of this article can be found at <http://www.jove.com/video/51276/>

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