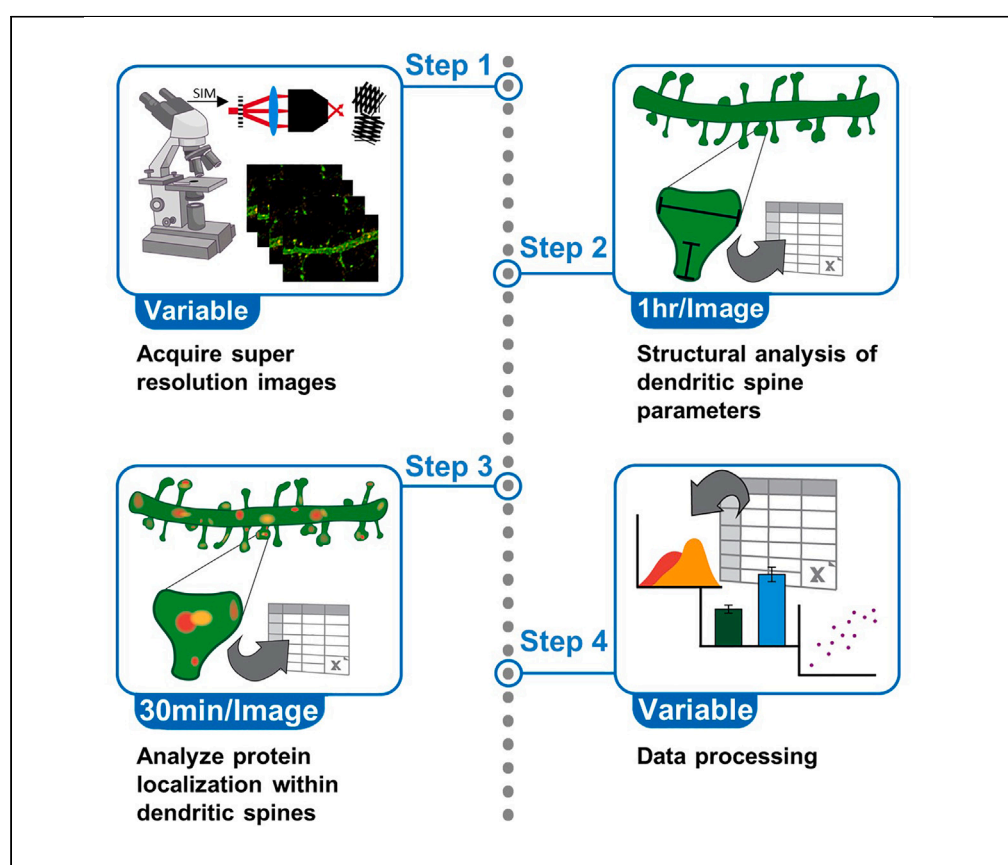


## Protocol

# Assessing protein distribution and dendritic spine morphology relationships using structured illumination microscopy in cultured neurons



Dendritic spines are protrusions on dendrites forming the postsynaptic aspect of excitatory connections within the brain. Spine morphology is associated with synaptic functional strength and the spatial regulation of protein nanodomains within dendritic spines is an important determinant of spine structure and function. Here, we present a protocol to resolve the nanoscale subcompartmentalization of proteins within dendritic spines using structured illumination microscopy. We describe steps for the structural analysis of dendritic spine parameters, protein localization analysis, and data processing.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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### Highlights

Assess protein spatial localization in dendritic spines via super-resolution microscopy

Assess the compartmentalization of proteins across spine morphology

Determine the effects of protein localization/interaction on spine structure

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## Protocol

## Assessing protein distribution and dendritic spine morphology relationships using structured illumination microscopy in cultured neurons

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## SUMMARY

Dendritic spines are protrusions on dendrites forming the postsynaptic aspect of excitatory connections within the brain. Spine morphology is associated with synaptic functional strength and the spatial regulation of protein nanodomains within dendritic spines is an important determinant of spine structure and function. Here, we present a protocol to resolve the nanoscale localization of proteins within dendritic spines using structured illumination microscopy. We describe steps for the structural analysis of dendritic spine parameters, protein localization analysis, and data processing.

For complete details on the use and execution of this protocol, please refer to Bjornson et al.<sup>1</sup>

## BEFORE YOU BEGIN

Dendritic spine structure and function are chiefly regulated by signaling pathways that culminate in the reorganization of the actin cytoskeleton, and spine morphology and the nanodomain localization of proteins within spines are important determinants of synaptic strength.<sup>2–6</sup> Although changes in protein levels can undoubtedly impact the morphology and function of individual dendritic spines, spine structure and function are also influenced by the spatial distribution of individual proteins within spines. Further, this relationship is seemingly reciprocal with changes in synaptic activity influencing the spatial distribution of select proteins within spines, and changes in this spatial distribution, in turn, modifying subsequent responses to changes in synaptic activity.<sup>3</sup>

Synaptic elements are generally smaller than the diffraction limit of light microscopy; however, super resolution microscopy enables the ability to determine the localization of proteins within small compartments. Studies using super resolution techniques have revealed that many molecules known to shape synaptic structure are not randomly dispersed throughout individual dendritic spines, but rather, they are spatially arranged and clustered. These clusters are referred to as nanodomains, and the nanodomains for some proteins are rearranged in response to altered synaptic plasticity.<sup>7</sup> Thus, assessments of the quantity and spatial location of nanodomains for an individual protein within dendritic spines advances knowledge of the biochemical architecture of dendritic spines, with implications for understanding broader synaptic function.

There is a growing body of literature examining the relationship between the nanodomain spatial distribution within spines and spine morphology. Thus, having consistent and established methods for examining both the amount and spatial location of an individual protein within dendritic spines across a spectrum of morphologies will help better illuminate the potential influence an individual



protein has on dendritic spine structure and function. This protocol describes a workflow for analyzing the sub-compartmentalization of protein nanodomains in dendritic spines as a function of spine morphology. This enables a quantitative assessment of whether the distribution of a particular protein is uniform across all spines or rather, if the distribution is strongly influenced by spine morphology. Further, this protocol describes the workflow for quantitatively assessing the spatial overlap between the spine nanodomains of two distinct proteins, and determining how the degree of spatial overlap is related to spine morphology. This protocol utilizes the Nikon-Elements AR software to analyze images captured with structured illumination microscopy (SIM); however, this pipeline can be modified for use with other super-resolution imaging techniques.

The protocol below starts with the acquiring of key parameters of individual dendritic spines from dissociated cultures of primary cortical neurons transfected with green fluorescent protein (GFP). The protocol will not detail the isolation of cultured primary neurons, or the specific methods used for transfecting plasmids as a variety of approaches are compatible with the imaging procedures detailed so long as they do not adversely impact neuron health. The presence of blebbing along dendrites (beads on a string appearance) is an indication of poor neuronal health, and such neurons should not be imaged or analyzed. The protocol below was optimized using dissociated cultures of rat primary cortical neurons that were plated onto glass coverslips, with plasmid transfection using lipofectamine 2000 following the manufacturer's instructions. Alexa Fluor 488, 555, and 647 were used to illuminate bound primary antibodies. The concentration of primary and secondary antibody needs to be empirically evaluated. Alternative approaches are also compatible with this protocol, such as the use of neurons from other brain regions or viral transfection, provided that the signal is of sufficient strength for SIM imaging. The use of appropriate controls is important for setting analysis parameters, such as the use of primary antibody delete, and secondary antibody delete conditions.

**Note:** For a detailed protocol on troubleshooting signal strength, SIM calibration, and a workflow for assessing quality, see: Demmerle, J., Innocent, C., North, A.J., Ball, G., Müller, M., Miron, E., Matsuda, A., Dobbie, I.M., Markaki, Y., and Schermelleh, L. (2017). Strategic and practical guidelines for successful structured illumination microscopy. *Nat. Protoc.* 12, 988–1010. <https://doi.org/10.1038/nprot.2017.019>.<sup>8</sup>

The example provided in this protocol involves the analysis of multichannel super resolution images that were acquired using a Nikon SIM ( $z = 0.12 \mu\text{m}$  images of secondary dendrites on pyramidal neurons with a  $100 \times 1.49$  NA objective). Acquisition was set to 5 MHz at 14 bit with EM gain and no binning. Auto exposure was kept consistent between images and within 250–300 ms, the EM gain multiplier was kept below 300, and conversion gain was held at 1x. Reconstruction was done using the built in Nikon Elements software and parameters (Illumination Modulation Contrast, Noise Suppression and Out of Focus Blur Suppression) were kept consistent across all images and achieved a score of 8 (see table for example parameters). Other reconstruction methods or deconvolution and/or background subtraction can also be used depending on need.

Reconstruction Parameters Used:			
	Channel 647	Channel 555	Channel 488
Illumination Modulation Contrast	3.32	3.32	3.24
Noise Suppression	0.60	1.59	1.67
Out of Focus Blur Suppression	0.44	0.42	0.38

**Note:** The use of SIMcheck, an ImageJ plugin, is highly recommended for assessing resolution and data quality. See: Ball, G., Demmerle, J., Kaufmann, R., Davis, I., Dobbie, I.M., and Schermelleh, L. (2015). SIMcheck: a Toolbox for Successful Super-resolution Structured Illumination Microscopy. *Sci. Rep.* 5, 15915. <https://doi.org/10.1038/srep15915>.<sup>9</sup>

△ **CRITICAL:** SIM is sensitive to refractive index mismatch, which can reduce axial resolution or produce unwanted artifacts. Ensure that the refractive index of the mounting media you use matches that of the immersion medium of your microscope objective (We use room temperature ProLong Glass Antifade Mountant (RI of 1.52 after curing) for an immersion oil with a refractive index of 1.515).

### Institutional permissions

All experiments using animals were approved by the University of Wisconsin at Madison Animal Care and Use Committee. If you are conducting this protocol institutional permissions from relevant bodies are necessary for the use of laboratory animals.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Alexa Fluor 488, 555, 647 secondary antibodies (1:400)	Thermo Fisher Scientific	Catalog number varies by host species of the primary antibody
Green fluorescent protein primary antibody (1:3,000)	Aves Labs	Cat# GFP-1010 (RRID:AB)
HA-Tag (1:500)	Cell Signaling Technology	
Anti-ARHGEF11 (1:250)	OriGene	
<b>Chemicals, peptides, and recombinant proteins</b>		
ProLong glass antifade mounting medium	Invitrogen	Cat# P36982
Lipofectamine 2000	Invitrogen	Cat# 11668019
<b>Software and algorithms</b>		
NIS-Elements AR (Version 5.2)	Nikon	NIS-Elements (RRID:SCR_014329)
Excel	Microsoft	

### STEP-BY-STEP METHOD DETAILS

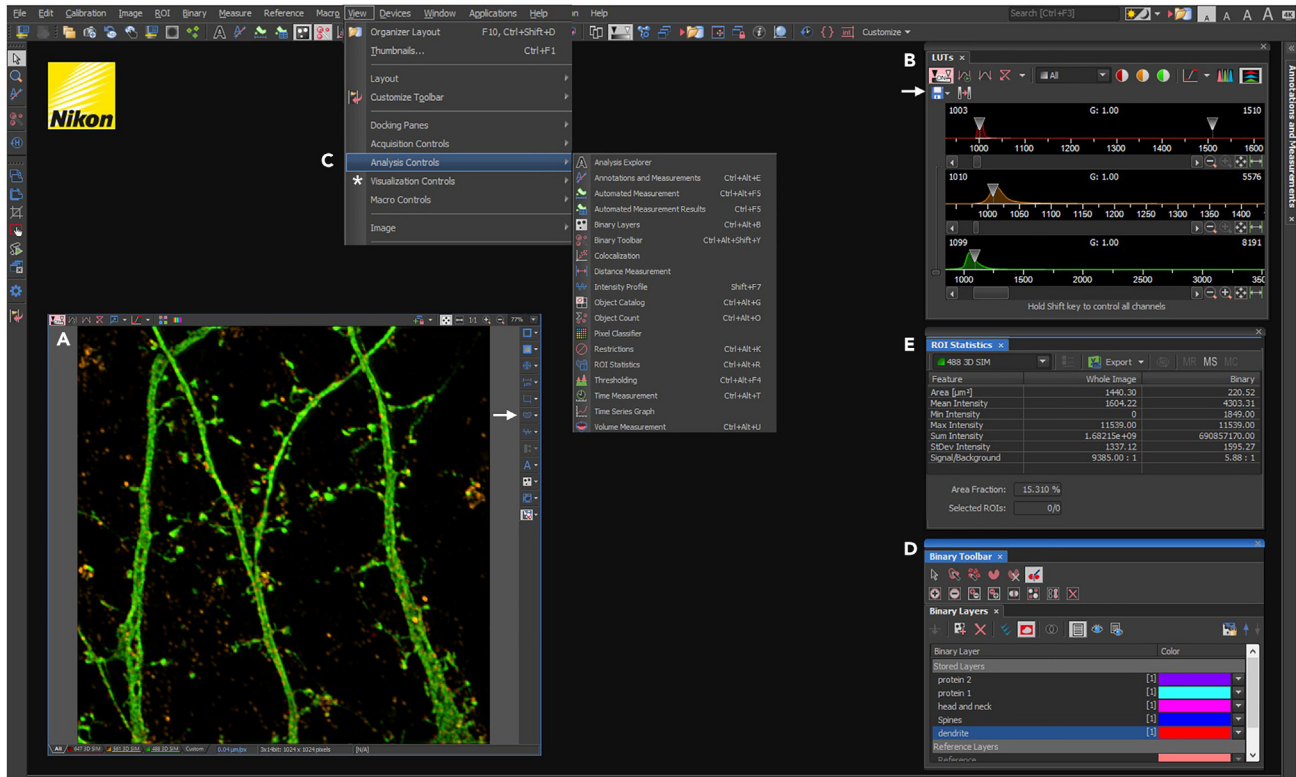
#### Structural analysis of dendritic spine parameters

⌚ **Timing:** roughly 1 h per image

This section describes the steps for acquiring relevant dendritic spine structural parameters including spine size, spine type, neck length, and head and neck size. This information was used to assess the effects of overexpressing a protein of interest on dendritic spine morphology.

1. Load a maximum intensity projection of the super resolution image in NIS-Elements AR (Nikon) (Figure 1A).
  - a. Max intensity projections can be created in the Nikon SIM imaging software used to capture the images (select the Image menu, ND Processing, Create Max intensity projection in Z).
2. Adjust the look up table (LUTs) for each channel (Figure 1B) and save the associated parameters using the floppy disc icon for use in subsequent images (Figure 1B arrow).
  - a. To open lookup tables, select View menu, visualization control, LUTs (Figure 1C, star).

**Note:** Here it is suggested that primary antibody delete, and secondary antibody delete conditions be used to help determine proper LUTs settings. If fluorescence is visible in these conditions, LUTs should be lowered. Additionally, the appearance of artifacts around nano-domain puncta (such as a halo-effect) can be used as an indicator that LUT levels are too high.

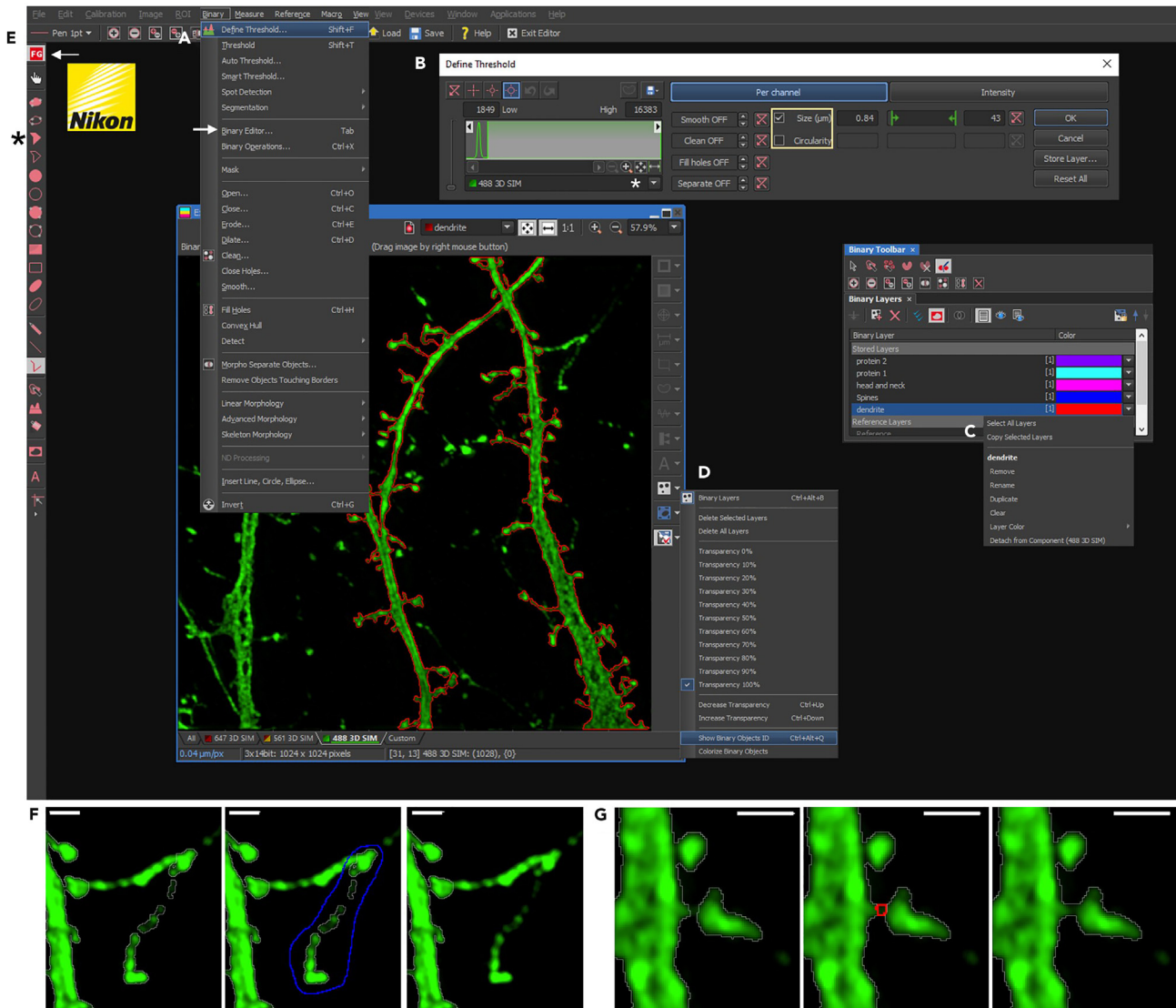


**Figure 1. Maximum intensity projection in NIS-Elements**

- (A) Structured Illumination Microscopy max intensity projection of rat primary cortical dendrites open in NIS-Elements. Arrow indicates “Turn ROI On/Off” dropdown menu. Use to show/ hide ROI labels.
- (B) Look up table (LUTs), used to adjust how the brightness of each pixel appears. This does not change the raw image data, only the way the pixels are displayed. Arrow indicates the floppy disc icon used to save LUTs settings.
- (C) Pathway for opening the tools used in this protocol. Star denotes the “Visualization Controls” menu used for opening the LUTs table.
- (D) Binary Toolbar (Top) and Binary Layers Toolbar (bottom).
- (E) ROI Statistics Toolbar used to export dendrite ROI data.

- b. Load the saved LUT settings for each image analyzed to maintain consistency.
3. Create a binary mask layer for GFP (or cell fill channel).
  - a. Open the Binary menu and select define threshold (Figure 2A).
    - i. In the Define Threshold window (Figure 2B), select per channel and use the dropdown menu (Figure 2B star) to select the cell fill channel (488).
    - ii. Adjust what is included in the segmentation by modifying how much of the histogram is covered with the opaque selection region, or by manually typing in low and high values (Hover over the histogram and use the scroll wheel to zoom in and out).
    - iii. To filter what's included in the binary segmentation by size or circularity, check the associated box (Figure 2B yellow box). Move the green bars to adjust the range or manually type values into the boxes.
    - iv. Increase or decrease smoothing, use the cleaning function, fill holes, or separate segments using the arrows next to the associated label.

**Note:** The LUT settings determined previously should be used to help set nanodomain segmentation parameters. If segmentation regions appear around areas without visible fluorescence, the settings encompass too much of the histogram and should be lowered. Additionally, if irregular shaped artifacts appear, this can also be an indication that settings are too high.



**Figure 2. Creating and editing a Binary Mask of the GFP (cell fill) channel**

(A) Pathway to open the Define Threshold Toolbar and Binary Editor menu indicated by the arrow.  
 (B) Define Threshold Toolbar used to create binary layer segmentations. Yellow box indicating tools used to filter the binary segmentation by size and circularity.  
 (C) Binary Layers Toolbar used to duplicate layers by right clicking on the layer to open more operations.  
 (D) Binary layer Tools dropdown menu used to change the transparency of the binary layers and turn off or turn on binary layer IDs.  
 (E) Binary Editor menu. Arrow indicating the button used to switch between the background (BG) and foreground (FG) editor. Star indicating the polygon fill selection tool used to edit binary segmentation masks.  
 (F) Using the binary editor to remove areas of the image from the binary layer segmentation and add them to the background. Blue outline indicating the area selected to be removed using the polygon fill tool while in BG setting.  
 (G) Using the Binary editor to add areas of the image to the binary segmentation layer. Red outline indicated the selection of pixels to be added using the polygon fill tool while using FG settings. Scale bar = 1 µm.

- v. Save the threshold settings using the floppy disc icon. Load and use these settings for subsequent images to maintain consistency.
- vi. A new layer will appear in the Binary Layers toolbar (Figure 1D) (to open the Binary Layers toolbar select the view menu, analysis controls, binary layers (Figure 1C)). Rename this layer "dendrite."



**Note:** The transparency level of the binary mask layers can be changed using the dropdown arrow indicated in [Figure 2D](#).

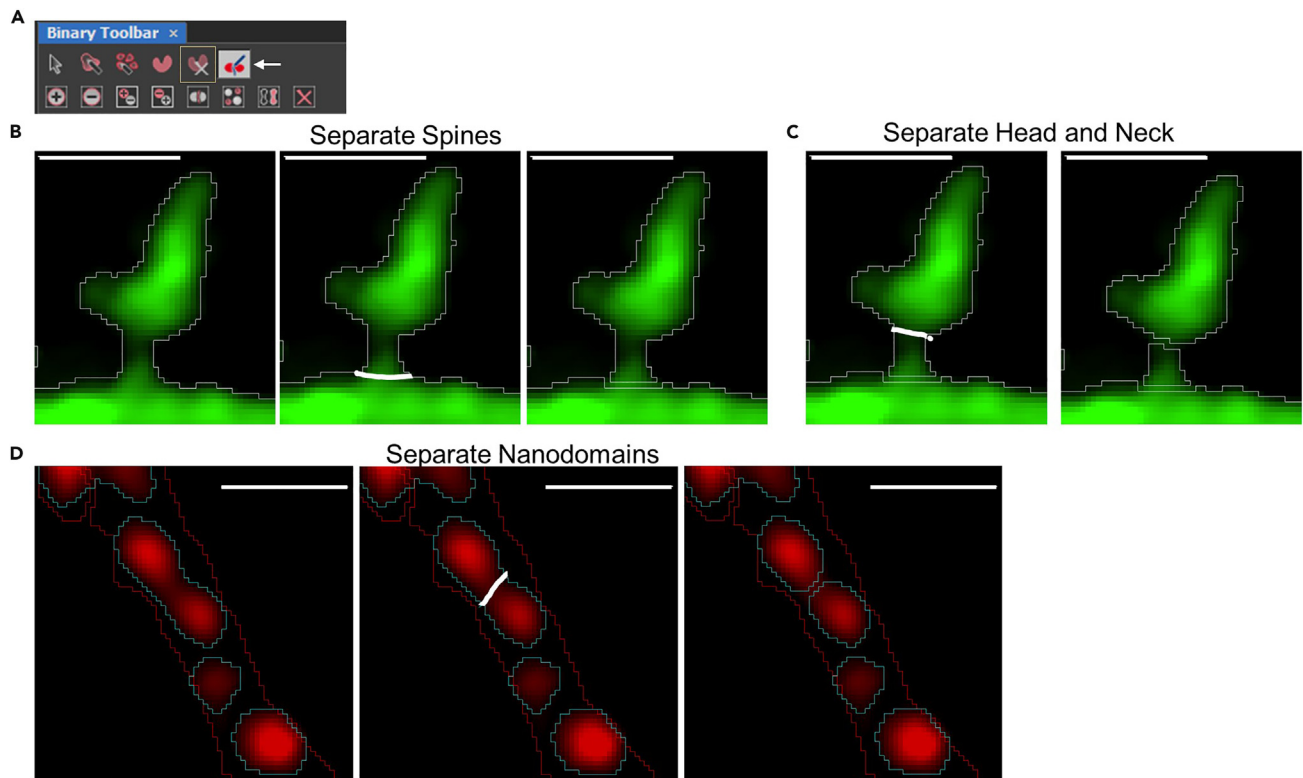
4. Use the Binary Editor to correct the binary mask ([Figure 2A](#) arrow).
  - a. The Binary Editor will open as a toolbar along the left and top panel ([Figure 2E](#)). Switch between editing what's included in the binary mask by clicking the red FG (foreground) or black BG (background) button ([Figure 2E](#) arrow).

**Note:** When FG is visible you are adding to the binary mask; when BG is visible, you are removing from the binary mask by adding it to the background.

- i. To remove areas from the binary mask, ensure that BG is visible and choose a selection tool (the polygon fill tool and the polyline tool are recommended).
  - ii. Using the polygon fill tool ([Figure 2E](#) star), encircle the area to be removed ([Figure 2F](#), blue line) and right click to finish drawing ([Figure 2F](#)).
  - iii. To add a section of the image to the binary mask, ensure that FG is visible and choose the polygon fill tool.
  - iv. Trace the area to be added and right click to complete the outline ([Figure 2G](#)). This is useful when the spine necks are not captured in the segmentation.
5. Create individual binary masks for each dendritic spine.
  - a. Duplicate the dendrite binary layer by right clicking on it in the binary toolbar and selecting duplicate ([Figure 2C](#)). Rename the new layer "spines."
  - b. Open the binary toolbar (select the view menu, analysis controls, binary toolbar ([Figure 1C](#))). Select the separate objects manually button on the binary toolbar ([Figure 3A](#), arrow).
  - c. With the newly created spine layer selected in the binary layer toolbar, separate each spine from the dendrite by drawing a line across the base of each dendritic spine where the separation should occur ([Figure 3B](#)).
6. Create an ROI from each binary spine mask.
  - a. Open the ROI toolbar then select copy binary to ROI. Save the ROI by selecting the ROI toolbar then selecting save ROI as.
    - i. If the ROI IDs are not visible, use the Turn ROI On/Off (Shift+Q) dropdown menu and select Show ROI Labels (Shift+Alt+Q) ([Figure 1A](#) arrow).
7. Export spine area parameters to Excel.
  - a. Open the ROI statistics menu (select the view menu, analysis controls, ROI statistics ([Figure 1C](#))) ([Figure 1E](#)).
  - b. Use the drop-down arrow next to export to select settings, then choose the data export tab.
    - i. Select the statistics to be exported (recommended: ROI ID, ROI Area. Optional: ROI intensity values within the ROI for each channel).
    - ii. Click the export button (an excel document should open automatically).
    - iii. Rename the sheet "Spine Statistics."
8. For thin and mushroom type spines, create a separate binary mask for the head and neck of each spine by separating them.
  - a. Duplicate the spine binary layer by right clicking on it in the binary toolbar and selecting duplicate ([Figure 2C](#)). Rename the new layer "head and neck."
  - b. With the newly created "Head and Neck" layer selected in the binary layers toolbar, separate the head from the neck using the separate objects manually tool ([Figure 3A](#)) by drawing a line through the head and neck boundary ([Figure 3C](#)).

**Note:** you can hide the ROI while completing this step for easier visualization (select the Turn ROI On/Off (Shift+Q) dropdown menu and choose Hide ROI labels (Shift+Alt+Q) ([Figure 1A](#) arrow))

**Note:** size parameters will be used later in the protocol to categorize spines into thin, mushroom, stubby, or filopodia type. For this step, unless it is very clear that a spine is stubby or filopodia



**Figure 3. Using the Separate Objects Manually tool to segment spine sub-regions and protein nanodomains**

(A) Binary Toolbar. Arrow indicating the separate Objects Manually tool. Yellow box indicating the Delete Objects tool.

(B) Separating each dendritic spine into individual binary segmentation layer. Red line indicated “cut” made using the Separate Objects Manually tool in the Binary Toolbar.

(C) Separating dendritic spine binary segmentation layers into head and neck sub-compartments. Red line indicated “cut” made using the Separate Objects Manually tool in the Binary Toolbar.

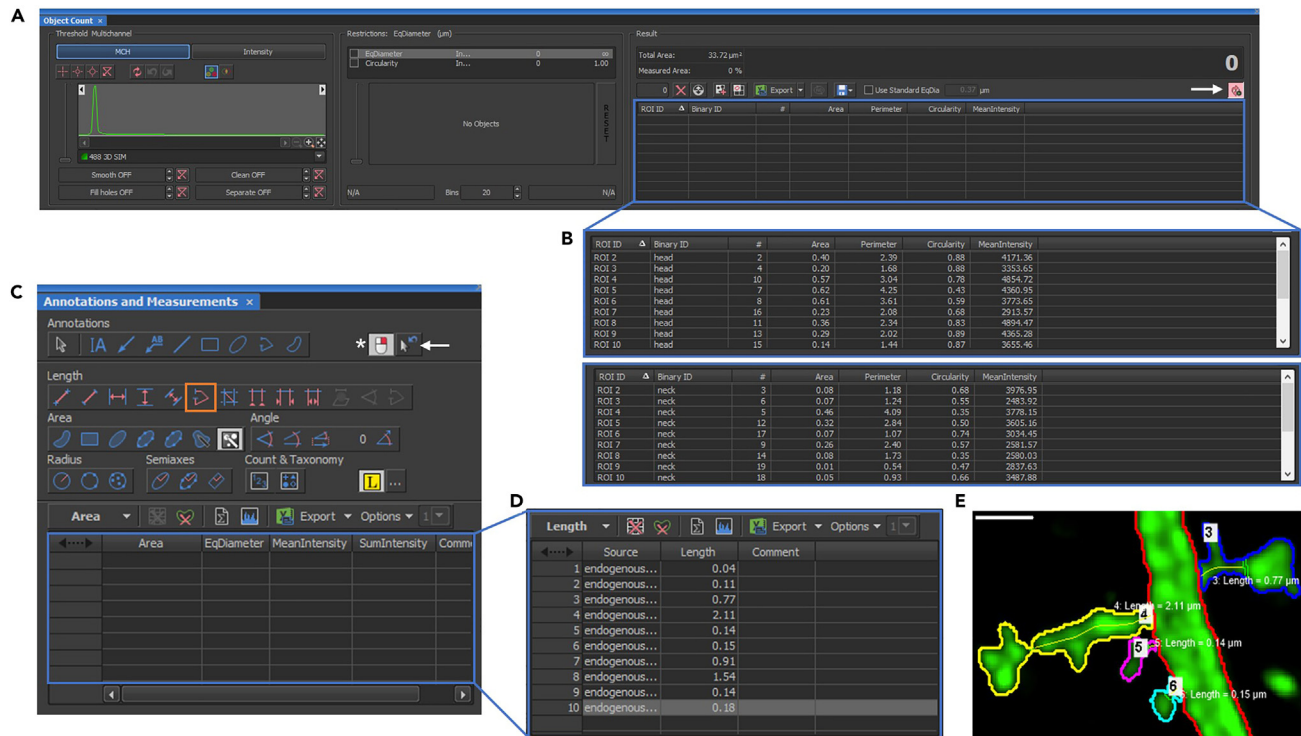
(D) Separating protein nanodomain. White line indicated “cut” made using the Separate Objects Manually tool in the Binary Toolbar. Scale bar = 1  $\mu\text{m}$ .

(i.e., it has no discernable neck for stubby spines and a very long neck but no discernable head for filopodia), it is best to separate spines into a head and neck region. If later categorization identifies a filopodia or stubby spine, the head and neck data for that spine can be discarded.

9. Measure the spine head and neck area (or total spine area for filopodia and stubby spines).
  - a. Duplicate the “head and neck” binary layer by right clicking on it in the binary toolbar and selecting duplicate (Figure 2C). Rename the new layer “head.”
  - b. Use the delete tool in the Binary toolbar (Figure 3A, yellow box) to delete all of the neck binary layers so that you are only left with the head regions.
  - c. Repeat steps a and b, this time renaming the duplicated layer “neck” and deleting all the head binary layers.
  - d. Ensure that the ROI is visible (select the Turn ROI On/Off (Shift+Q) dropdown menu and choose Show ROI labels (Shift+Alt+Q) (Figure 1A arrow)).
  - e. With the “Head” layer selected in the binary layers toolbar, open the Object count toolbar (select the view menu, analysis controls, Object count toolbar (Figure 1C)) (Figure 4A).
  - f. Select the “Keep updating count” button (Figure 4A arrow) to populate the binary head data into the object table (each binary will be an object in the table) (Figure 4B).

**Note:** When the ROI is on, only the objects contained within the ROI will be counted.





**Figure 4. Spine Head and Neck sub-compartment measurements**

(A) Object Count Toolbar used for taking area measurements of the head and neck dendritic spine sub-compartment. Arrow indicates the “keep updating count” button which should be selected.

(B) Object Count table in which binary segmentations selected from the Binary Layers toolbar will be populated. Examples shown from head and neck binary segmentations.

(C) Annotations and Measurements Toolbar used for taking neck length measurements. Orange square indicates Polyline tool used for measuring. To right click to finish drawing, select the button indicated by the star. Un-select the button indicated by the arrow to ensure “Switch to Pointing Tool After Drawing” is off.

(D) Annotations and Measurements Table in which measurements will appear in the order they are created.

(E) Example image of spine neck measurements. Yellow line indicates measurement segment drawn using the polyline tool. The length of this line appears in the white text and is numbered according to the order the measurements are taken. This number assignment should correspond to the ROI ID of the dendritic spine. Scale bar = 1  $\mu\text{m}$ .

- g. Export the spine head area and other head parameters to Excel.
  - i. Use the drop-down arrow next to export to select settings, then choose the data export tab.
  - ii. Select the statistics to be exported (required: Binary name, Object, ROI ID, and Area. Optional: perimeter and diameter).
  - iii. Click the export button. An excel sheet will be added to your open excel document.
  - iv. Rename the sheet “Spine head Statistics.”
- h. Select the “neck” binary in the Binary Layer toolbar.

**Note:** If the “Keep updating count” button is highlighted in pink, this should automatically populate the binary neck data into the object table (each neck binary will be an object in the table) (Figure 4B).

- i. Export the spine neck area as directed in step fi. Rename the sheet created “Spine Neck Statistics.”

10. Measure the neck length (or total spine length for filopodia and stubby type spines).

- a. Select the “head and neck” layer in the binary toolbar so that the boundary between sub-compartments is visible along with the spine ROIs.
- b. Open the Annotations and Measurements menu (select the view menu, analysis controls, annotations and measurements (Figure 1C) (Figure 4C)).
- c. To measure the neck length of each spine, select the polyline tool (under the length heading) in the Annotations and Measurements toolbar (Figure 4C, orange box).
- d. Starting with the spine that has an ROI ID of 1 (and taking measurements in the order of their spine ID numbers), create a measurement path along the length of the spine neck (Figure 4E, yellow lines) starting from the base of the spine and continuing to the binary outline marking the head region (Figure 4C, green lines).

**Note:** Use multiple clicks to follow any curve of the spine neck and ensure that the line being drawn remains in the middle of the neck.

- e. Right click to finish the path (if the “click to finish” button is selected (Figure 4C, star)). The length measurement will appear in table (Figure 4D). Do this for all of the dendritic spines in the order of their ROI ID.
  - i. This process can also be used to measure the length of the entire spine. Rather than stopping at the boundary between the head and neck region, continue measuring through the head region, ensuring that the line remains in the middle of the head region.

**Note:** Ensure that “switch to pointing tool after drawing new object” button (Figure 4C, arrow) is not selected, to avoid having to re-click the tool between each measurement.

**△ CRITICAL:** measure the areas in the order of their ROI ID. Do not skip numbers. For example, the dendrite will also be associated with an ROI ID, even if you do not need a measurement for this section, create a measurement of some kind anyway and note or remove it from the spreadsheet later. Failing to do so will result in incorrect numbering of the measurements which will no longer correspond to the correct spine ROI ID.

- f. Export the neck lengths to Excel. Use the drop-down arrow next to export to select settings, then choose the data export tab.
  - i. Select the statistics to be exported (required: ROI ID, Length).
  - ii. Click the export button and an excel sheet will be added to your open excel document.
  - iii. Rename the sheet “neck length.”

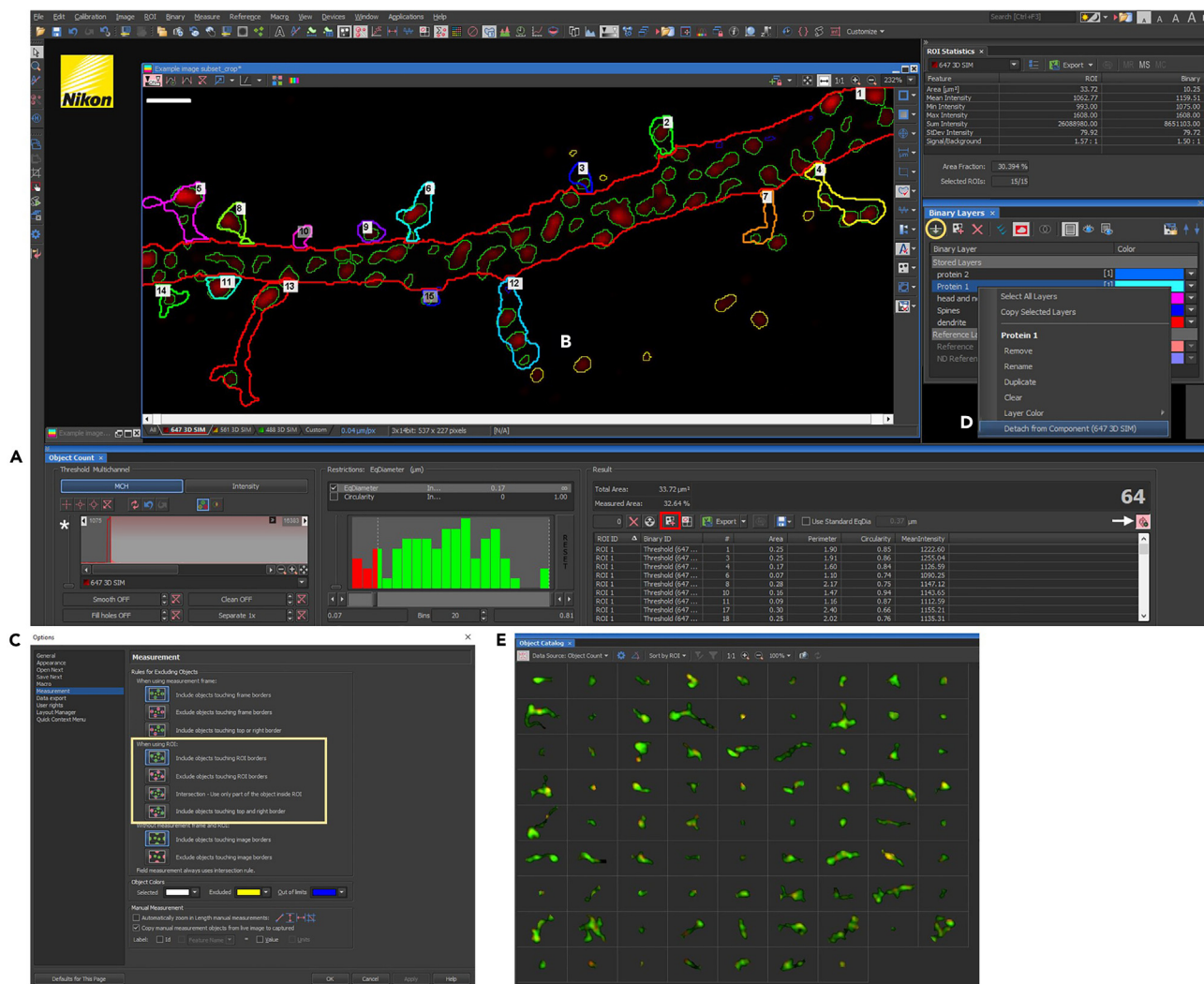
**Note:** If you measured the areas in an order corresponding to their ROI ID, the “item” column will correspond to the same number ROI ID.

### Analyzing nanodomain localization within dendritic spine compartments

⌚ Timing: roughly 30 min per image

This section describes the steps for acquiring sub-compartment localization of protein nanodomains within thin and mushroom spines as well as nanodomain spatial association with other proteins of interest. This information can be used to assess the effects of protein-protein interactions on protein localization and dendritic spine morphology.

11. Create object binary segmentations for the protein nanodomains located within dendritic spines.
  - a. Open the object count toolbar (Select the view menu, analysis controls, object count (Figure 1C)) (Figure 5A).



**Figure 5. Creating protein nanodomain objects**

(A) Object Count Toolbar used for object segmentation of protein nanodomains within the dendritic spine ROIs. The red box indicates the "Generate Binary" mask button used to create a new binary segmentation of only the object protein nanodomains that are within the dendritic spine ROIs. Star indicates look up table used to set maximum and minimum intensity to be included in the object segmentation.

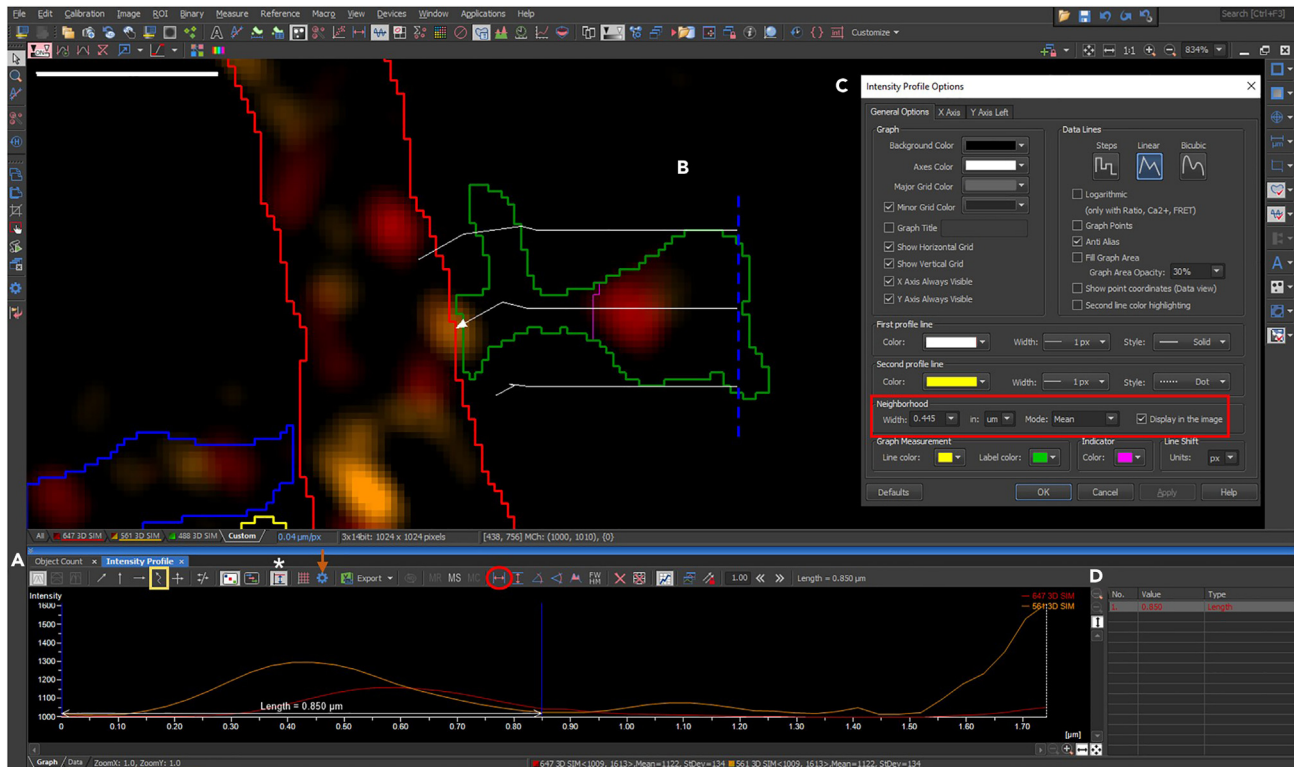
(B) Example image of Object segmentation. Overexpressed Protein in red, green segmentation outline indicates objects that will be included in the binary (within the ROI). Yellow segmentation outline indicates objects that will not be included when the binary is generated.

(C) Measurement options menu for selecting inclusion criteria for what is within the ROI (indicated by the yellow box) and thus what gets included in the generated object binary. Menu to indicate whether to include objects touching the ROI boundary.

(D) Menu used to detach binary layer from a particular channel. Right click the binary layer to open the menu and select "Detach from Component." Yellow circle indicates the button used to move binary layers from "Working layers" to "Stored Layers."

(E) Object Catalog Toolbar used to display all objects of the selected category. Example shows each ROI. Use this tool to quickly look at each segmentation and assign nanodomain location and/or protein overlap/ colocalization. Scale bar = 1  $\mu\text{m}$ .

- Select the channel of interest from the drop-down menu and use the look up table to set threshold boundaries by adjusting what gets included within the opaque selection region (Figure 5A, star). Hover over the look up table and use the scroll wheel to zoom in or out.
- To filter what's included by size or circularity, check the EqDiameter or circularity box and use the histogram below to assign maximum and minimum values for inclusion, or type them in manually.



**Figure 6. Intensity Line Scan for assessing spatial localization of protein nanodomains**

(A) Intensity profile Toolbar. Distance is located along the x-axis while intensity is located along the y-axis. Each channel selected is indicated by a corresponding line color. The graph appears for the distance created by the line scan drawn using the polyline tool indicated by the yellow box. Measurements can be taken of the graph using the measurement tool indicated by the red circle. Blue lines indicate the bounds of the measurement area. The orange arrow indicates the Intensity Profile Options menu.

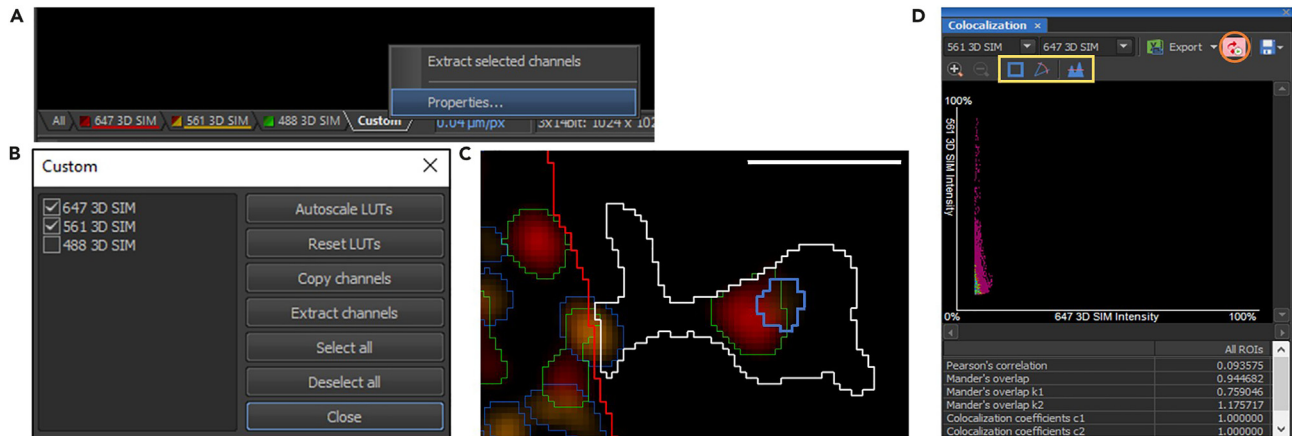
(B) Example image of a line scan drawn with the polyline tool through the dendritic spine. The line scan is indicated by the white arrow, whereas the neighborhood bounds selected in the Intensity Profile Options menu are indicated by the white lines on either side. The dashed blue line corresponds to the first boundary of the measurement being created in the Intensity Profile graph. The pink line indicates the boundary between the head and neck region indicated by the "head and neck" binary layer.

(C) Intensity Profile Option menu used to change the neighborhood width of the line scan to encapsulate the entire spine indicated by the red box. (D) Measurement table for measurements created in the Intensity Profile Graph. Scale bar = 1  $\mu$ m.

- iii. Increase or decrease smoothing, cleaning, fill holes, or separate using the arrows next to the associated label.
- iv. Save threshold settings using the floppy disc icon.
- v. Use the separate objects manually tool in the binary toolbar (select the view menu, analysis controls, binary toolbar (Figure 1C)) (Figure 3A), to separate nanodomain puncta (Figure 3D).

**Note:** Again, it is suggested that primary antibody delete, and secondary antibody delete conditions for each channel be used to help determine proper LUTs settings. If fluorescence is visible in these conditions, LUTs should be lowered. Additionally, the appearance of artifacts around nanodomain puncta (such as a halo-effect) can be used as an indicator that LUT levels are too high. Load the saved LUT settings for each image analyzed to maintain consistency.

- b. Generate a binary mask that includes only the nanodomain objects within the ROI.
  - i. Ensure that the ROI is visible (select the Turn ROI On/Off (Shift+Q) dropdown menu and choose Show ROI labels (Shift+Alt+Q) (Figure 1A arrow)) and that the object count toolbar is open (Figure 5A).



**Figure 7. Tools for assessing the spatial relationship between two proteins**

(A) Custom channel combinations can be created by right clicking the sheet located at the bottom of the image named Custom.  
(B) The Custom toolbar used to select the channels to be displayed together.  
(C) Example image of two protein binary segmentations within the ROI area indicating overlap.  
(D) Colocalization Toolbar used for Mander's overlap or Pearson's correlation. Yellow box indicated the thresholding tools for selecting what is included in the colocalization. Orange box indicates the "keep updating count" button. Scale bar = 1  $\mu$ m.

- ii. Objects will be outlined in green when they fall within the ROI, and yellow when they are outside the ROI and not included in the binary mask (Figure 5B).

**Note:** you can change the inclusion criteria by opening the Edit menu, selecting options, then measurement from the left menu bar (Figure 5C). Change the settings under "When using ROI" (Figure 5C, yellow box). Here, you can choose whether objects touching the border of the ROIs will be included, excluded, or partially included.

- iii. Save the settings using the floppy disc icon. Load and use these settings for subsequent images to maintain consistency.
- iv. Select generate binary (Figure 5A, red box) in the Object Count toolbar, then select store current working layer using the Binary Layers toolbar (Figure 5A, yellow circle).
- v. Rename the binary layer to the protein name.

**Note:** If an action causes the object table to become empty, reload it using the keep updating count button (when selected, the table will update automatically) (Figure 5A, arrow).

- c. Export object measurement parameters.
  - i. Use the drop-down arrow next to export to select settings, then choose the data export tab.
  - ii. Select the statistics to be exported (ensure that ROI ID is selected to identify which dendritic spine each nanodomain is located within).
  - iii. Click the export button and an excel sheet will be added to your open excel document.
  - iv. Rename the sheet to correspond to the name of the protein.
- d. If applicable, repeat this process for a second protein.

**Note:** To view binary layer outlines in other channels, right click on the channel in the binary toolbar, then select "Detach from Component" (Figure 5D).

12. Manually assign nanodomain objects to spine sub-compartment (head or neck) in thin and mushroom spines.
  - a. Using the nanodomain object excel data exported in step 1, sort the excel sheet by ROI ID.



- i. To do this, highlight the data and choose the insert menu, then select table. Use the drop-down menu next to the ROI ID column header to sort A to Z.
  - b. Add a column to the data table for nanodomain sub-compartment location.
  - c. In NIS-elements, select the head and neck binary to visualize the cut off between sub-regions.
  - d. Select show binary object ID in the View Overlay (Ctrl+V) menu (Figure 2D) to help distinguish between the objects exported to the excel sheet.
  - e. Visually assign the location of nanodomain objects to either head or neck on the excel sheet under the newly created column.
    - i. Optional: Use the object catalog toolbar for easier visualization and assignment. (select the view menu, analysis controls, object catalog (Figure 1C)) (Figure 5E). Select the Head or Neck binary layer in the binary toolbar to populate each binary into the object catalog (Figure 5E). Select Sort by ROI.
13. Optional: Assess the spatial localization of nanodomains using line scan intensity profiles.
  - a. Open the intensity profile toolbar (select the view menu, analysis controls, intensity profile (Figure 1C)) (Figure 6A).
  - b. Using the polyline tool (Figure 6A, yellow box), insert multiple points along the dendrite keeping the line in center of the spine (Figure 6B, white arrow). Start at the top of the spine head and draw the line toward the base of the spine neck.
  - c. Open settings in the intensity profile toolbar (Figure 6A, orange arrow) and change the neighborhood width (Figure 6C, red box) until the spine is captured by the boundary lines displayed in the image (Figure 6B, white lines). Note the width used for each spine.

**△ CRITICAL:** When analyzing this data, be sure to correct for the size of the spine, as the amount of extra space captured along with the spine will differ between spines.

- d. Using the intensity graph created, take intensity profile measurements for each sub-domain of the spine (Select vertical auto scale to adjust graph (Figure 6A, star).
    - i. Select measure vertical lines (Figure 6A, red circle).
    - ii. Insert the first measurement point on the left edge of the graph by clicking (Figure 6A, blue line). This should correspond with the top of the spine head and a measurement line will appear on the image (Figure 6B, dashed line).
    - iii. Move the second measurement point along the graph (the line in the image will move in correspondence to the position of your mouse along the graph) until you reach the boundary between the head and neck as indicated by the "head and neck" binary layer (Figure 6B, pink line) in the image.
    - iv. Insert the second measurement point at the boundary by clicking on the graph. The measurements will appear in the table next to the graph (Figure 6D).
  - e. Repeat this measurement process for the neck region, inserting the first measurement where you left off at the boundary along the head and neck region (Figure 6B, pink line) and the second on the right edge of the graph.
  - f. Use the export drop down menu to select "export all". Click export and rename the sheet with the corresponding spine ROI ID.

**Note:** Length 1 in the Excel sheet will correspond to the head and length 2 will correspond to the neck.

14. Assess the spatial relationship between two proteins (if applicable).
  - a. Create a custom channel using the sheet located at the bottom left of NIS-Elements.
    - i. Right click custom, select properties (Figure 7A), then check the channels corresponding to the proteins being assessed (Figure 7B).



- b. View the object binaries for both proteins at once by holding ctrl while selecting the name of each binary mask from the binary toolbar.
- c. Manually assign spatial overlap or use the colocalization tool.
- d. To manually assign:
  - i. Add a column to the object data excel sheet for spatial overlap.
  - ii. Sort the excel sheet by spine ROI ID for organizational ease and ensure that object ID labels are showing in NIS-Elements (Figure 2D).
  - iii. Using the binary object masks created for each protein or the object catalog view described in step 2E (Figure 5E), visually assess the overlap between object masks (Figure 7C) and note this for each object on the excel sheet by adding an x in the overlap column in excel.
- e. To use the colocalization tool:
  - i. Open the colocalization menu (select the view menu, analysis control, colocalization (Figure 1C)) (Figure 7D).
  - ii. Ensure that the keep updating binary button is selected (Figure 7D, orange circle) so that colocalization measurements are restrained to the ROI.

**Note:** A scatter plot will appear in which the selected channels (chosen in the dropdown menus at the top) will appear on the x and y axis. Depending on the brightness of the image, these points will extend further into the graph.

- iii. To create a binary mask segmentation by colocalization, use one of the three selection tools (Figure 7D, yellow square).

**Note:** As you adjust the selection tool on the scatter plot, the corresponding colocalization threshold will be adjusted on the image.

- iv. Pearson's correlation values and Mander's overlap values are located below the scatterplot. Use of these will depend on your biological question. Both colocalization methods have pros and cons.

**Note:** See: Dunn, K.W., Kamocka, M.M., and McDonald, J.H. (2011). A practical guide to evaluating colocalization in biological microscopy. *Am. J. Physiol. Cell Physiol.* 300, C723-742. <https://doi.org/10.1152/ajpcell.00462.2010>,<sup>10</sup> or other publications for a helpful resource on colocalization.

**Note:** Use Save as and rename the file to save the binary masks created and the ROI. To save thresholding parameters or LUT parameters, use the save (floppy disc) icon on the respective toolbars to ensure consistency between images being analyzed.

## Data processing

⌚ Timing: h

This section describes potential steps for processing and organizing the data collected above. Specifically, this workflow combines the dendritic spine morphology data into one spreadsheet and integrates this information with the protein localization and interaction data. Here we describe example data in which we look at the relationship between nanodomain location and dendritic spine type and size parameters. Additionally, we are interested in the effect of overexpressing a protein (Protein 1) on the localization of an endogenously expressed protein (protein 2) and what effect the spatial overlap/ colocalization of the two proteins has on dendritic spine morphology and protein localization.

15. Combine the spine measurement spreadsheets (Spine Area, Head data, neck data, length measurements) exported from Nikon-Elements into one excel document using the ROI ID (for head and neck parameters this will be labeled ROI # and for neck length this will be item).

**Note:** The ROI ID is the dendritic spine identifier. The goal of this step is to organize each parameter under the associated dendritic spine so that there is one row for each spine with associated spine variables in columns.

- a. Reorder the excel sheets with the head and neck parameters by ROI # to make combining the sheets easier.
  - i. To do this, select the insert tab and choose table. Then sort the table by selecting the drop-down menu beside ROI # and choose "Sort Smallest to Largest."

**Note:** As you will likely be analyzing more than one image, be sure to add a column for the cell or image number.

**Note:** The dendrite section will also have a corresponding ROI, you can remove this from the data table if it is not needed, not it on the spreadsheet, or create a column parameter with this information.

16. Assign dendritic spine types based on size parameters.
  - a. Here we assigned spines with no discernable neck region as stubby spines, spines with a skinny neck of length 1.5  $\mu\text{m}$  or greater with no discernable head as filopodia, spines with a head diameter greater than 0.4  $\mu\text{m}$  as mushroom spines, and others as thin spines. Additionally, a category of filopodia can be used for spines that have no discernable head region but have long neck lengths.
    - i. Neck width can also be collected using the same method for collecting neck length described above. This can provide an additional parameter with which to determine spine type classifications.
    - ii. Ensure that spines classified as stubby or filopodia do not contain head and neck variables, as these are not applicable (delete any placeholder measurements on the excel sheet that might have been made).
  - b. Classification can be automated using a decision tree structure in programs like R (i.e., "if else" statements).
17. Use the nanodomain object excel sheets to count values and create columns for the number of nanodomains in each spine, in the spine head, and in the spine neck.
18. If applicable, you can also create columns for the number of nanodomain objects that are colocalized within each of these regions.

**Note:** Pivot tables in excel or programs like R can be used to automate this step (this is highly recommended).

- a. If applicable, you can also create columns for the combined area of nanodomains within each spine or sub-region, intensity values, or other parameters you wish to evaluate.
19. If applicable, line scan data can be analyzed in a separate Excel sheet.
  - a. For the measurements, the data is exported with columns associated with the values and type that correspond to the measurement number.

**Note:** There will be a value for the length of the measurement taken and for the area of each channel, for both the head and the neck (head will correspond to measurement 1, neck to measurement 2). The area value corresponds to the area under the curve of line profile graph (which is intensity per length). This data can be used to compare areas for the protein of interest within the head vs the neck sub-compartment for each spine, as an assessment of protein localization.

△ **CRITICAL:** If the value used for the neighborhood area (width of the line scan) was changed for each spine, this should be taken into account if doing comparisons between spines.

- b. Line profile data can also be exported for each spine. This data will provide a value for each channel at set intervals along the entire line-scan drawn. Use the measurement data from the line scan to separate these intervals into head and neck regions.
20. In single protein experiments, this data can be used to analyze a range of questions regarding nanodomain localization between spine types and within spine sub-compartments. A few examples include:
- a. Does the number of nanodomains per area differ by spine type?
  - b. Is there a difference in the concentration of nanodomains within the spine head or spine neck region by spine type?
  - c. Correlations can be made between nanodomain parameters and spine size parameters.
21. In multi-protein experiments, such as the example used here, this data can be used to assess the relationship between the two proteins and their effects on spine structure. For example, we compared cells that received overexpression of protein 1 vs. those that received mock transfection. We used immunocytochemistry to label the overexpressed GFP fill, an endogenous protein 2, and where applicable, the overexpressed protein 1. The analysis described above allowed us to assess:
- a. Changes in the spine size and the sub-compartment size with or without overexpression of protein 1 by spine type.
  - b. To compare how the sub-compartment localization of the endogenously expressed protein changed with or without overexpression of protein 1 in different spine types.
  - c. Assess changes in spine size and sub-compartment size when there is spatial overlap/ colocalization between the two proteins compared to when there is not.

## EXPECTED OUTCOMES

The completion of this workflow should provide you with dendritic spine morphology data including (but not limited to) spine area, head area, neck area, and neck length, which can be used to determine spine type. This workflow will also provide protein nanodomain information including (but not limited to) area, channel intensity, location (head or neck sub-compartment), and if applicable, whether it is colocalized with another protein. This data can provide information about the number of protein nanodomains per spine, the number of colocalized protein nanodomains per spine, the number of protein nanodomains in each spine sub-compartment (head and neck), and the number of colocalized protein nanodomains in each spine sub-compartment. This information can then be analyzed against the spine structure parameters including spine size and spine type. Overall, this protocol creates a large dataset that can be analyzed in several ways to assess the effects of protein localization or protein interaction on dendritic spine structure.

## LIMITATIONS

Some limitations to this protocol include the use of 2D rather than 3D analysis. The lack of volume information limits the degree of accuracy of spatial information in the z-plane. Additionally, the analysis requires subjective decisions to be made by the user to determine nanodomain puncta separation, LUT, and threshold values. Although this is also a limitation of most dendritic spine analyses, here, the user must define the boundary of the dendritic spine as well as where the delineation is between the head and neck region. Lastly, this protocol is effective for comparing levels of proteins within dendritic spines as a function of spine morphology or classification. However, it does not provide any information about absolute values of a given protein.

### TROUBLESHOOTING

The Nikon NIS-Elements AR user guide can be found online and the Nikon Instruments Learning Center (<https://training.nikoninstruments.com>) and provides useful information for troubleshooting any issues with the tools described in this protocol.

#### Problem 1

The appearance of a 'ghosting' artifact or structures displaying 'echo shadows' in reconstructed images.

#### Potential solution

This might be due to a refractive index (RI) mismatch. Ensure that the refractive index of the mounting media matches that of the immersion medium of your microscope objective.

#### Problem 2

The protein of interest does not appear as distinct nanodomain structures, rather it is diffusely spread throughout the dendritic spine.

#### Potential solution

Assess spatial localization using line scan intensity profiles described in step 3 of the [analyzing nanodomain localization within dendritic spine compartments](#) section rather than manually assigning nanodomain objects to spine sub-compartments.

#### Problem 3

Nanodomain signal appears too weak.

#### Potential solution

Alter the labeling strategy; use brighter, more photostable dyes; reduce background caused by nonspecific labeling; avoid auto-fluorescence by using orange/red dyes.

Increase the laser power or exposure time in acquisition of the images (ensure that this does not lead to photobleaching).

#### Problem 4

Nanodomain signal artificially extends beyond the boarder of the dendritic spine.

#### Potential solution

Reduce the gain to avoid over-enhancing the intensity appearance. Change LUT settings to ensure that nanodomain puncta are not artificially inflated.

#### Problem 5

Difficulty determining boundaries when creating or adjusting binary segmentation masks.

#### Potential solution

Load the associated Z-stack image and utilize the 3D information available by scrolling through the Z-stack to get a better sense of the image compared to the flattened maximum intensity projection.

#### Alternative approach

Rather than creating individual binary masks for the head and neck region as suggested in step 9 of [structural analysis of dendritic spine parameters](#), use the binary area measurement in the Annotations and Measurements toolbar (Figure 4C).

- Select the head and neck layer in the binary toolbar so that the boundary between sub-compartments is visible along with the spine ROIs.

- Open the Annotations and Measurements tool (View, analysis controls, annotations and measurements).
- To measure the head area of each spine, choose 'select binary' located under the area heading.
- Click on just the head region of each spine in order of their ROI ID. The area measurement will be added to the table after each click. Similar to the neck length measurement, it is critical that these area measurements are taken in the order of the ROI labels.
- Export the data to Excel. If you measured the areas in the order corresponding to their ROI ID, the measurement number will correspond to the correct spine ID number.
- To measure the neck area of each spine, reset the table using the red x button and repeat the steps listed above, clicking each neck region rather than the head in order of the ROI ID.

Other analysis software with similar capabilities can also be used. For example, an adapted version of this protocol was also performed in Imaris. Imaris has the benefit of working in 3D, having this additional spatial information can make the subjective decisions easier, however, the tools used for separating the segmented masks are more difficult to use and much more time consuming.

NIS-Elements can also be used with most common file types including JPG, BMP, AIV, TIFF, JIFF, JPEG2000, JTF, LIM, AND ICS/IDS.

Other versions of NIS-Elements can also be used they have all of the basic capabilities required for this protocol.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Michael Cahill ([michael.cahill@wisc.edu](mailto:michael.cahill@wisc.edu)).

### Technical contact

Technical issues should be directed to the technical contact, Kathryn Bjornson ([kbjornson@wisc.edu](mailto:kbjornson@wisc.edu)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

The published article includes example figures and datasets. Source data is available in:

Bjornson, K.J., Vanderplow, A.M., Yang, Y., Anderson, D.R., Kermath, B.A., and Cahill, M.E. (2023). Stress-mediated dysregulation of the Rap1 small GTPase impairs hippocampal structure and function. *iScience* 26, 107566. <https://doi.org/10.1016/j.isci.2023.107566>.<sup>1</sup>

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## AUTHOR CONTRIBUTIONS

K.J.B. completed the experiments, conceived and designed the analysis, and analyzed and interpreted the data. K.J.B. and M.E.C. wrote the paper and M.E.C. supervised the original experiments.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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