Appendix 1

Structural synaptic signatures of contextual memory retrieval-reactivated hippocampal engram cells

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Protocol for reconstructing spines and dendrites in Imaris

This protocol described methodology to reconstruct spines and dendrites in Imaris (version 9.9.1, Oxford Instruments, Abingdon, United Kingdom). The obtained data can subsequently be used to investigate parameters of structural synaptic connectivity, such as the number, distribution and morphology of spines.

This protocol was developed based on membrane-tagged engram cells using myrRFP and labelled synapses originating from the CA3 and forming synapses on CA1 engram cells using eGRASP(Choi et al., 2018). Z-stacks of the region of interest were obtained using a widefield microscope with a 100x oil objective (NA 1.45) while adhering to Nyquist criteria. Prior to reconstruction, all images were deconvolved using Huygens Professionals (Scientific Volume Imaging B.V., The Netherlands). Channel 1 corresponds to DAPI signal, Channel 2 to myrRFP signal, Channel 3 to YFP eGRASP, and Channel 4 to cFos signal (Alexa 647).

Note: images taken on a confocal microscope can also be used.

After importing images into IMARIS, the eGRASP signal is first used to identify CA1 engram spines receiving CA3 input. The fluorescent signal of these eGRASP+ synapses is used to mark spines with a spot. The morphology of engram cells and their spines is identified using a membrane targeted RFP (myrRFP). Based on myrRFP fluorescence, dendrites and spines are reconstructed by creating filaments. For this study, we chose to only reconstruct eGRASP+ spines, i.e. those with both a pre- and postsynaptic element, on engram cells by only reconstructing filaments that co-localise with spots. If the aim is to reconstruct all spines, the first step of creating spots based on synaptic labels can be skipped.

Note: other fluorophores that allow for dendrite and spine morphology to be detected, such as Biocytin, can also be used.

From image acquisition to exporting data of reconstructed dendrites and spines, **special attention should be given to accurate and unique naming of files** as elaborated in VI.5d of this protocol. Obtained data of reconstructed dendrites and spines are then exported for further statistical analyses in R Studio (RStudio-Team, 2020). Scripts for subsequent creation of data sets based on IMARIS and identification of spine clusters and spine classification are available in a GitHub repository: (https://github.com/PantheaNemat/structural_dendrite_spine_analysis).

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I. Starting up Imaris

Prepare data for analysis

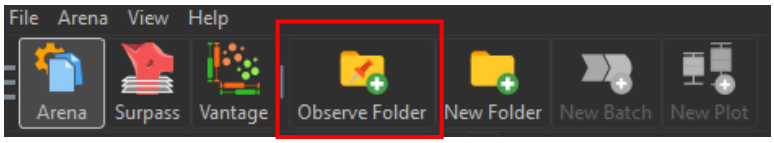
1. Store your deconvolved images in a folder

Opening the program and selecting licenses

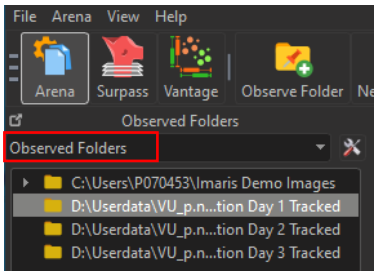
1. Double-click to open Imaris
    a. The **Initial Settings** window will appear
2. Select the following licenses: **FilamentTracer**, **Imaris Lineage** and **Imaris Track** and click OK

Observe folders

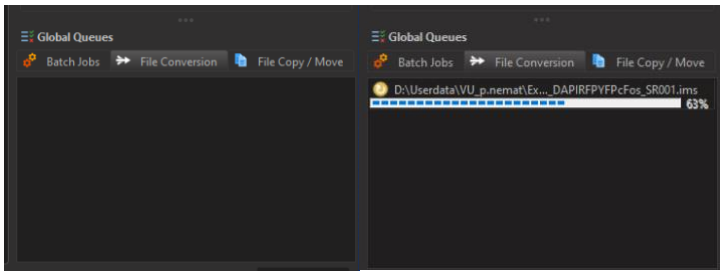
1. Click **Observe Folder** in **Arena** view



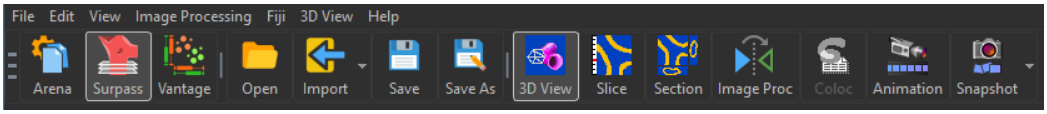
2. Locate the folder storing the images
    a. The folder will appear below **Observed Folders**



3. Click select to open the folder
    a. The images will appear in the middle tab
4. Double-click images to convert files into Imaris format
    a. The **File Conversion** will be visible below **Global Queues**

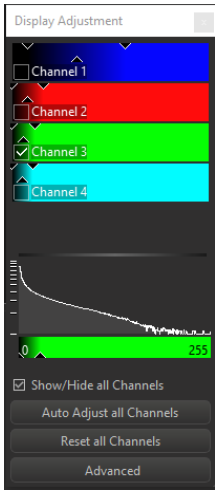


5. Double-click an image to open it after it has been converted into Imaris format
    a. This will switch the view from **Arena** to **Surpass**
    b. Ensure to have **3D view** selected for optimal reconstructions later

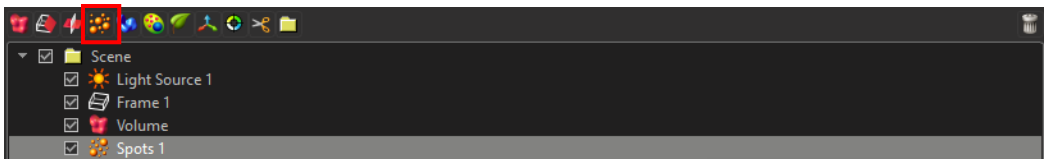


II. Spine spot creation

1. **Edit > Show Display Adjustment**
   Deselect all channels except for the channel capturing the fluorescent synaptic tag in the **Display Adjustment** window (here: Channel 3 – YFP eGRASP)



2. Click **Add Spots** () and deselect **Classify Spots** in **Create**
   a. **Object-Object Statistics** can remain selected
   b. The newly created **Spots** object will appear below **Scene**

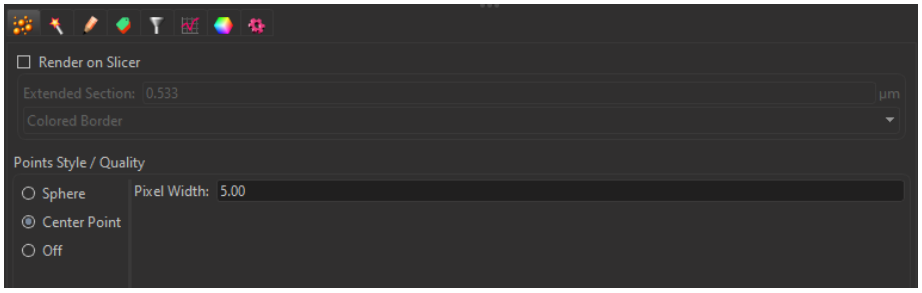


3. Click the blue arrow button ()
4. Scroll down to select the channel capturing the fluorescent synaptic tag (here: Channel 3 YFP-eGRASP)
   a. Thereby only synapses of this size will be marked with a spot.

5. Use the automatically calculated diameters or fill in measured diameters (using **Measurement Tool**) of fluorescent synaptic tags

   a. "Estimated XY diameter" (here: 0.400 µm)
   b. "Estimated Z diameter" (here: 0.800 µm)

6. Click the green double arrow button () to finish **Spot Creation**
    a. Spots will be placed based above the automatic threshold of the **Quality** Filter Type. The 'Quality' is the intensity at the center of the spot in the channel the Spots was detected (*p.* 432, Imaris 9.2 Reference Manual). Thereby only synapses with sufficient signal will be marked with a spot.
7. Select **Center Point** below **Point Style** in **Spots Settings** to optimize visibility
    a. **Pixel Width** can be adjusted to enhance visibility of the spots



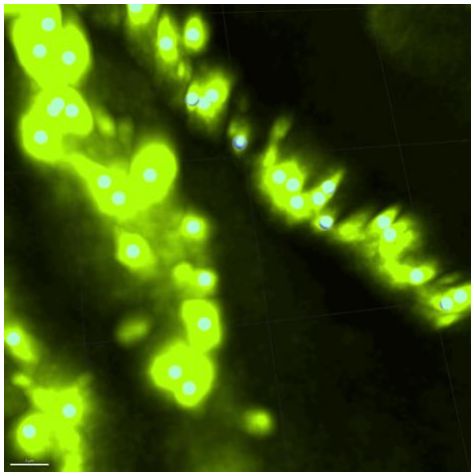
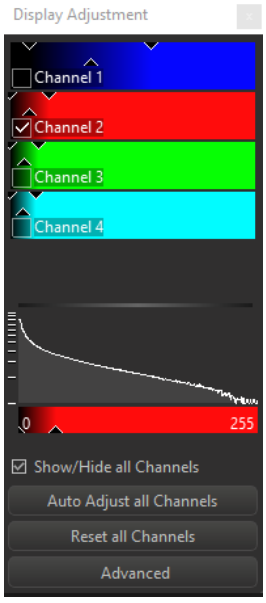


Fig. A1. Example image of automatic spot detection. YFP eGRASP+ synapses are automatically detected based on size and signal intensity. Scale bar = 2 µm. Green – YFP eGRASP, blue – spheres.

8. Deselect Spots object

III. Dendritic filament creation

1. Deselect all channels except for the membrane-dye channel in **Display Adjustment** window (here: Channel 2 - myrRFP)



2. Find a **free-standing secondary or tertiary dendrite** that matches the following criteria:
   e.g.
   a. ***Must be at least 50 µm away from soma***
      ← differences in hippocampal spine density were only found at a distance from at least 50 µm from the soma (Abate, Colazingari, Accoto, Conversi, & Bevilacqua, 2018)
      i. Use **Measurement Tool** to estimate distance from soma
      ii. Left-click to make a beginning point and again left-click to make an end point

   b. ***Must be a dendritic segment of 25-50 µm in length***
      ← due to high density of dendrites in the CA1, we found most dendrites in this range to fulfil reconstruction criteria while ensuring sufficient data quality
   c. ***Must have a clearly visible soma located in pyramidal layer***
      → to later determine reactivation status

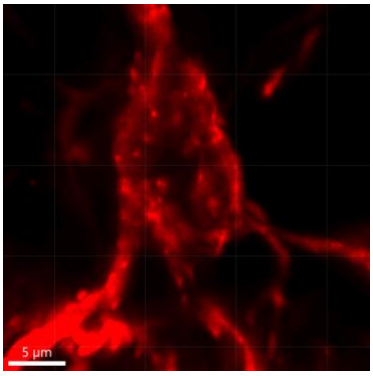
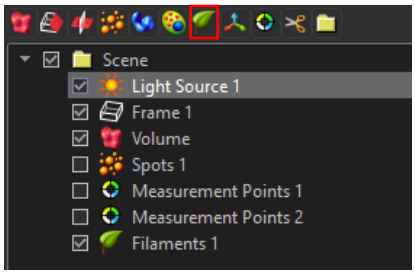


Fig. A2. Example image of soma. Scale bar in image. Red – myrRFP.

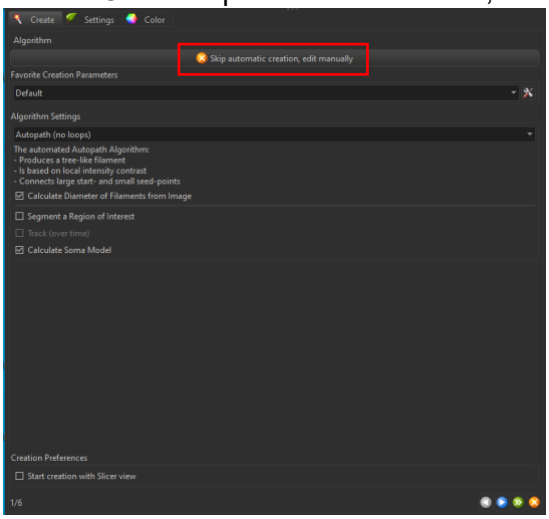
d. ***Avoid:***
   i. ***branching dendrites***
      ← branching points are typically spine-free and are therefore anatomically different than continuous parts of dendrites
   ii. ***crossing dendrites or other dendrites in close vicinity***
      ← impairs clear identification of the origin and orientation of the fluorescent signal
   iii. ***dendrites located at borders of the 3D volume***
      can be recognised as fluorescent signal of a dendrite is cut-off
e. ***Do not reconstruct primary dendrites***
   - primary dendrites directly branch off from soma → secondary and tertiary dendrites are the thinner dendrites after the first and the second branching points, respectively
   - can have a larger diameter and therefore be anatomically different to secondary and tertiary dendrites
f. ***Do not reconstruct interneurons***
   typically recognised as by shape/size and soma lying outside of the CA1 pyramidal layer

These criteria can/should be adjusted to the specific research question or brain region

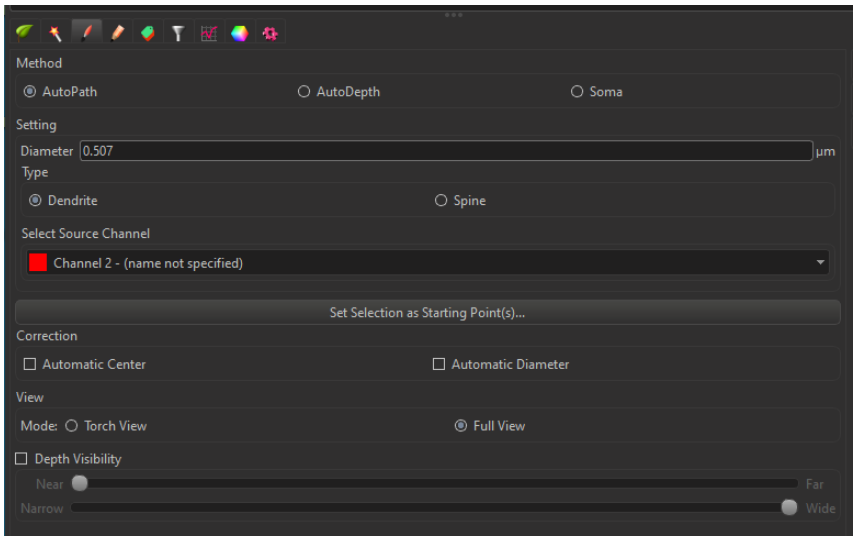
3. Click **Add new Filaments** ( )
   a. The newly created **Filament** object will appear below **Scene**



4. Click “Skip automatic creation, edit manually” and (de)select the following options in



5. the **Draw** tab
    a. Select the source channel of the membrane-dye (here: Channel 2 – myrRFP)
    b. Ensure that **Dendrite** is selected for **Type**



6. Shift + right-click to set the **Dendrite Beginning Point** visualized as a blue sphere

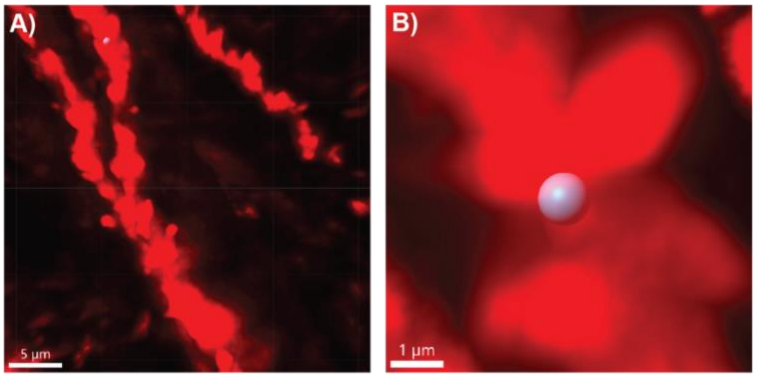


Fig. A3. Example images of dendrite beginning point placement. A) Sphere indicates dendrite start point along tagged engram cell. B) Close up of engram cell with dendrite beginning point. Scale bar in image. Red – myrRFP, blue – dendrite start point.

7. Move the cursor along the dendrite until it reaches roughly 25-50 µm in length
    a. Dendrite length will be checked in step III.8
8. Shift + left-click to set the **Dendrite End Point**
    a. Imaris creates a line tracing the dendritic segment along the membrane-dye channel

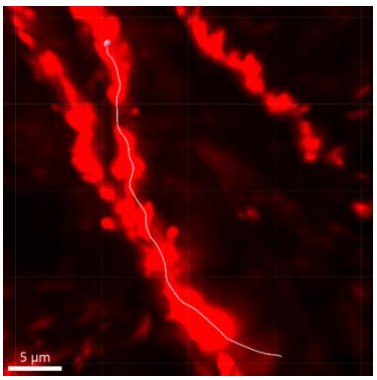
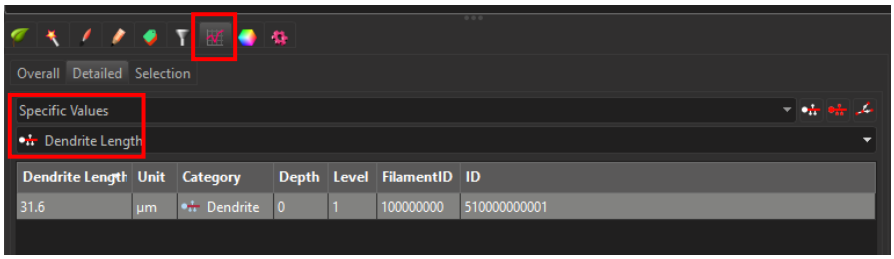


Fig. A4. Example image of manually traced dendrite. Sphere indicates dendrite beginning point. Line shows manual tracing of dendrite based on myrRFP fluorescence corresponding to tagged engram cell. Scale bar in image. Red – myrRFP, blue – dendrite beginning point, grey – filament.

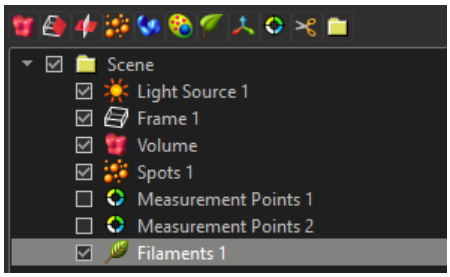
9. Go to **Statistics > Detailed > Specific Values** and scroll down to check **Dendrite Length** (25-50 µm)



10. Repeat steps 5 – 8 until all qualified dendritic segments within the 3D volume have been traced.

IV. Manual spine tracking

1. Select Spots object



   a. Now Filament object and Spots object are visible

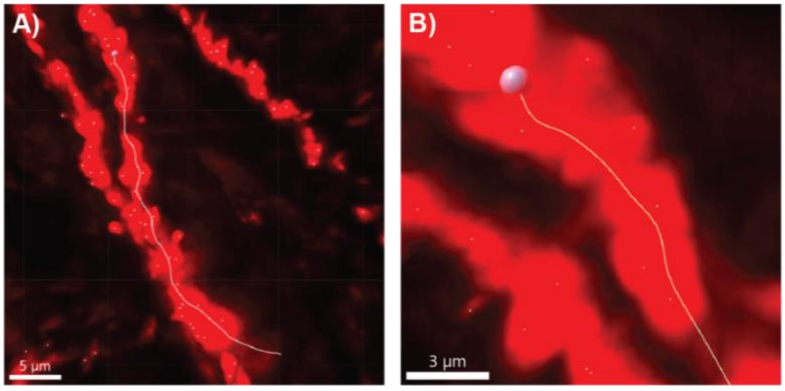
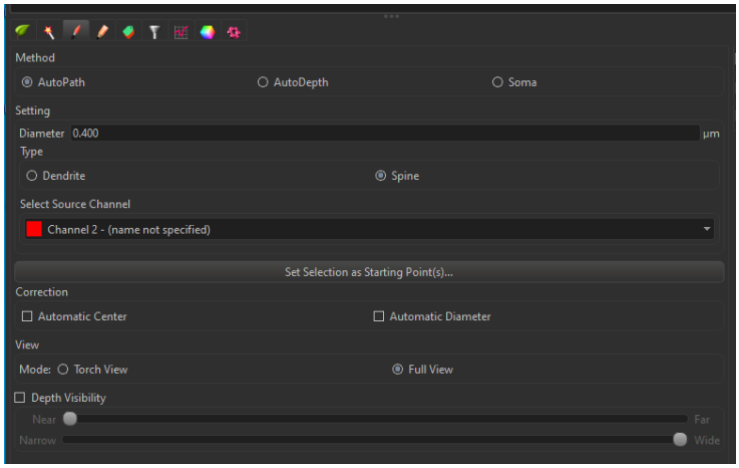
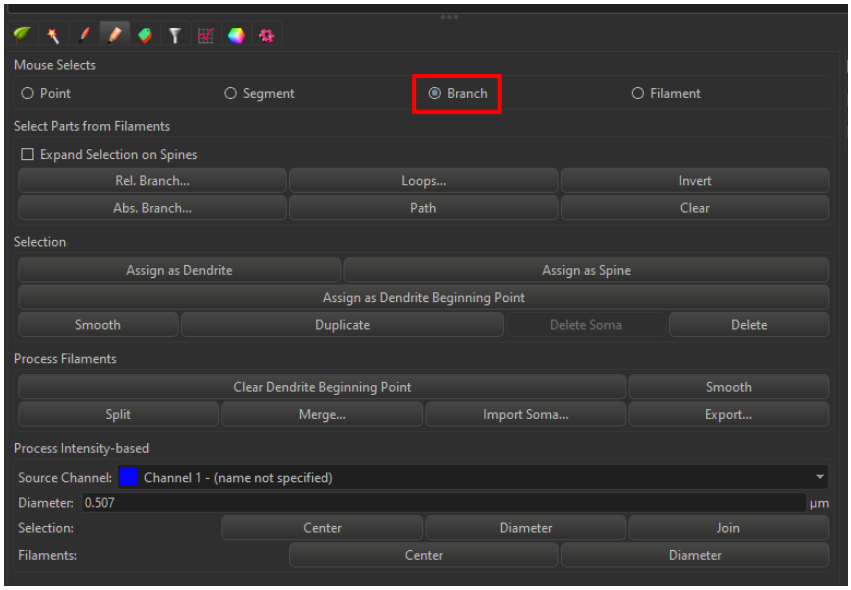


Fig. A5. Example images of manually traced dendrite together with spots objects indicating spines to be reconstructed. A) Large blue sphere indicates dendrite beginning point. Line shows manual tracing of dendrite based on myrRFP fluorescence corresponding to tagged engram cell. Smaller spots indicate previously identified spines to be reconstructed, see section II Spine spot creation. B) Close up of engram cell with dendrite beginning point, manually traced dendrite, and automatically detected spots. Scale bar in image. Red – myrRFP, blue – dendrite beginning point, grey – filament, white – spots.

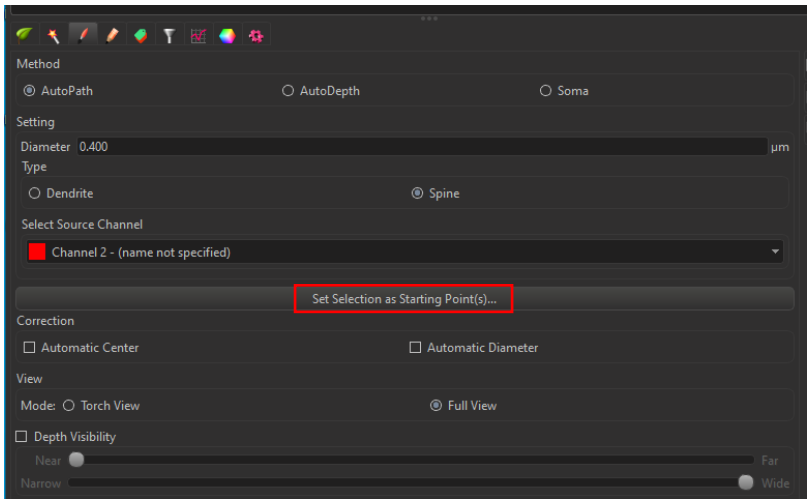
2. Go to **Draw** and adjust **Type** to **Spine**
   a. Ensure selection of the source channel of the membrane-dye (here: Channel 2 – myrRFP)
   b. Drawn spines will be depicted in blue on a (unselected) white dendrite
      i. Selecting an object turns it yellow to indicate current selection



3. Switch to **Edit** and adjust **Mouse Selects** to **Branch**



4. Left click on the dendrite to select it
    a. The dendrite will turn yellow
5. Go back to **Draw** and click “Set Selection as Starting Point(s)…”
    a. All drawn spines will now originate from the dendritic branch



6. Find a spine on the dendritic segment filament in which the fluorescent signal of the membrane dye overlaps with a spot generated in section II Spine spot creation of this protocol
    → Only eGRASP+ spines previously identified with a spot object will be reconstructed
7. Move the cursor to trace the spine until you localize its orientation and length
    a. Use the membrane-dye channel to identify spine orientation
    b. Make sure to do the tracing of the spine only in the membrane-dye channel

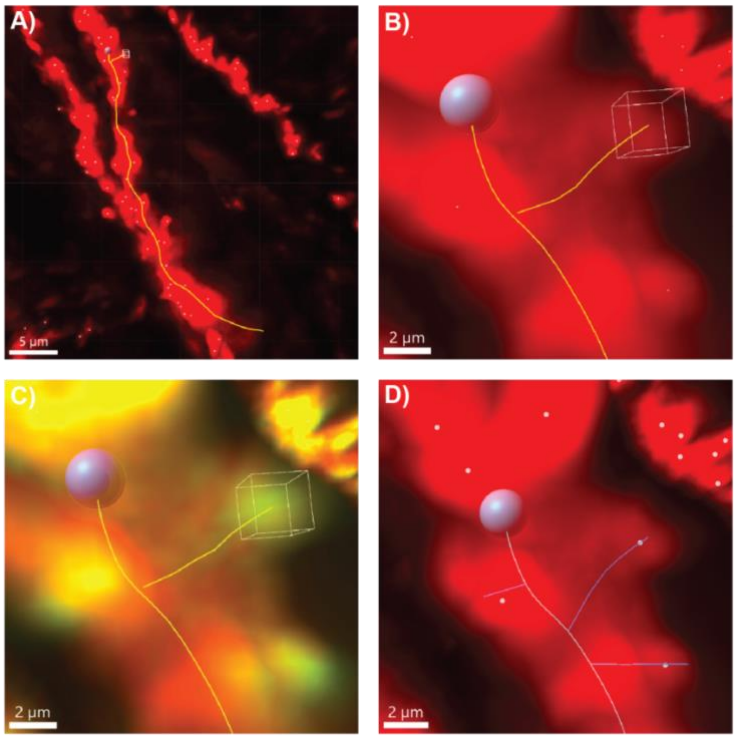


Fig. A6. Example images of manual spine tracing. A) Large blue sphere indicates dendrite strat point. Yellow line depicts manual tracing of selected dendrite and spine based on myrRFP fluorescence corresponding to tagged engram cell. Smaller spots indicate previously identified spines to be reconstructed, see section II Spine spot creation. B) Close up of manual tracing of spine starting from dendrite until end of spine previously labelled with spot. Engram cell with dendrite beginning point, manually traced dendrite, and automatically detected spots. Cube indicates cursor to manually trace spine. C) Same close-up as in B), but now also indicating YFP eGRASP signal overlapping with myrRFP, demonstrating overlap of labelled synapses along engram cell. D) Result of manually traced spines. Scale bar in image. Red – myrRFP, green – YFP eGRASP, yellow – overlap of myrRFP and YFP eGRASP, blue – dendrite beginning point and spines, grey – filament, white – spots.

8. Shift + left-click to set the **End Spine Point**
    a. Spines should be consistently traced until the end of the fluorescent signal
    b. The longest direction from dendrite to end of spine is chosen for spines that seem to go in multiple directions
9. Repeat steps 6-9 until all spines with a spot of all dendrites have been manually traced

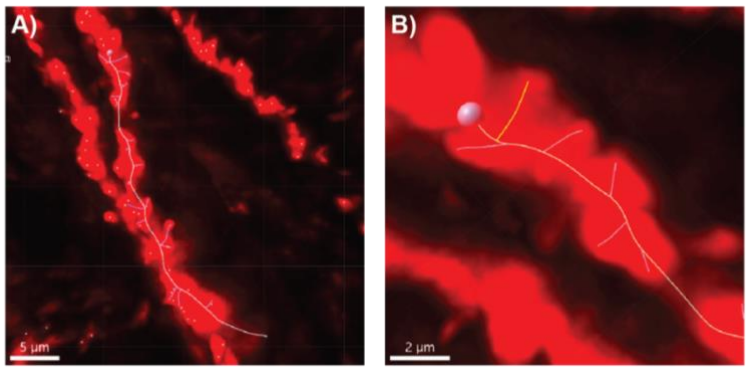
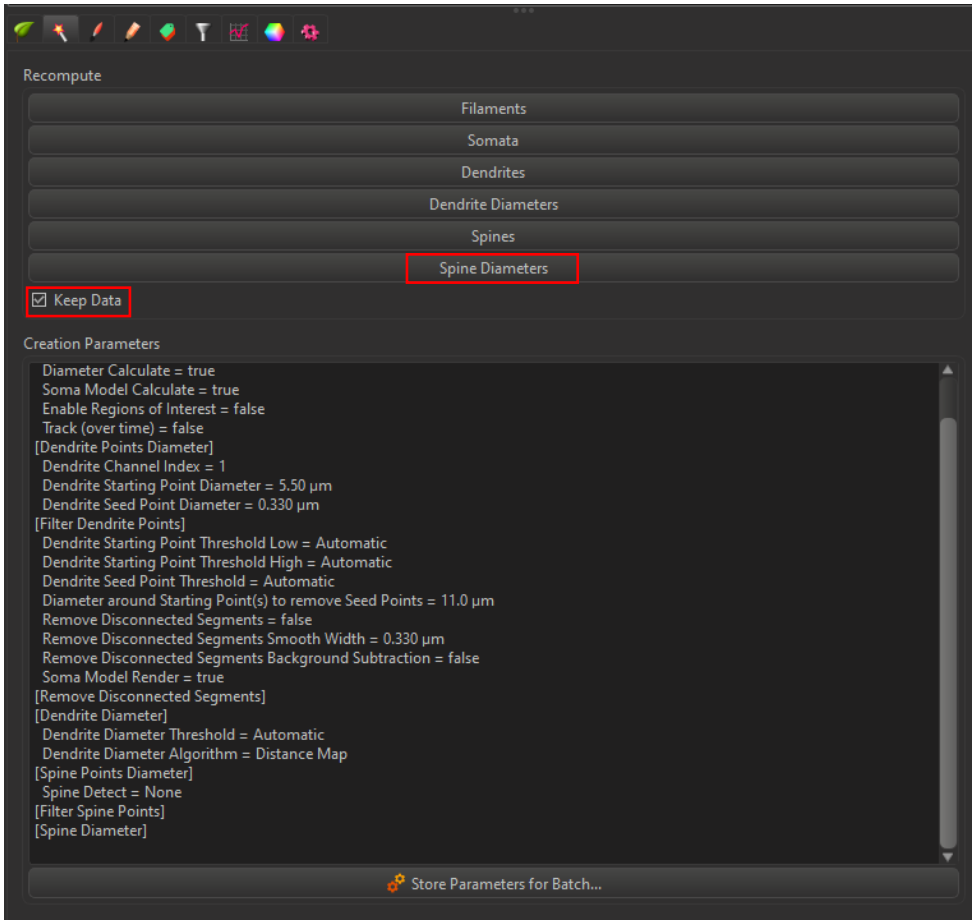


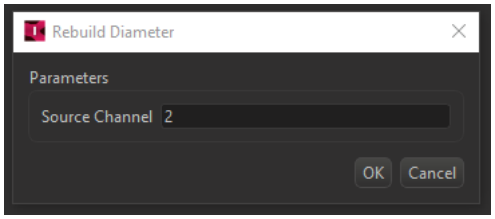
Fig. A7. Result of manual dendrite and spine tracing. A) Large blue sphere indicates dendrite beginning point. Line shows manual tracing of dendrite and spines based on myrRFP fluorescence corresponding to tagged engram cell. Smaller spots indicate previously identified spines to be reconstructed, see section II Spine spot creation. B) Close up of final result of manually traced dendrites and spines. Red – myrRFP, blue – dendrite beginning point and spines, grey – filament, white – spots.

V. Reconstructing dendrites and spines

1. Go to **Creation**
2. Ensure selection of **Keep Data**
3. Click on **Spine Diameters**



4. Fill in the membrane-dye channel number (here: Channel 2 – myrRFP) in the **Rebuild Diameter** window as spine shape is best represented by membrane-dye



5. Keep threshold **Quality Filter** on default settings

6. Click the green double arrow button () to finish **Automatic Spine Diameter Creation**
7. Check for all dendrites whether the spines created were reliably reconstructed

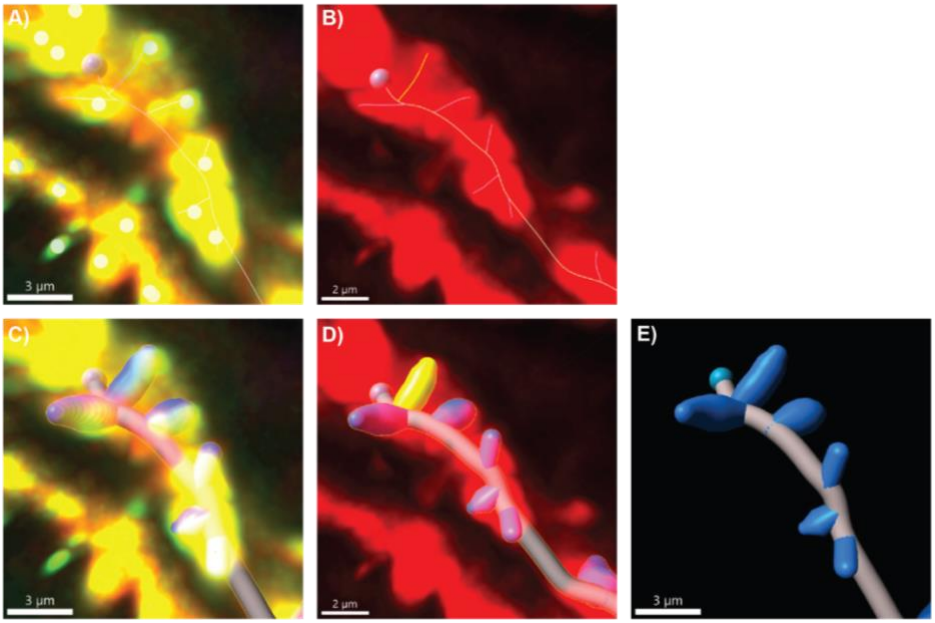
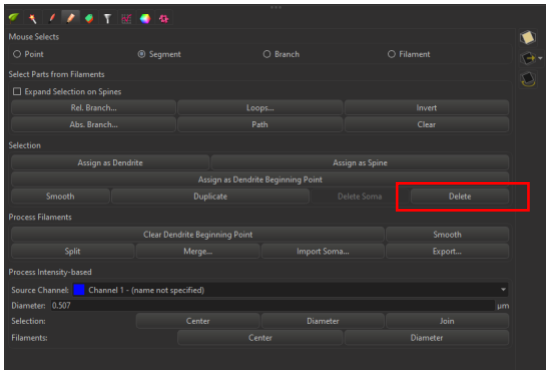
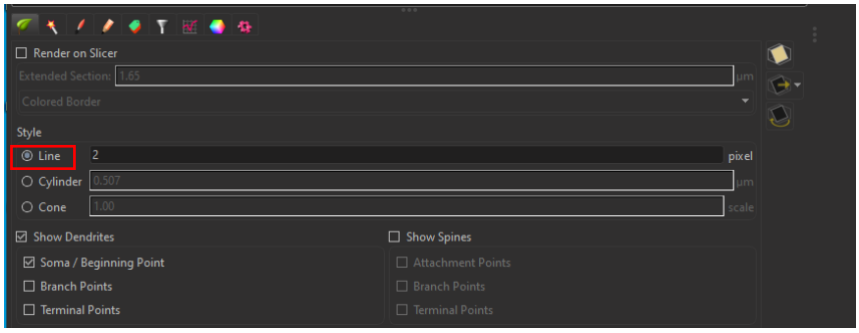


Fig. A8. Overview from automatically detected labelled eGRASP+ synapses, over manually traced dendrite and spines, to final result of automatically reconstructed dendrite and spines. A) Manually traced dendrite and spine together with myrRFP and YFP eGRASP signal. Large blue sphere indicates dendrite start point. Line shows manual tracing of dendrite and spine based on myrRFP fluorescence corresponding to tagged engram cell. Spots indicate previously identified spines to be reconstructed, see section II Spine spot creation. B) Result of manually traced dendrite with spines. C) Result of automatically reconstructed dendrite overlapping with myrRFP and YFP eGRASP signal. D) Same close-up as in C), but now with only myrRFP signal. E) Final result of automatically reconstructed dendrite with spines. Scale bar in image. Red – myrRFP, green – YFP eGRASP, yellow – overlap of myrRFP and YFP eGRASP, blue – dendrite beginning point and spines, grey – filament, white – spots.

8. Correct any mistakes in orientation or length:
   Go to **Edit** and select spine to be corrected
9. Click **Delete** below **Selection** in **Edit**



10. Return to from **Cone** to **Line Style** in **Filament Settings**

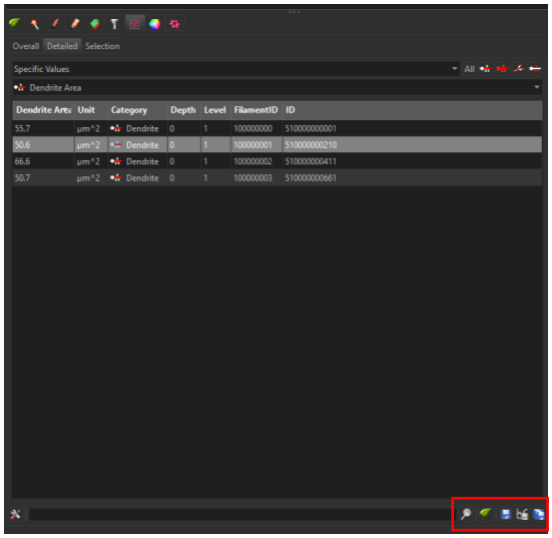


11. Retrace the spine as described in protocol section “IV. Manual spine tracking”

VI. Export data of reconstructed dendrites and spines

Export data for number and distribution of spines

1. Export all statistical data after all dendrites and spines have been reconstructed
2. Go to **Statistics > Detailed**
3. Scroll to select **Specific Values**



4. Click on “Export All Statistics to File” ()
5. Rename the file and click **Save**
    a. If multiple dendrites were traced, data of all dendrites can be exported together, as dendrites can be distinguished in the exported data file based on their filament number.
    b. Make sure to note reactivation status of cell in the file name by adding an extension (here: cFos+RFP+ or cFos-RFP+) Only if dendrites with different reactivation status were reconstructed, then data should be exported separately by creating separate Filament objects as explained in step VI.7
    c. Give your file name a unique name to differentiate it from the spine selection in the next step of the protocol by adding another extension – such as AllS (All Spines)
    d. **Important: To allow for the most straightforward processing in Imaris and subsequent data analysis, attention should be paid to accurately naming files – ideally starting during image acquisition.**
    File names should be chosen to give unique codes to each image (here: ExperimentNumber_AnimalNumber_SliceNumber_Hemisphere_ImagedFluorophores_AnatomicalLayer_cFos-RFP+/cFos+RFP+_AllS/SelS)
6. Return to the **Filament** object

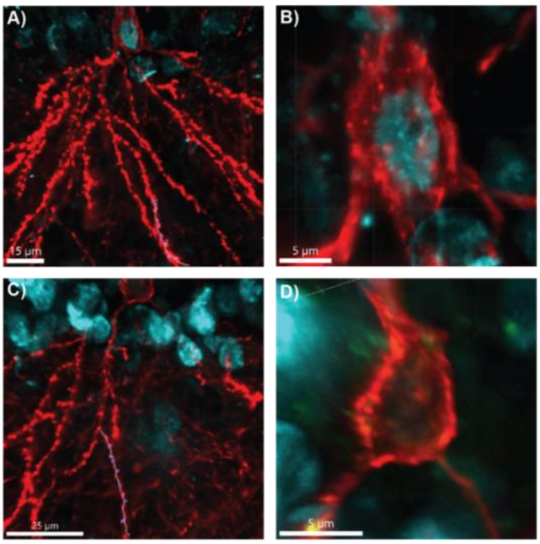
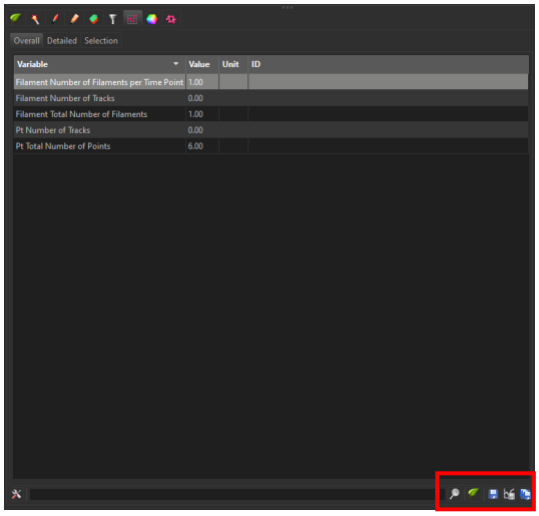


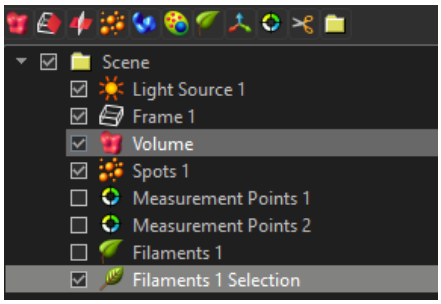
Fig. A9. Example images of reactivation status. A) Example of reactivated engram cell as soma is cFos+RFP+. B) Close up of reactivated engram cell. C) Example of non-reactivated engram cell as soma is cFos-RFP+. D) Close up of reactivated engram cell. Scale bar in image. Red – myrRFP, turquoise – cFos.

Export data for morphological analysis

7. Ctrl + left-click to select all dendrites and spines in XY direction ← as lateral resolution is higher than axial resolution, select only spines in XY direction for morphological analysis (*see* Methods)
    a. Ensure all dendrites and spines to be exported to be yellow, i.e. selected by Imaris
    b. Go to **Edit** and adjust **Mouse Selects**: Segment
8. Go to **Statistics > Duplicate Selection to new Filaments** ( )



a. The newly created **Filament Selection** will appear below **Scene**



9. Again save the Filament selection:

   Statistics > Detailed by clicking on **Export All Statistics to File** ()
   a. Change the extension of your file name to differentiate these selected spines from data of all spines that were previously stored – such as SelS (Selected Spines)
10. To return to other images click on Arena view
    a. The “Switch View – Save changes?” window will appear
    b. Click **Yes** if you want the tracked dendrites and spines to be saved

11. *Generate one data file of all exported single data files and determine spine clusters and spine types by using provided R scripts in Github repository.*

References

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