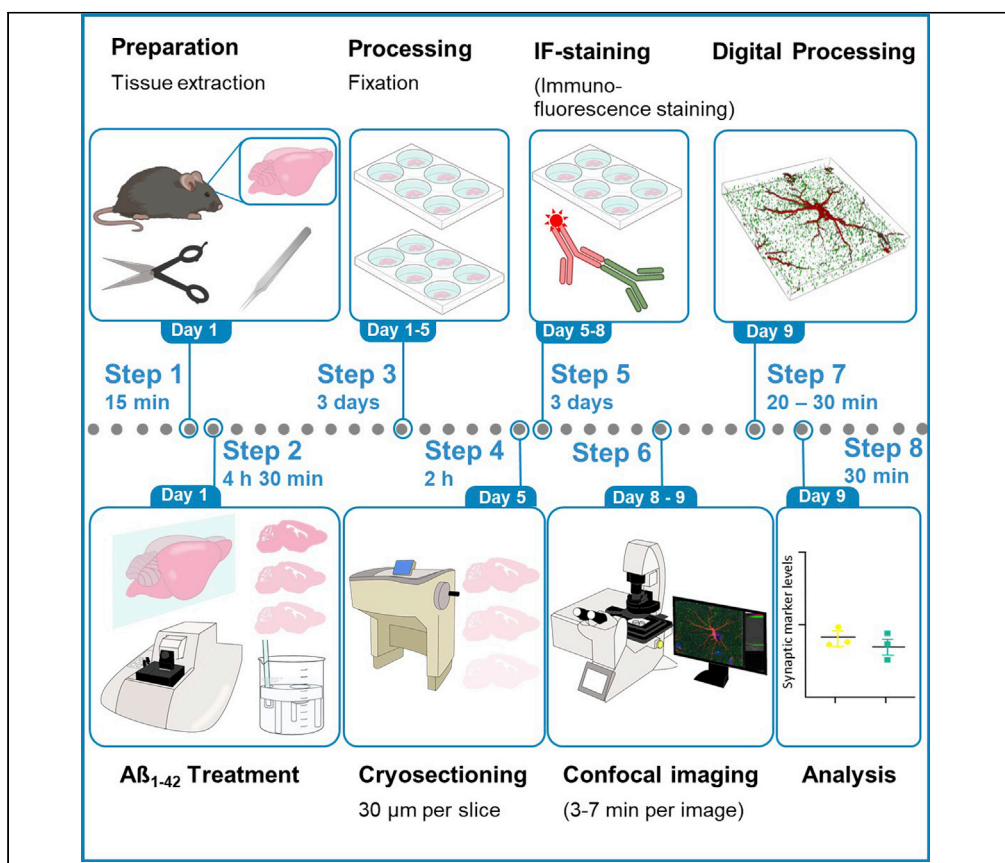# STAR Protocols

Protocol

Quantification of astrocytic synaptic pruning in mouse hippocampal slices in response to *ex vivo* Aβ treatment via colocalization analysis with C1q

Preparation — Tissue extraction — Processing — Fixation — IF-staining (Immunofluorescence staining) — Digital Processing

Day 1 — Step 1 — 15 min
Day 1-5 — Step 3 — 3 days
Day 5-8 — Step 5 — 3 days
Day 9 — Step 7 — 20 – 30 min

Day 1 — Step 2 — 4 h 30 min
Step 4 — 2 h — Day 5
Step 6 — Day 8 - 9
Step 8 — 30 min — Day 9

Aß₁₋₄₂ Treatment
Cryosectioning — 30 μm per slice
Confocal imaging — (3-7 min per image)
Analysis

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Highlights

Measurement of synaptic engulfment in response to treatment with Aβ peptide

Volumetric reconstruction of high-resolution individual astrocyte

Colocalization analysis of astrocyte and complementary "eat-me" protein C1q

Quantification of synaptic engulfment is an indirect measurement of synaptic pruning. Here, we provide a detailed protocol for the volumetric rendering of individual high-resolution astrocytes in the CA1 region of hippocampus in an *ex vivo* model of amyloid-beta (Aβ) treatment. The protocol includes the treatment of free-floating sections with Aβ peptide and confocal imaging of individual astrocytes. We also provide a comprehensive analysis for 3D rendering of astrocytes and assessment of synaptic engulfment via "eat-me tag" C1q protein.

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

Protocol

Quantification of astrocytic synaptic pruning in mouse hippocampal slices in response to *ex vivo* Aβ treatment via colocalization analysis with C1q

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SUMMARY

Quantification of synaptic engulfment is an indirect measurement of synaptic pruning. Here, we provide a detailed protocol for the volumetric rendering of individual high-resolution astrocytes in the CA1 region of hippocampus in an *ex vivo* model of amyloid-beta (Aβ) treatment. The protocol includes the treatment of free-floating sections with Aβ peptide and confocal imaging of individual astrocytes. We also provide a comprehensive analysis for 3D rendering of astrocytes and assessment of synaptic engulfment via "eat-me tag" C1q protein.

BEFORE YOU BEGIN

Synaptic pruning, which is primarily mediated by the glial cells, is an essential step in modulation and shaping up of synapses and fine-tuning of neuronal connection (Tierney and Nelson, 2009; Tau and Peterson, 2010; Perez-Catalan et al., 2021). Astrocytes, which form a bigger subset of glial cells, play a significant role in restructuring of synapses by regular crosstalk with microglia (Chung et al., 2015). This crosstalk is mediated by a diverse range of cytokines (Garland et al., 2022). C1q of the complement cascade is one such essential tags, which is also referred to as the "eat-me" signal and plays a critical role as a signaling molecule in the pruning of neurons (Iram et al., 2016) (Kovács et al., 2021). The pruning of synapses in the brain occurs via both C1q dependent and independent pathways (Györffy et al., 2018) (Druart and Le Magueresse, 2019; Gomez-Arboledas et al., 2021). Upon detection of redundant synapses, astrocytes release tumor growth factor-β (TGF- β), which increase the expression of C1q tags (Allen and Eroglu, 2017). Microglia upon recognizing this tag release inflammatory cytokine to engulf the synapse through phagocytosis.

Alzheimer disease (AD), often associated with memory deficit and cognitive decline, is a progressive neurodegenerative disorder with amyloid plaques and neurofibrillary tangles as key histopathological hallmarks and hypothesized as essential players driving the pathophysiology. Pruning of synapses has often been implicated in the early pathological advances of Alzheimer's. Previously, it has been reported that C1q expression is increased and associated with synapses before plaque deposition and is necessary for mediating the toxic effects of soluble Aβ oligomers on synapses and long-term potentiation (LTP) in hippocampus (Hong et al., 2016). From previous studies including one from our lab, the treatment of hippocampal slice with 50 nM $A\beta_{42}$ caused a significant decline in the CA1-LTP of the hippocampus (Li et al., 2011; Rammes et al., 2018). Therefore, we recently aimed to look at the molecular level changes, particularly at the level of synapse, after $A\beta_{42}$ incubation. We recently developed a protocol to analyze and systematically quantify synaptic engulfment of materials by the astrocytes in the hippocampus after *ex vivo*

slice treatment with Aβ$_{42}$ oligomers. The hippocampus slices were obtained from 6-8 weeks old C57BL/6 mice. To look at the synaptic involvement, we also tried to look at the C1q engulfment in the astrocytic volume. Although this protocol has been optimized in the stratum radiatum layer of CA1 region of hippocampus, it can also be applied to other brain regions. This protocol of synaptic engulfment determination using *ex vivo* slice treatment of Aβ$_{42}$ oligomers can be combined with different treatment regimens of drugs/small molecules, which affect the binding of Aβ$_{42}$ oligomer. Since, aberrant pruning of synapses may play a quintessential role in the development of AD, this protocol would help understand the therapeutic relevance and the underlying working mechanism of different drugs/small molecules tested.

Institutional permissions

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Klinikum rechts der Isar, Technical University of Munich. All the experiments performed confirm to the institutional regulatory standards. The researchers following this protocol should seek approval from their own institutional regulatory agencies before starting the experiment.

Making of preparation ringer and mess ringer solution

⏱ Timing: 30 min

1. Prepare 1 L of mess ringer/artificial cerebrospinal fluid (aCSF) solution.
   a. Dissolve the following reagents in 800 mL of milliQ water by constantly stirring the content with a magnetic stirrer and bring up the level to 1 L after the solid has completely dissolved. Store the mess ringer solution at 4°C for a maximum of seven days.

Mess ringer (aCSF)		
Reagents	Final concentration [M]	Amount [g]
NaCl	0.125	7.305
KCl	0.0025	0.186
NaH$_2$PO$_4$-Monohydrate	0.00125	0.172
D-(+) Glucose-Monohydrate	0.025	4.954
NaHCO$_3$	0.025	2.100
MgCl$_2$-Hexahydrate	0.001	0.203
CaCl$_2$-Dihydrate	0.002	0.294

Add MgCl$_2$ and CaCl$_2$ separately. Adjust pH to 7.4 After 10 min of oxygenation with carbogen (95% O$_2$ and 5% CO$_2$), the actual pH value can be measured, it should be 7.30 (7.20–7.40).

⚠ CRITICAL: The mess ringer solution should be freshly prepared before the starting of the week and should be immediately stored at 4°C for a maximum of 7 days. Past this period, do not use this solution, and prepare a fresh batch.

2. Making 2 L of preparation ringer solution.
   a. Dissolve the following reagents in 1.5 L of milliQ water by constant stirring with a magnetic stirrer and make up the volume to 2 L.
   b. Divide the solution into four plastic bottles of 500 mL each and store the bottles at - 80°C for a maximum of one month.

Preparation ringer		
Reagents	Final concentration [M]	Amount [g]
NaCl	0.125	14.610 g
KCl	0.0025	0.373 g

(Continued on next page)

Continued

Preparation ringer		
Reagents	Final concentration [M]	Amount [g]
NaH$_2$PO$_4$-Monohydrate	0.00125	0.345 g
D-(+) Glucose-Monohydrate	0.025	9.909 g
NaHCO$_3$	0.025	4.201 g
MgCl$_2$-Hexahydrate	0.006	2.440 g
CaCl$_2$-Dihydrate	0.00025	0.074 g

⚠ CRITICAL: Do not fill the plastic bottles to the top, which would cause cracks in the bottle due to the expansion in volume. In an alternate scenario, where researchers intend to use the preparation ringer solution within a span of seven days, the 2 L solution can directly be stored at 4°C and placed for 40 min at −80°C before starting the sacrifice procedure.

Preparation of amyloid-beta (Aβ$_{42}$)

⏱ Timing: 3–4 h

3. 1 mg of Aβ$_{42}$ is dissolved in hexafluoro-2-propanol (HFIP) (400 μL) and is incubated at room temperature (RT) (21°C to 24°C) until clear solution forms.

   Note: This could take up to 90 min at RT (21°C to 24°C). Alternatively; one can warm up at 37°C for 15–20 min.

4. Aliquot the solution into 20 micro centrifuge tubes with 20 μL each.

   ⚠ CRITICAL: Use siliconized (low protein binding) tubes! Put the tubes directly on dry ice in a box.

5. Place the tubes in lyophilizer for up to 2 h until white pellets form at the bottom of the tubes.

   ⚠ CRITICAL: Before placing the tubes into the lyophilizer, open them and close the tubes with parafilm while the lid is open. Gently poke several holes into the parafilm to make the lyophilization process possible. This is to ensure a smooth evaporation of HFIP leaving Aβ$_{42}$ at the bottom of the tube.

6. After the completion of lyophilization, the tubes should be tightly closed, labeled and placed at −80°C.
7. For the preparation of monomers, one Aβ aliquot was thawed and freshly dissolved in DMSO (anhydrous). 100 μM of Aβ$_{42}$ is made by adding 111 μL DMSO and then sonicating the tube on an ultrasonic bath (35 kHz) for 15 min (Russ et al., 2022).

   ⚠ CRITICAL: Use only freshly prepared Aβ$_{42}$ in DMSO. The solution can be kept at −20°C (no longer than a month) and should not be kept out at RT (21°C to 24°C) for longer than 20–30 min.

Preparation for slice incubation

⏱ Timing: 15 min

8. Before sacrificing the mice, make sure that the microtome setup is working and running. Switch on the water bath and keep it at 35°C.
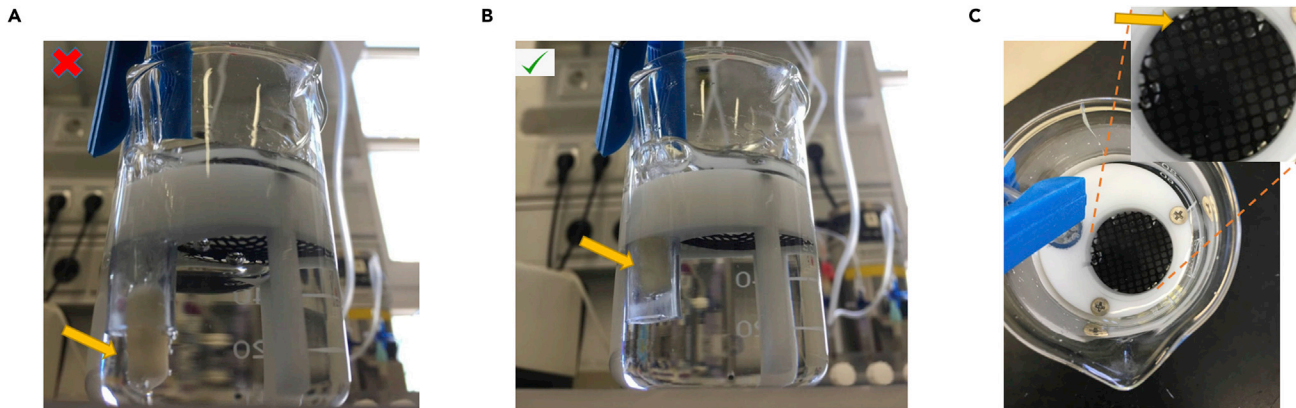
Figure 1. Positioning of micro filter candle for carbogen oxygenation
(A) Representative image of wrong positioning of the tube inside the beaker. Placing the tube completely inside the beaker will result in air bubble formation on the net, which affects the health of the slice. The micro filter has been indicated using an arrow.
(B) Correct positioning of the tube inside the beaker.
(C) Air bubble formation in the net should always be avoided.

9. Prepare two beakers (control and treatment) with 70 mL of mess ringer solution and oxygenate (95% O_2 and 5% CO_2).

   △ CRITICAL: Make sure that the glass filter is properly inserted inside the beaker containing mess ringer solution. If it is inserted too deep, bubbles can form on the net, which potentially hampers the health of the slices. Additionally, make sure that the glass filter is not on the surface, which would lead to unequal distribution of carbogen. Ideally, the glass filter should be inserted in a way in which it is placed at three-fourth the position of the stand (Figure 1). Figure 1A shows the wrong insertion whereas Figure 1B shows the right way of placing the microfilter. Figure 1C shows the bubbles on the net, which should be avoided.

10. Prepare a petri dish with mess ringer solution and oxygenate it constantly.
11. Make sure to surround the microtome box by ice all the time (this is to keep the preparation ringer solution in slushy state for smooth cutting of the slices). Inside the microtome box fill in the preparation ringer solution (around 6 tablespoon) and start fumigating it with carbogen.

   △ CRITICAL: The preparation ringer should be always in a slushy state. It should not be completely in a liquid state or in a hard ice state. From our experience, we recommend placing a preparation ringer bottle kept initially in $-80°C$, in $4°C$ refrigerator a day before starting the experiment. In an alternate scenario, if the preparation ringer is kept in $4°C$, 500 mL of it can be separated and kept in $-80°C$ for 40 min to bring it into a slushy state.

Preparation of buffer solution for immunofluorescence labeling

   ⊙ Timing: 2 h

12. Prepare 10× phosphate buffered saline (PBS). Dilute it 1:10 to obtain 1× PBS. This can be stored at RT (21°C to 24°C) for up to 6 months.
13. Prepare 4% (w/v) paraformaldehyde (PFA) in 1× PBS and store it at 4°C for a maximum of 7 days. We recommend making a fresh preparation of PFA before starting the staining procedure.
    a. Dissolve 20 g of PFA powder in 500 mL of 1× PBS solution on a hot plate heated up to 50°C and stirred with a magnetic stirrer continuously until the powder dissolves completely. After

dissolving, keep the solution in the RT (21°C to 24°C) to bring down the temperature and adjust the pH value to 7.4.

> △ CRITICAL: PFA is a hazardous chemical. The solution should be prepared inside a fume hood.

14. Prepare 30% (w/v) sucrose solution (for cryoprotection) in 1× PBS.
    a. Dissolve 150 g of sucrose powder in 500 mL 1× PBS and mixing with a magnetic stirrer.
15. Preparation of blocking solution (10% (v/v) normal goat serum).
    a. Dissolve 1 mL of 100% normal goat serum (NGS) in 9 mL of 1× PBS. Add 0.3% (v/v) triton-X 100 solution.

> △ CRITICAL: The blocking solution should always be prepared fresh before every staining and should not be stored for more than 24 h.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat Anti-Mouse IgG H&L (Alexa Fluor® 647); 1:500	Abcam	ab150115
Goat Anti-Rabbit IgG Fc (Alexa Fluor® 488); 1:500	Abcam	ab150089
Mouse GFAP; 1:800	Cell Signaling Technology	3450
Rabbit Anti-C1q; 1:250	Abcam	ab182451
Rabbit PSD95; 1:400	Cell Signaling Technology	3670S
Chemicals, peptides, and recombinant proteins		
10× PBS	PanReac AppliChem	A0965,9050
Aß$_{42}$	Bachem	H-1368-1000
CaCl$_2$-Dihydrate	Sigma-Aldrich	2971347
Carbogen	Linde	10021938
D-(+) Glucose-Monohydrate	Sigma-Aldrich	14431-43-7
DMSO	Sigma-Aldrich	67-68-5
Frozen section media	Leica	3801480
HFIP	Sigma-Aldrich	920-66-1
Isoflurane	CP-Pharma	400806.00.00
KCl	Sigma-Aldrich	7447-40-7
Leica Type F Immersion liquid	Leica	195371-10-9
MgCl$_2$-Hexahydrate	Sigma-Aldrich	7791-18-6
MilliQ Water	Merck	F4HA55065B
NaCl	Sigma-Aldrich	7647-14-5
NaH$_2$PO$_4$-Monohydrate	Sigma-Aldrich	10049-21-5
NaHCO$_3$	Sigma-Aldrich	144-55-8
Normal Goat Serum	Sigma-Aldrich	S26
Paraformaldehyde powder	Millipore	30525-89-4
ProLong™ Glass Antifade Mountant with NucBlue™ Stain	Thermo Fisher Scientific	P36985
Sucrose	Sigma-Aldrich	57-50-1
Tissue Adhesive	3M	1469SB
Triton X	Sigma-Aldrich	9036-19-5
Experimental models: Organisms/strains		
Mouse: C57BL/6, male and female, wild type, 9–10 weeks old	Charles River Laboratories	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
IMARIS 9.7 (or higher) for Neuroscientists	Oxford Instruments, Bitplane	https://imaris.oxinst.com/microscopy-imaging-software-free-trial
LAS X (Acquisition software)	Leica Microsystems	https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/
Other		
Brush	Artisti	155 Set
Confocal Microscope	Leica	Leica TCS SP8 X equipped with special pulsed (78 MHz) "super continuum white light laser" (WLL) (470 nm–670 nm) - as well as an additional 405 nm diode laser, with HyD detector, pinhole size at 1 Airy, 10×/0.40, 20×/0.75, and 63×/1.40 NA objective
Cryotome	Thermo Fisher Scientific	CryoStar NX70
Glass Beakers	Duran	1008839
Microcandle filter with glass tubes	ROBU	25103
Microscope Cover slips (24 × 50 mm). Thickness: 0.15 mm	VWR	630-2747
Microscope Slides	Epredia	ISO 8037/1
Microtome	Leica	Leica VT100S
Microtome blade	Wilkinson Sword	B0012Y1DE0
Parafilm	Bemis	13-374-10
Pasteur Pipette	VWR	612-2851
Petri dish	Merck	Z231525-1EA
Protein loBind Tubes 1.5 mL	Eppendorf	22431081
Spatula	NeoLab	XC-0168
Standard pattern forceps with curved and serrated tips	Dumont	11271-30
Surgical scissors	Fine Science Tools	14508-15
Tissue culture test plates	TPP	Y92406
Water Bath	JULABO	SW22
3D Shaker	Hettich Benelux B.V.	4064343311816

MATERIALS AND EQUIPMENT

Following are the settings of the microscope used for our acquisition of astrocytes (The settings, however, could vary greatly upon the region of interest, proteins for colocalization and the staining protocol):

Imaging Setup: Leica Confocal SP8.

Frame size: 1024 × 1024 pixels.

Pinhole: 1 AU.

Lasers: 499 nm (C1q, PSD95 Intensity: 5%–10%), 405 nm (NucBlueTM, Intensity: 10%) and 653 nm (GFAP, Intensity: 1%–2%), laser power depends on staining efficacy. The power output was fixed at 70% of maximum output (1.5 mW).

Gain (Master, analog): 800–1,000 V.

Digital Offset: 0 for 405 channel (PMT), (HyD doesn't require a digital offset).

Scan Area: Zoom according to the focused astrocyte. On a general note, since we are trying to image individual astrocytes of diverse size and shapes, we recommend to using the following range of pixel size for X/Y plane:

X/Y pixel dimension: 0.04–0.08 μm.

Z step size: 0.27–0.30 μm.

Averaging: 2 lines.

Bit Depth: 8-bit, bidirectional scanning.

Scanning speed: 200 Hz (lines per second).

Z-stack scanning: System optimized (z-step size normally 0.3 μm), scanning from the top to bottom of the focused astrocyte. On an average we collect 30–40 stacks over 6–8 μm (Z axis).

Objective: 63× oil (Leica Type F Immersion liquid n_e^{23} = 1,5180, v_e = 46). NA= 1.40.

Selection of channels: C1q (Alexa 488, Abcam, green, 499 nm laser).

PSD95 (Alexa 488, Cell Signaling Technology, 499 nm laser).

GFAP (Alexa 647, Cell Signaling Technology, red, 653 nm laser).

NucBlue™ Stain (405 nm laser).

Emission Wavelength: For C1q and PSD95 (510 nm–530 nm).

GFAP (663 nm–693 nm).

NucBlue™ Stain (415 nm–457 nm).

Processing: Lightning Function: Adaptive.

Refractive Index: 1.44.

> *Note:* The laser power settings are determined by the balance between over saturation and under saturation by using the quick look-up table (LUT) icon provided in the LAS X software. It is essential to keep in mind that while optimizing the setting, the investigator uses these settings in a non-essential region, to avoid photo bleaching in the experimental region of interest. Once the settings are finalized, for the subsequent imaging of brain slices, the same image settings (Laser power) are applied by using the "Apply image settings option".

> *Note:* Parameter settings depend on image quality/staining intensity, background etc. It is essential to make sure that the user tries to stay away from the over saturated pixels to avoid excessive background signals. A guide on how to use the deconvolution parameter has been provided below in step 33. If the confocal microscope does not have an inbuilt deconvolution software, one can use the deconvolution function from the Imaris package.

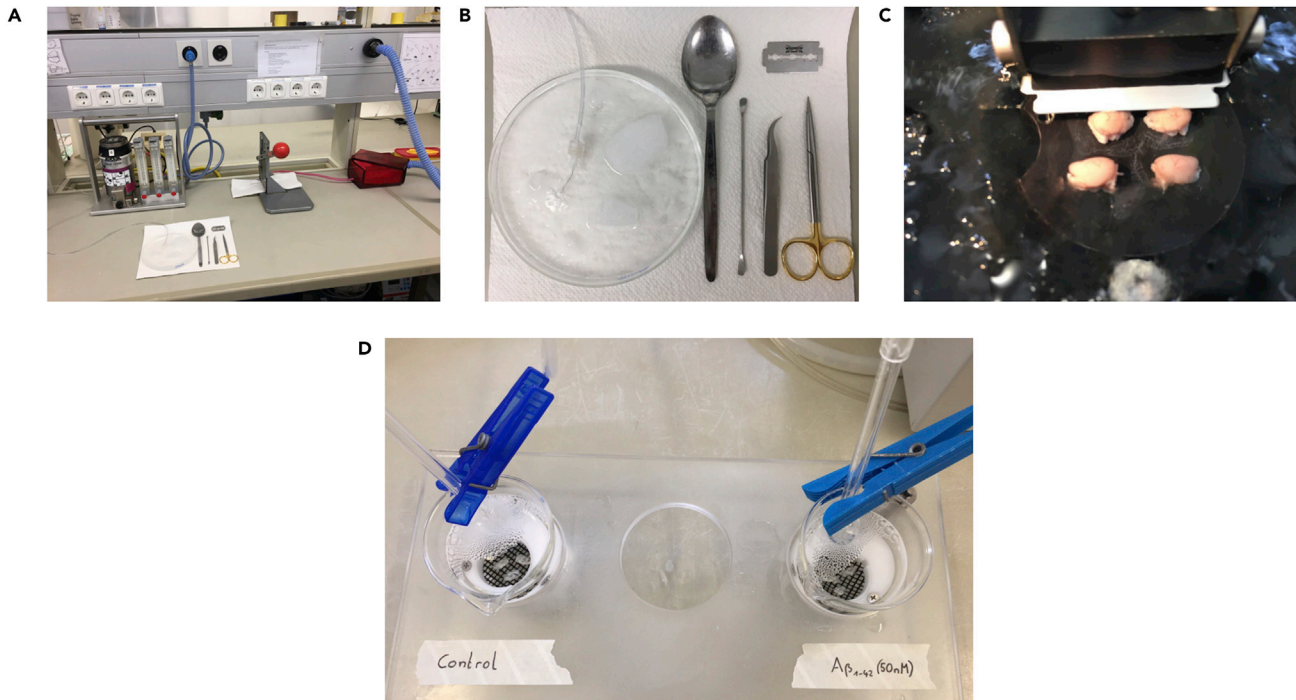Mounting Medium: ProLong TM Glass Antifade Mounting medium with NucBlueTM Stain.

Figure 2. Preparation of brain slices and incubation with Aβ₄₂ peptide

(A) Overview of the setup required for the sacrifice of the mice. Isoflurane (5%) was used as the anesthetizing agent.

(B) Close overview of the surgical set required for the dissection of mice brains. From the left, spoon, spatula, standard pattern forceps with curved tips and surgical scissor.

(C) Microtome cutting through the sagittal section of the mouse brain.

(D) Organotypic incubation of slice with Aβ₄₂ peptide.

STEP-BY-STEP METHOD DETAILS

Preparation: Decapitation and tissue extraction [part 1]

⏱ **Timing: 20 min**

The mouse brain is dissected after decapitation with a guillotine and placed inside the preparation ringer solution, which is constantly oxygenated. Note that to keep the slices healthy for a longer duration, the respective preparation ringer or mess ringer solution should be constantly oxygenated with carbogen. The preparation of microtome and oxygenation is to be done prior to sacrificing the animals.

1. Prepare the surgery site with surgical scissors, standard pattern forceps with curved and serrated tips, fresh blades, and a spatula (Figures 2A and 2B).
2. Keep a beaker with the preparation ringer solution continuously oxygenated on the side.
   a. Make sure to keep the beaker in a box full of ice, to keep the preparation ringer in a slushy state.
   b. Keep another petri dish with oxygenated preparation ringer solution, which serves as a platform to dissect the brain from the decapitated head.
3. Anesthetize the mouse by placing it in an isoflurane chamber with 5% isoflurane concentration and at a flow rate of 2 L/min. Wait until the animal is unresponsive. The standard and a reliable way to test whether the mouse is aptly anesthetized is by doing a twofold check.
   a. Check for the instance when the mouse loses the righting reflex.
   b. Pinch the tail with the forceps and see if the mouse does not respond. Once, checked for these measures, bring the mice to the guillotine and decapitate the head.

4. Quickly transfer the decapitated head to the petri dish containing preparation ringer solution.
   a. With the help of sharp surgical scissors, cut open the scalp through the midline along the sagittal suture.
   b. Make a lateral cut on both the sides of skull and remove the top of the skull to expose the brain.

   ⚠ CRITICAL: Be careful while cutting through the joints of the skull particularly the bregma. If the bone does not fall off completely, it can be slowly removed using fine-tip forceps.

5. From the caudal side, gently lift out the brain using the spatula. Quickly transfer the brain to iced-cooled preparation ringer solution in the beaker. Discard mouse body and head in the freezer, disinfect and clean guillotine, anesthetizing chamber and workspace.
6. Bring the brain to the microtome setup.

Treatment: Sectioning (350 μm slice) and incubation with Aβ$_{42}$ [part 2]

🕐 Timing: 5 h

Section the brain in the sagittal plane into 350 μm thick slices for incubation and treatment with the Aβ peptide. Sagittal sectioning best preserves the stratum radiatum layer of CA1 region of hippocampus. This section includes the incubation of slice for 30 min in water bath warmed to 35°C and then in the aCSF at RT (21°C to 24°C) for 1 h. This allows sufficient time for the recovery of slices.

7. Transfer the brain to a small flat platform and cut off the cerebellum with a razor.
   a. Separate the two hemispheres with a sharp razor blade.
   b. Put two streaks of tissue adhesive on the microtome cutting plate.
8. Dry both hemispheres of the brain by first transferring them on a filter paper for 2–3 s.
   a. Transfer the brain hemispheres to the tissue adhesive on the microtome plate and place the plate inside the holder.
   b. Carefully lock the plate and insert the razor blade inside the microtome.
9. Fix the cutting window in the microtome by setting the starting and the final position of brain.
   a. Set the thickness to 350 μm. Start trimming the hemispheres until anterior hippocampus starts to appear (Figure 2C).
   b. Start collecting the slices and divide them into the respective beaker: control and treatment (Aβ$_{42}$).
   c. Cut slices and carefully transfer them to oxygenated mess ringer filled beakers in the water bath with a pipette (Figure 2D).

   ⚠ CRITICAL: Pay attention to the bubbles in the nets holding the slices. They directly affect the longevity of the slices. Also, make sure that the brain slices are not overlapping on each other. This would affect the health of the slices.

   ⚠ CRITICAL: To reduce the overcrowding of slices inside the beaker, one can trim off the rest of the brain area from the sagittal section of the brain keeping the hippocampus and cortex intact. This allows more slices to fit in the respective beakers.

10. After completing the sectioning, place the beakers containing the slices in the water bath at 35°C for about 30 min, then remove it from the water bath, and incubate at RT (21°C to 24°C) for 1 h. This allows sufficient time for the revival of slice.
11. Follow the incubation scheme as shown in the table below this step.
    a. Pipette out 35 μL of Aβ$_{42}$ in 70 mL of the aCSF solution into the treatment beaker to obtain 50 nM concentration of the Aβ$_{42}$ and incubate the slices for a duration of 90 min at RT (21°C to 24°C). The Aβ$_{42}$ would evenly mix by the continuous oxygenation.

b. Leave the slices in the control chamber in the aCSF with continuous oxygenation for the same 90 min duration.

Control	30 min at 35°C	1 h at RT (21°C to 24°C) in aCSF	1.5 h (RT) (21°C to 24°C)
Aβ$_{1-42}$ Treatment	30 min at 35°C	1 h at RT (21°C to 24°C) in aCSF	Aβ$_{1-42}$ (50 nM) 1.5 h (RT) (21°C to 24°C)

⚠ CRITICAL: The Aβ$_{42}$ stock solution will begin to degrade at RT (21°C to 24°C). Twenty min prior to the starting of incubation with the amyloid beta oligomer, the Aβ$_{42}$ can be brought to the RT (21°C to 24°C) to allow the contents to thaw, then place it back in the −20°C freezer.

Processing of brain slices [part 3]

⏲ Timing: 3 days

This step is crucial for fixation and further cryopreservation. Hard fixatives like PFA allow covalent cross-linking between molecules and stabilizes the proteins. Place the slices in 30% sucrose solution for cryoprotection. It helps to prevent the ice crystal formation in the tissue and thereby avoid freeze-damage.

12. Divide the slices into a 6-well plate and pour in adequate amount of 4% PFA to cover the slices. Incubate the slices overnight (16–20 h) on a 3D shaker at 4°C in 4% PFA solution.

⚠ CRITICAL: PFA is hazardous. Take adequate care while handling it. It should be freshly prepared and should not be reused multiple times. Dispose of properly in hazardous waste.

13. On the following day, wash the slices three times with 1× PBS for 10 min each.
14. Add 2 mL of 30% sucrose solution into each well and incubate for 2 days on 3D shaker at 4°C.

Cryosectioning: 30 μm brain section [part 4]

⏲ Timing: 2–3 h per brain

The brain slices are further sectioned into 30 μm thick sections for better staining and effective immunofluorescence protocol. This step is crucial for obtaining good free-floating sections. Cracks or tear in the sections would directly affect the staining and mounting onto the slides.

15. Pour in a small amount of the optimal cutting temperature (OCT) media on the sample disk and allow it to solidify. Carefully place the disk in the holder and trim the OCT compound until it becomes a flat surface.

⚠ CRITICAL: Make sure that the surface of the OCT is completely flat. The brain slices (350 μm) need to be kept on a flat surface to ensure uniform cutting of the brain sections.

16. Take the specimen disk out, mark the position where you intend to place the brain slice. For our purpose, we marked the OCT with positions resembling the hands of a clock.
    a. Pick up the brain slice by a spatula and place it horizontally on the specimen disk. Now cover the surface of slice with ample amount of OCT media and place the specimen disk back in the holder.
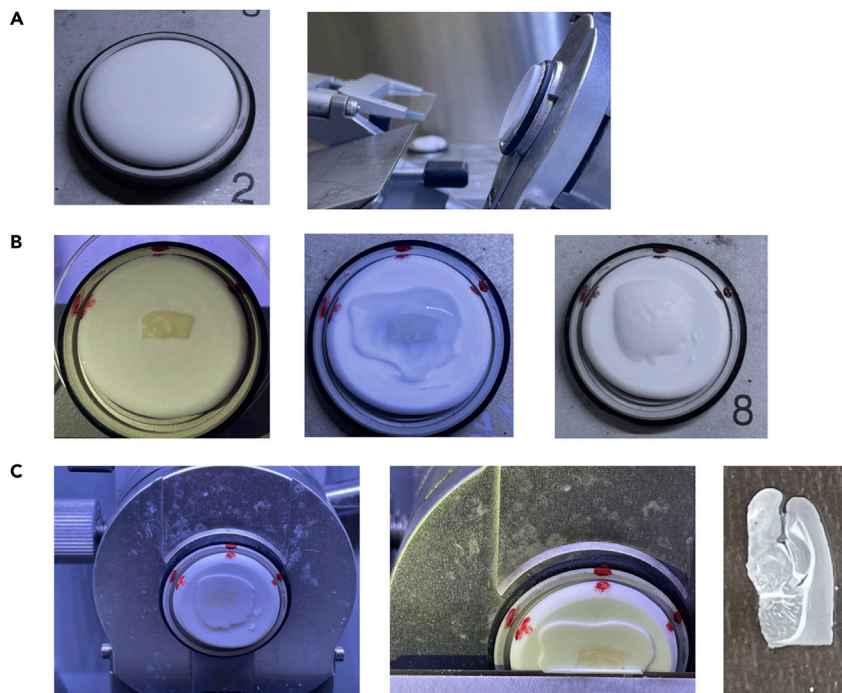
Figure 3. Cryosectioning of brain sections (30 μm)

(A) The first step is to cover the specimen disk with the OCT mounting media. Flatten the surface by initially trimming off the OCT media and creating a smooth flat surface.

(B) Place the brain slice (350 μm) horizontally on the surface of flattened OCT mounting medium. If not flat, gently press the brain slice to the surface and cover the surface with OCT.

(C) Start trimming of extra parts of OCT before reaching the brain slice. Thin straight 30 μm sections as seen in the last image of the panel is produced at the end of this procedure.

b. Start trimming until the slices are reached. Once the slices are reached, set to desired thickness (30 μm) and start collecting the sections. (Figure 3) See Methods video S1 for detailed demonstration.

⚠ CRITICAL: It is essential to make sure that the slices are laid flat on the OCT media. If necessary, it can softly be pressed using a brush/spatula to make the surface flat.

17. Carefully cut 3–4 sections, take them up all at once with a metal pick and transfer them into 6-well plate filled with 1× PBS (the sections will unroll in the 1× PBS solution).

Note: In an alternative scenario, if one wishes to do the staining not in a free-floating section, rather on a slide, the brain section can be made flat using an anti-roll plate and can directly be mounted on the slide. These slides can be stored in -80°C for a longer duration.

18. It is possible to obtain 6-7 good 30 μm sections from a single 350 μm sagittal slice of hippocampus. Collect enough sections in 1× PBS from both control and treatment groups.

Immunofluorescence staining [part 5]

⏱ Timing: 3 days

To assess the synaptic engulfment of the "eat me signal" C1q protein, we perform the immunofluorescence on a sagittal section. The synaptic engulfment is further verified using a post-synaptic

marker PSD95. The astrocytes are marked by using GFAP. Pre-select intact hippocampus sections using a brush and place them in the 6-well plate. Avoid putting too many sections into a single well, which would lead to overcrowding. A maximum of 3–4 sections per well is recommended.

19. Wash the sections three times with 1× PBS using a plastic Pasteur pipette. Handle the sections carefully.
20. Blocking: Add 1 mL/well of blocking solution (10% NGS in 1× PBS + 0.3% triton-X) and block for 2 h at RT (21°C to 24°C) on a 3D shaker.
21. Remove the blocking solution. Incubate with primary antibody (800 μL/well, diluted in 10% NGS) for 2 days at 4°C. Use Rabbit Anti C1q (1:250) for staining C1q protein, mouse anti GFAP (1:800) for staining astrocytes and rabbit anti-PSD95 (1:400).
22. Remove the primary antibody and put it in a separate tube marked as recycled. These reused antibodies can be stored in −20°C and can be used for another round of staining.
23. Wash the sections three times with 1× PBS solution for 10 min each. Meanwhile prepare the secondary antibodies.
24. Incubate the sections with respective secondary antibodies anti-mouse 647 (1:500) and anti-rabbit 488 (1:500) for 2 h at RT (21°C to 24°C). Cover the plate with a shielded cover (like aluminum foil) to keep it dark.
25. Wash the sections three times with 1× PBS solution for 10 min each.
26. Take a petri dish filled with 1× PBS and transfer 3–4 sections from the 6-well plate into petri dish and carefully mount them flat on the slide. Use a brush to transfer and flatten them.

    Note: For an efficient and easy transfer of the section onto the slide, keep the slide tilted with half of its surface inside PBS. Carefully move up the section where the slide touches PBS and slowly pull out the slide. The section sticks onto the surface. If there are still small folds on the section, use a soft fine-ended brush and carefully adjust the section to make it flat.

27. Keep the slides tilted on filter paper in the dark to dry. Mount the sections with a NucBlueTM mounting media.

    △ CRITICAL: Each slide should not contain more than 4 brain sections. Extreme care should be taken while putting coverslip on the section. Put adequate amount (10 μL per hippocampus section) of the mounting media to avoid putting pressure on the section, which could affect the structure of the proteins under study, and thereby affecting rendering process in the further steps. Also, make sure not to introduce any air bubble into the specimen. An easier way to accomplish this would be to put a thin pipette head between the coverslip and the slide and slowly removing the pipette allowing the mounting media to slowly but evenly distribute above the sample. In any case, if there is still air bubble, avoid scratching the coverslip or applying direct pressure from the top, instead start removing the bubble by slowly applying pressure from the sides, particularly from the areas, which are distant from the brain specimen.

    △ CRITICAL: For preserving the fluorescent signals for a longer period, keep the slides at −20°C. When kept at RT (21°C to 24°C), the fluorescent signal decays. Before imaging the slides on a confocal microscope, they can be briefly kept in at RT (21°C to 24°C) (20–30 min).

Confocal imaging: "Hunting for astrocytes" [part 6]

⊙ Timing: 2 h per brain

We image high-resolution individual astrocyte from the stratum radiatum layer of CA1 region of hippocampus. The slides are double-immunofluorescent stained for astrocytes and either the

complementary tag C1q protein or a postsynaptic marker PSD95 for reference. The images taken during the process are further deconvolved with the lightning function in the Leica software, to improve image resolution and contrast. For effective volumetric analysis of the synaptic engulfment by the astrocyte, it is essential to reduce the background noise. To effectively categorize the astrocyte, the nucleus/DNA is labeled with NucBlueTM present in the mounting media (nuclear stain). Since we are particularly focused on the CA1 area of hippocampus, it is also crucial to mark distinct layers and regions of the hippocampus, to effectively identify our region of interest and pick the astrocyte from the layer. Imaging is performed with three active fluorescent channels. Leica Microscope SP8 provides the user an advantage to have 2-hybrid detectors (HyD) which produce less noise. Apart from these HyD sensors, they also have an additional photomultiplier tube (PMT) sensor. Since the Leica microscope, has a maximum of two HyD sensors and the rest PMT sensors, it is essential to pick the correct proteins for the respective sensor. To make things easier, it is always advisable to keep large, ubiquitous or uniformly expressed proteins in the PMT sensor and the proteins of interest for colocalization and for the analysis of the synaptic engulfment to be kept in the HyD sensor. In our case, we detect the nuclear (NucBlueTM) signal with the PMT sensor and GFAP and C1q/PSD95 signals using with the HyD sensors.

28. Switch on the confocal 30 min prior to imaging. Care should be taken to calibrate the stage before imaging.
    a. While starting the system the LASX provides the option to initialize the calibration of the stage. This should always be switched on. Non-calibration will interfere while taking the z-stacks.
    b. The laser power should also be stabilized before imaging. On a first-hand rule we switch on the laser after the confocal has been switched on for at least 15–20 min.
29. The primary step here is to locate the CA1 area of the hippocampus. Using the widefield fluorescence imaging mode, start from a lower magnification (10×/0.40 NA) and (20×/0.75 NA). Having a nuclear stain in the regiment of fluorophores greatly helps in identifying the region of interest (CA1) (Figure 4).

    Note: In our experience, it is easier to locate the dentate gyrus (V shaped structure) in the hippocampus first and then track down the CA1 area of the hippocampus. While finding the stratum radiatum of the CA1 can be tricky, it can be eased by locating the pyramidal layer, which is densely packed with nuclear bodies as seen in the NucBlueTM channel. The stratum radiatum layer (marked by a red box) is directly below the densely stained pyramidal layer (Figure 4D).

30. Once the region has been identified, switch to 63× (oil)/1.40 NA for single cell acquisition of astrocytes. Put one drop of immersion oil.
31. Adjust all three channels: Blue, Green and Red with the parameters as suggested above. Slowly move across the XY plane and look for well-processed and structured astrocytes. The z-stack is set according to the GFAP channel in a way that it covers the entire volume of the astrocyte. The step size can be changed as per needs and demands of the experiment (Figure 5). The green channels (C1q/PSD95) are imaged in this z stack range.

    Note: The step size to be chosen greatly depends on what analysis; the researcher wants to perform with the astrocytes. For instance, if someone were looking to characterize the structural analysis or the branching complexity of the astrocyte we would recommend having a smaller step size (0.2 μm–0.25 μm) to have a detailed structural outlook of the astrocytes.

32. Click on "Start Experiment" to start taking acquisition of all the three channels. For an individual astrocyte, this could take between 5-8 min depending on the step size and the volume of astrocyte under consideration.
33. Deconvolution: To improve resolution and signal to noise (S/N) ratio, we recommend performing deconvolution of the images. The lightning function from the Leica Confocal SP8 allows one to deconvolve the images before they are exported to Imaris for further analysis.
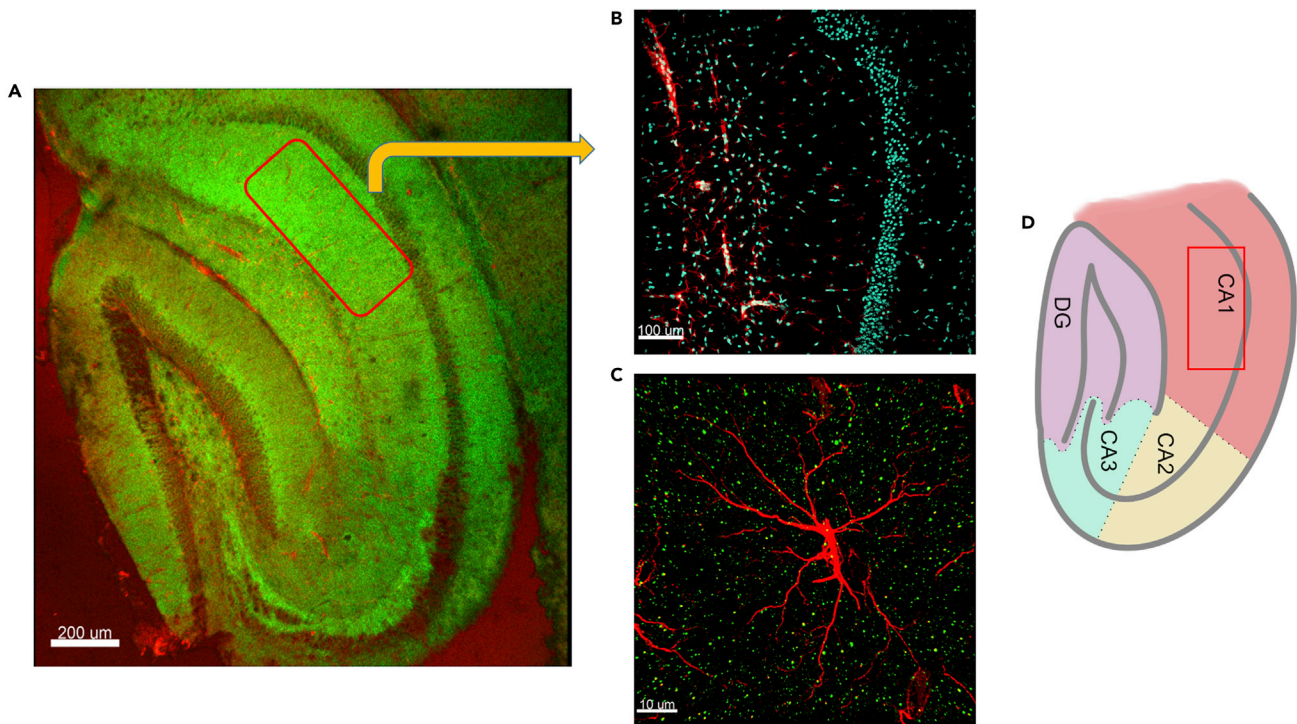
Figure 4. Overview of the C1q-GFAP interaction in the hippocampus

(A) 10×/0.40 magnification of a sagittal hippocampal section detailing through the different region of hippocampus stained with C1q (green) and GFAP (red). *Scale Bar 200* μm.

(B) 20×/0.75 magnification focusing on the CA1 region of the hippocampus stained with NucBlueTM (cyan) and astrocyte (red). Just below the densely stained area (pyramidal layer) is the stratum radiatum layer where we pick the individual astrocyte. *Scale Bar 100* μm.

(C) 63×/1.40 magnification of a high-resolution individual astrocyte (red) stained alongside with C1q (green). *Scale Bar 10* μm.

(D) Schematic representation of different regions of hippocampus. The astrocytes are selected from the stratum radiatum layer, which is illustrated in magenta and is marked using a red rectangular box.

Note: The lightning function uses Richardson-Lucy deconvolution algorithm and a physically modeled Point Spread Function, which is adapted to the respective imaging method (confocal, STED, multiphoton, etc.). The parameters to be considered are regularization and smoothing. The Regularization parameter determines how strongly to average over a certain radius around a voxel. For the highest possible gain in resolution (with good signal-to-noise (SNR) in the raw data), this value should be set as low as possible (e.g., to 0.01 or even 0), with a low SNR this value should be set as high as possible (e.g., to 0.7) to avoid artifacts. The settings used for our image acquisition are found in Figure 6.

⚠ CRITICAL: Switch off the smoothing function for the GFAP channel, which might cause changes in the astrocyte structure and therefore affect the morphometric analysis.

⚠ CRITICAL: The regularization parameter is paramount to having a better signal to noise ratio. It represents to what extent a signal is interpreted as background or noise. Having an accurate adjustment of this parameter is essential to avoid false positives. The higher the value, the more severe constraints are applied to avoid the risk of deconvolution artifacts. However, this also increases the chance that information carrying signal will be discarded and thus the highest resolution enhancement might not be achieved. We use the default settings of the LASX regularization parameter for the deconvolution of the image. The Regularization parameter determines how strongly to average over a certain radius around a voxel. This value is proportional to the full width at half maximum of the point
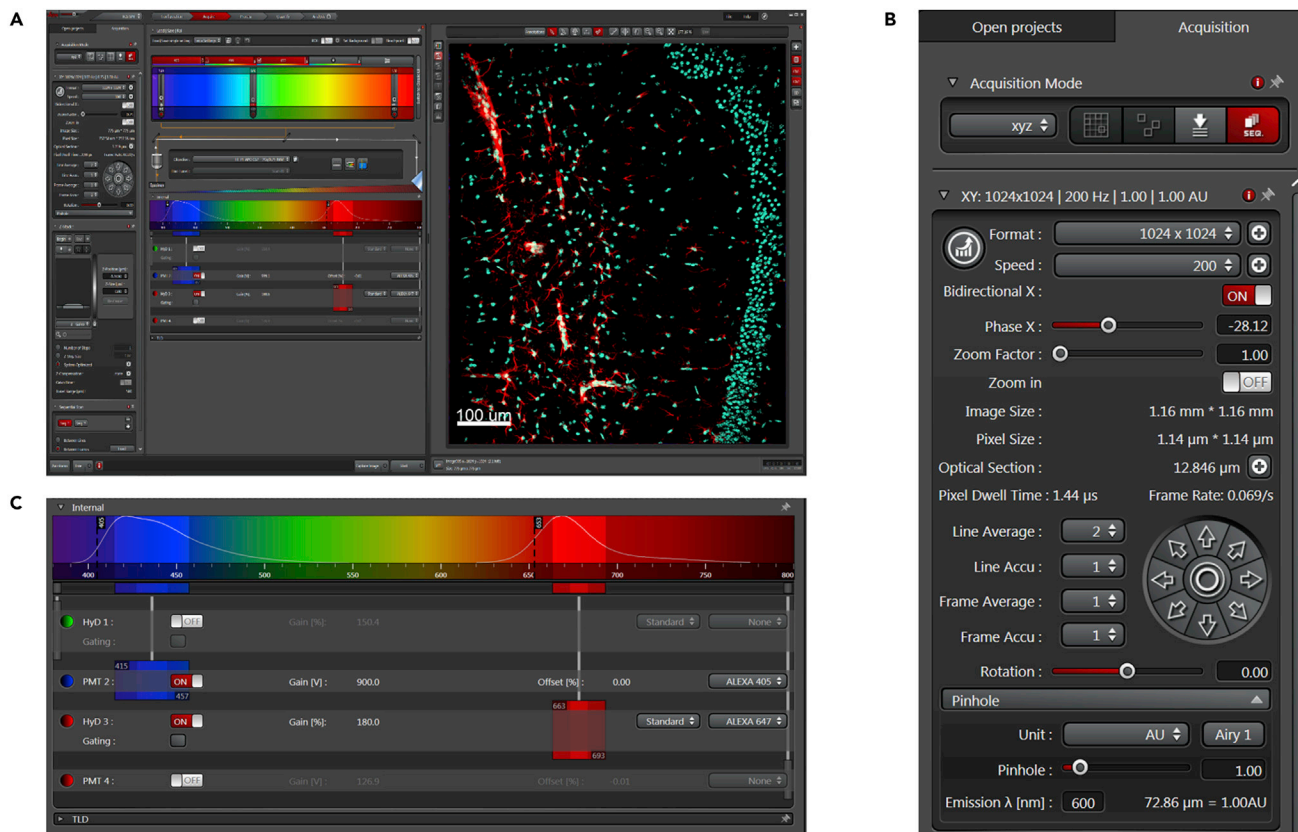
Figure 5. Representation of acquisition settings used with the Leica Confocal SP8 system (version 3.5.7.23225)
(A) Parameters set during the acquisition of 20× (NA 0.75) magnification images focusing on the CA1 area stained with GFAP (Red) and NucBlueTM (Cyan). *Scale Bar 100* μm.
(B) Detailed view of the settings for acquiring the image.
(C) Lasers used for acquiring the image.

> spread function (PSF) of the optical system. The regularization parameter also depends on the SNR ratio (Dey et al., 2004). With a good SNR the regularization value should be set at the lower side and vice versa (described in step 33).

Note: If the confocal microscope does not have an inbuilt deconvolution software, one can use the deconvolution function from the Imaris Package.

34. Files can be saved in the appropriate "xxx.lif" format and can be exported to Imaris directly for quantification of synaptic pruning.

Digital processing: Measurement of synaptic engulfment [part 7]

🕐 Timing: 15 min per astrocyte

This part is essential for the measurement of the synaptic engulfment of the C1q protein by the astrocytes. The procedure involves 3D volumetric reconstruction of individual astrocytes and co-localization analysis to calculate the C1q protein inside the entire volume of the astrocyte. PSD95, postsynaptic marker has also been used as a reference to calculate the synaptic engulfment. Correct rendering of astrocyte volume forms a major step in the subsequent colocalization analysis.
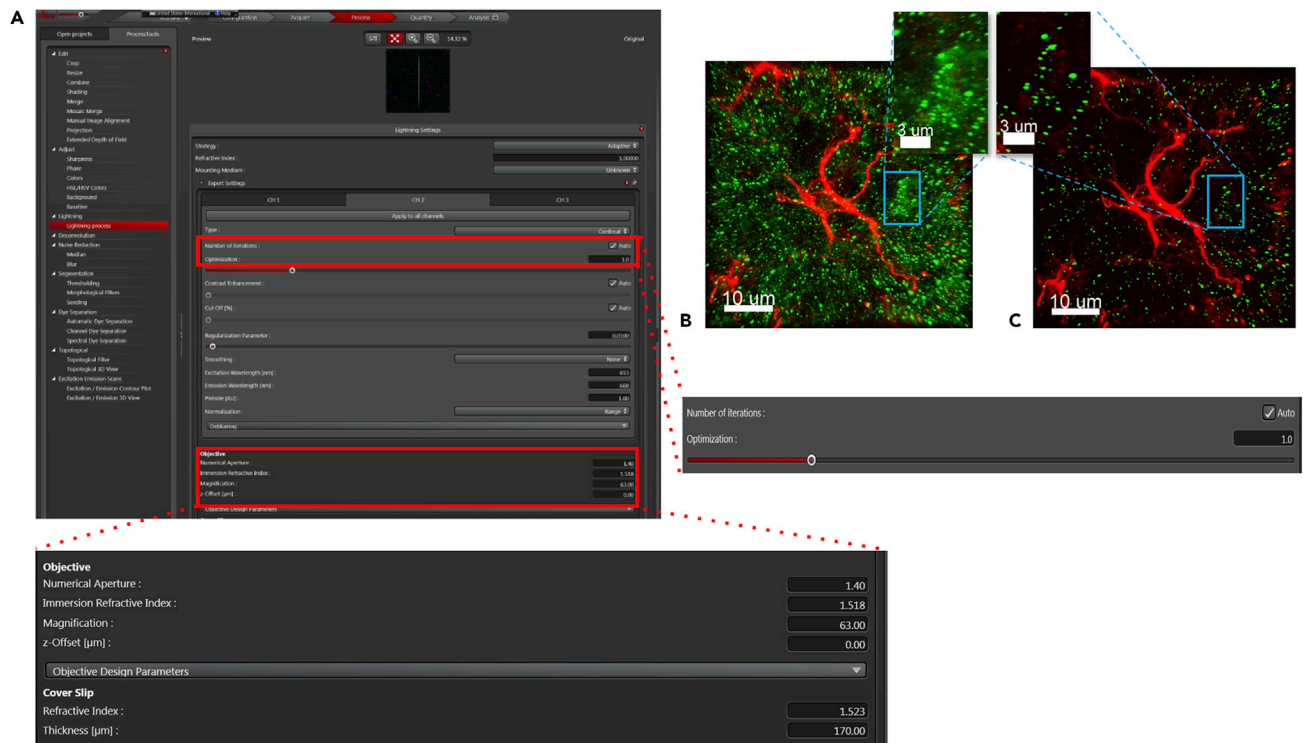
Figure 6. Deconvolution using the lightning function of the images acquired

(A) Deconvolution parameters set during the acquisition of the GFAP channel. Of importance are the parameters marked using the red boxes. Switch off the smoothing function for the GFAP channel.

(B) 63× magnification astrocytes stained with C1q (Green) before deconvolution.

(C) After deconvolution images of the astrocyte. Deconvolving the image highly reduces the background and improves the signal to noise ratio. *Scale Bar 10 μm. Scale Bar for zoomed in section (inset) 3 μm.*

> ⚠ CRITICAL: For proteins expressed uniformly throughout the tissue, it might be necessary to perform a background subtraction, with a filter width suitable to the spatial distribution of the protein. This improves the signal to noise ratio. The background subtraction can be done in Imaris by clicking on the image processing and selecting background subtraction for the respective channel. In our case, the deconvolution using the lightning function from the Leica Confocal SP8 was sufficient to obtain high-resolution imaging for all the channels.

35. Open the file in the Imaris software. Select "Add new surface" (blue oval like structure ◥) from the tab menu.

36. The creation of the surface around the astrocyte takes place in 4 steps. To segment individual astrocyte, select "Segment only a region of Interest" under the "Algorithm Settings" box.

    Note: Select the region of interest by dragging in through the X-Y plane. One can use pull arrows to restrict the region of interest (step 1 of 4).

37. In the next step, unselect the smooth option for the astrocyte. The smooth function as described before can affect the morphology of the astrocytes and thereby interfere with the final analysis (2/4). An overall visual guide to surface construction around the astrocyte is provided in Figure 7A.

38. Next, we render the surface of the astrocyte by adjusting the signal threshold corresponding to the GFAP channel. One can manually slide the bar, under the "Threshold" tab to select the surface. Although there is an automatic threshold which is suggested by the Imaris, we suggest
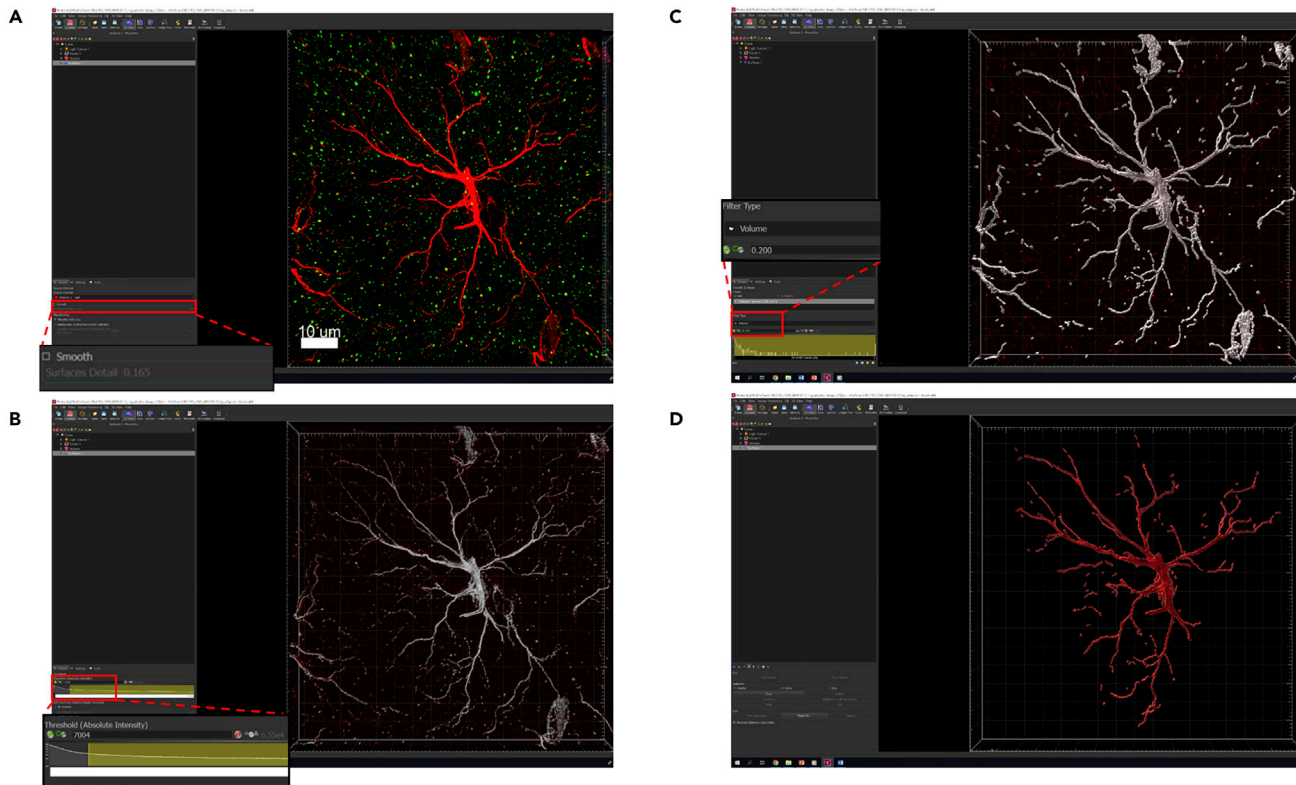
Figure 7. A visual guide through the four different steps of surface building on the astrocyte

(A) In step 2 select the red channel (GFAP), disable the smoothing option to avoid changes to the morphology of astrocytic surface.

(B) In step 3 the intensity threshold is selected for the red channel (For a detailed understanding of how to balance the threshold refer to Figure 8). While Imaris provides an automatic threshold for the surface reconstruction, we recommend adjusting the threshold manually for a more consistent and better rendering of the astrocyte.

(C) While the surface construction of the astrocyte, there are small unwanted disjoint signals produced in the background. In step 4, we filter these small disjoint signals using a volume threshold filter.

(D) The surface of the astrocyte is rendered. *Scale Bar 10* μm.

using the manual way to set the threshold in a way that the surface covers the complete astrocyte (3/4) (Figure 7B).

⚠ CRITICAL: This step is crucial and plays an essential role in directly affecting the colocalization analysis. Correct rendering of astrocyte is essential to quantify the synaptic engulfment. Both over rendering and under rendering can provide false sets of data. We therefore recommend an iterative approach (or trial and error approach) and, once a surface is created, compare with the original image, see if the surface is rendered in the proper way (Figure 8). If not, come back to step 39 and repeat it again, until the surface encloses all visible processes!

39. In the next step, we can adjust the additional rendering required for achieving the volumetric rendering of astrocyte. For this step, set the filter to volume and manually adjust the threshold to remove small particle. We used a volume filter of 0.2 μm^3, to exclude unwanted particles.

Note: However, if the experimenter feels that additional small disjointed particles need to be removed, this parameter can be changed accordingly. However, to avoid any kind of bias leave the same setting for all the astrocytes (4/4) (Figure 7C).
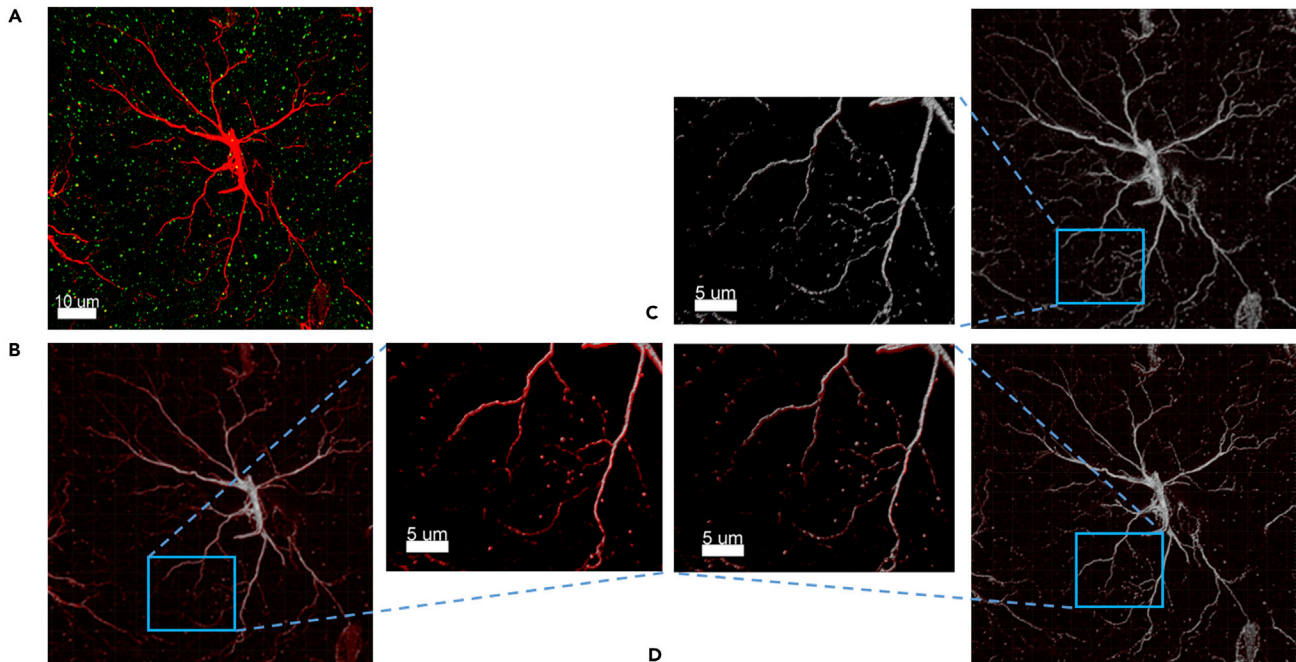
Figure 8. Creating the balance for a correct threshold for 3D rendering of astrocyte

(A) Original image showing astrocyte (red) and C1q (green).

(B) Example of under representation of the surface of the astrocyte. In this case, the threshold is not adequately selected, and several astrocytic filaments are not covered by the surface function.

(C) Over rendering of the surface. Having the threshold set too high, would introduce unnecessary and unreliable processes on the astrocyte.

(D) A balanced threshold should always be selected for the correct rendering of the astrocyte. The rendered astrocyte is now processed to further remove any other background signals. *Scale Bar 10* μm. *Scale Bar for zoomed in section (inset) 5* μm.

40. Click the green arrow button on step 4 to complete the rendering of the astrocyte. If desired rendering is not achieved, the user is advised to start again from step 39. This loop is to be followed until the desired rendering of astrocyte is achieved (Figure 7D). For a demonstration of steps, 37–40, see Methods video S2.

    Note: If a user intends to perform morphometric analysis of the astrocytes along with the co-localization study, Imaris provides an option to export all the statistics regarding the structural complexity and the branching details of the astrocyte. Click on the "Statistics" tab. Select all the parameters that you intend to study in the dropdown list "Export Statistics on Tab Display to file". If the user intends to study all the parameters, select "Export all statistics to file". The file can either be exported in "xxx.csv" or in "xxx.xls" format. These sets of data can be further analyzed.

41. The next step is to process the rendered astrocyte (Figure 9A). Usually even after careful surface reconstruction of the astrocyte, there are several unwanted signals or sometimes processes from other astrocytes, which need to be filtered out before proceeding to the next step.
    a. This is achieved by selecting all the unwanted background signal (Figure 9B) using the "Circle Selection Mode" (Figure 9D).
    b. Choose the delete option under "Edit" tab (Figure 9E). These unwanted signals are now filtered from the astrocyte (Figure 9C). For a demonstration of this step, see Methods video S3.

42. The next step is to mask the surface of the astrocyte. To achieve this, click on the "Edit" tab under a newly built surface. Click "Mask All" option (Figures 10A and 10B).
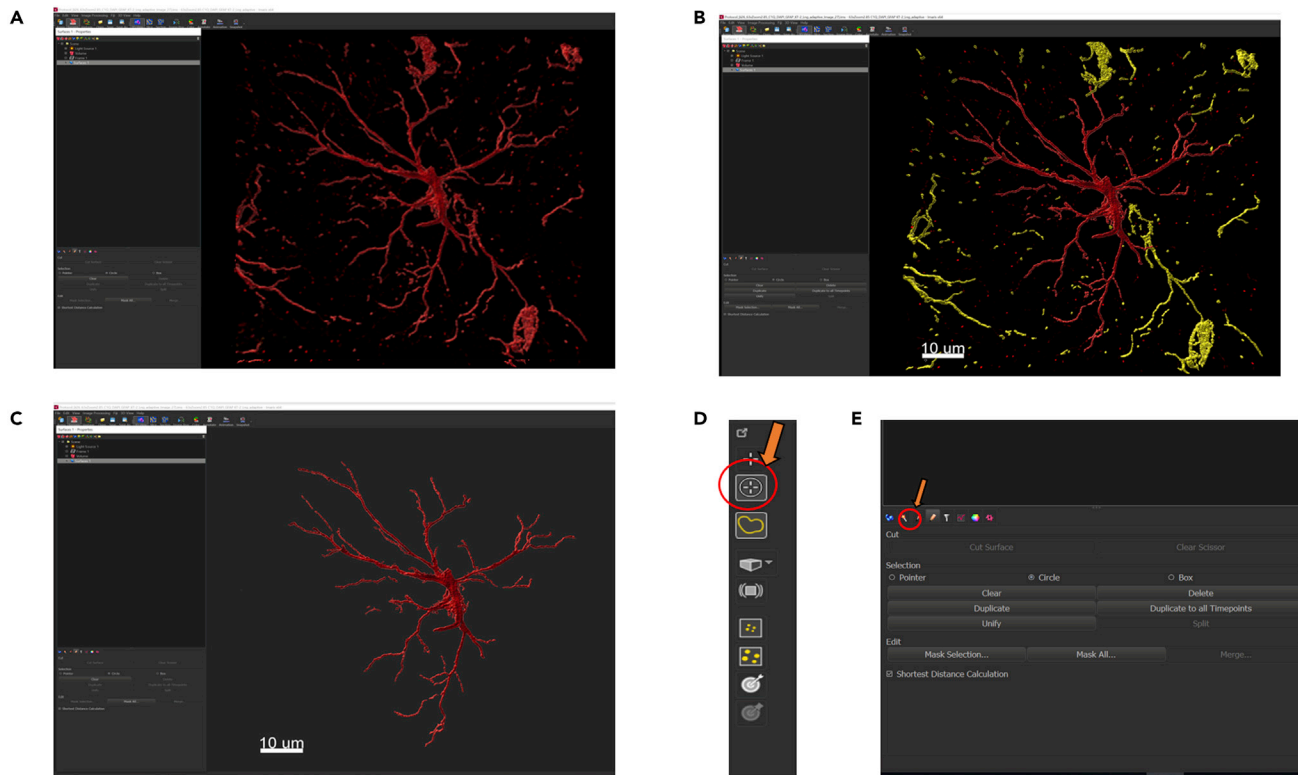
Figure 9. Processing of rendered astrocyte

(A) The rendered astrocyte produced after step 4/4 of surface creation. There are several other background signals as well as processes from other astrocytes. These need to be filtered out before performing the colocalization analysis.

(B) The unwanted signals and processes are selected and are represented in yellow.

(C) Processed astrocytes.

(D) The unwanted signals are selected by using the "Circle Selection Mode" shown by orange arrow in the upper right corner.

(E) After selecting all the unwanted background signal we select the edit option (pencil like icon on the lower left side indicated by the orange arrow) and click on the delete option. *Scale Bar 10* μm.

43. The mask channel box opens. Under the channels, select GFAP (Red) channel. Set voxels outside the surface to 0. This is to eliminate any signal, which is outside the surface that has been rendered. This step further reduces any false positives resulting from the background signal (Figures 10C and 10D). For a demonstration of steps, 42–43, see Methods video S4.

    Note: To quantify the synaptic engulfment, we use the colocalization analysis. The coloc analysis of Imaris analyzes the contact between the C1q protein (Green) and the astrocyte surface. Using the masked red channel, we can calculate the colocalization between the C1q and the astrocyte. The percentage of region of interest (Astrocyte) colocalized with the neuronal tag can be exported in a separate file in .csv format. In this part, we are focusing on the colocalization between C1q and GFAP (Astrocytes). However, this analysis can be extended to different post-synaptic and pre-synaptic markers.

44. Select the Coloc tab in the Imaris (Figure 11A).

45. Imaris allows the selection of 2 channels to be analyzed for the colocalization. In the first channel (A) select green (C1q) (Figure 11A). The next step is to set the threshold intensity for the green channel.

    Note: While Imaris provides an automatic way to determine threshold, we have found this approach gives inconsistent and unreliable results (Figure 11D). The better way to analyze the threshold for green channel is by setting a manual threshold.
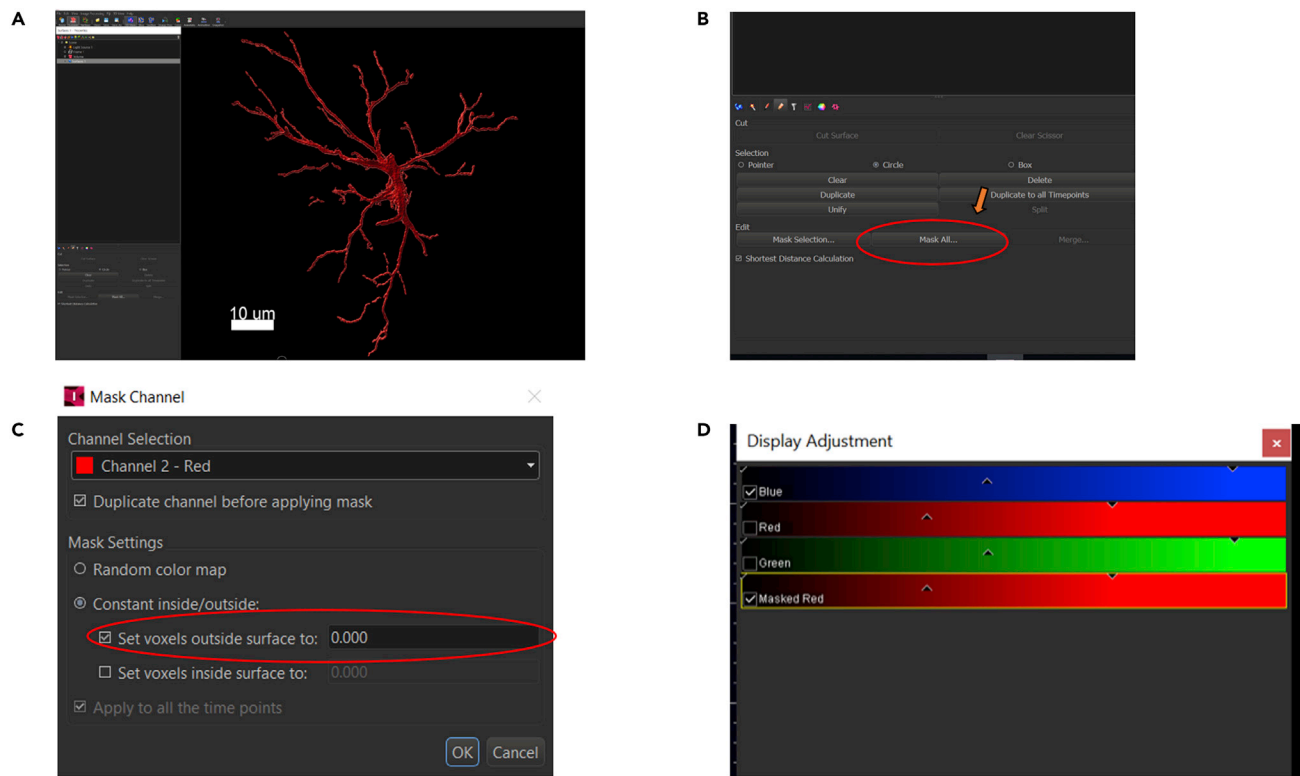
Figure 10. A visual guide to mask the surface of the astrocyte
(A) Rendered and processed astrocyte.
(B) On the edit option (pencil like icon), select ''Mask All'' option (Shown by the arrow).
(C) A drop down selection box appears. Select the channel in which the astrocyte is present (red) and set the voxels outside surface to 0.
(D) The masked surface of the astrocyte appears and in the display adjustment box, in addition to blue, red and green channel, the masked red channel appears. *Scale Bar 10* μm.

46. Select the manual threshold for the green channel by randomly selecting 10 unambiguous green dots and averaging the intensity to set the threshold for the green channel.
    a. Left click and drag on a green punctum in a way that the boundary set (yellow as shown in Figure 11B) by Imaris completely covers the signal (Figure 11B).
    b. Find the mean value of the 10 randomly selected C1q protein signals and set the threshold intensity of channel A. Averaging the intensity from multiple puncta ensure a balanced threshold for the image.

    ⚠ CRITICAL: While Imaris recommends setting the threshold for both the channels, using the 2D histogram, in our experience it is better to proceed with manual threshold instead of using the 2D histogram, as it produces inconsistent results.

    ⚠ CRITICAL: While encircling the boundary of a positive C1q signal, care should be taken to completely encircle the boundary. Wrong acquirement could produce false results in the colocalization analysis.

47. In the channel B select the masked GFAP and set the intensity threshold for the channel B (Figure 11C). This is relatively easy as compared to the setting of the threshold for channel A. During the masking protocol, we eliminated the background signal by threshold masking of the original GFAP channel. Therefore, the threshold intensity of this channel can be directly set at 1.
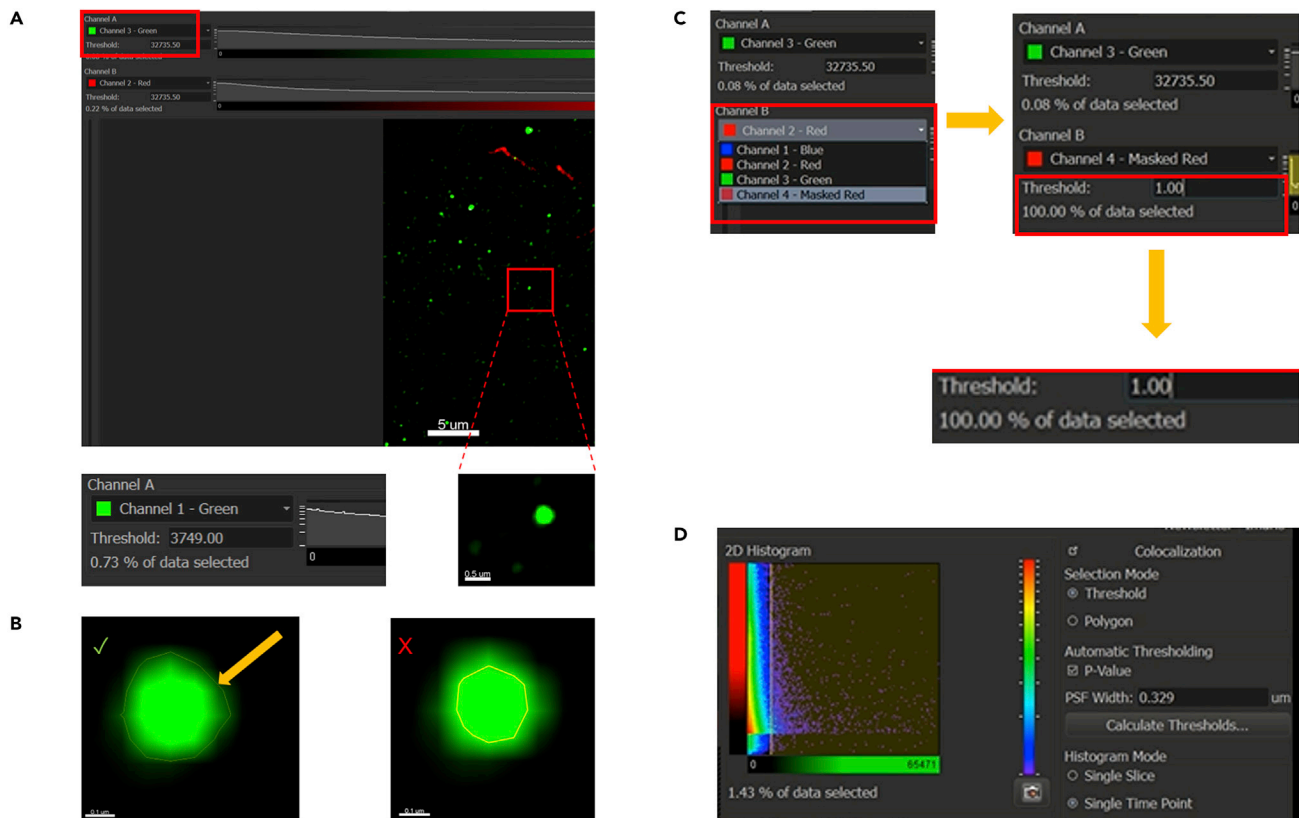
Figure 11. Guide for the colocalization analysis with Imaris

Open the Coloc tab (▣) which is present on the side of "Image Processing" option.

(A) For setting the threshold of channel A select unambiguous punctum as is shown in the figure. *Scale Bar for zoomed in section (inset) 0.5 μm.*

(B) Right click on each punctum and drag to create a boundary around each punctum. The boundary is shown by an arrow. Make sure that the yellow boundary covers the entire individual green signal. The intensity threshold for channel A (green) is selected by averaging the intensities of 10 unambiguous green puncta selected by setting the boundary around each signal. A correct boundary is shown with a tick on the left and a wrong boundary setting is shown on the right with a cross sign. *Scale Bar for zoomed in section (inset) 0.1 μm.*

(C) For the channel B (masked red), the threshold is set at 1.

(D) Imaris does provide an option for automatic thresholding which can be done clicking on the calculate threshold option as shown in the figure. However, this produces inconsistent results and therefore we do not recommend using this method for the colocalization.

48. Towards the bottom in the right side, select the define "Region of Interest" tab. This is a crucial step in the analysis of the final set of the data. Since we intend to calculate the colocalization over the whole volume of astrocyte, we select the masked GFAP channel and set the threshold as 1 (Figure 12A).

49. The last step of the analysis is to "Build Coloc Channel".
    a. A new tab opens with different parameters analyzed.
    b. The whole file can be exported as a "xxx.csv" file. To our interest, is the % of ROI colocalized, where we have defined the ROI as the astrocyte mask (Figure 12A). This gives us an estimation of the amount of C1q tag present in the whole volume of the astrocyte.
    c. This can be transferred to a master excel file consisting of data from several other astrocytes and in both control and treatment samples for the final statistical analysis (Figure 12B).

EXPECTED OUTCOMES

In this paper, we present a fast and efficient method to characterize the synaptic engulfment of neuronal tag in an *ex vivo* slice treatment of amyloid beta oligomers by the astrocyte (Figures 12, 13, 14). The colocalization analysis protocol serves as an indirect measure for the synaptic pruning
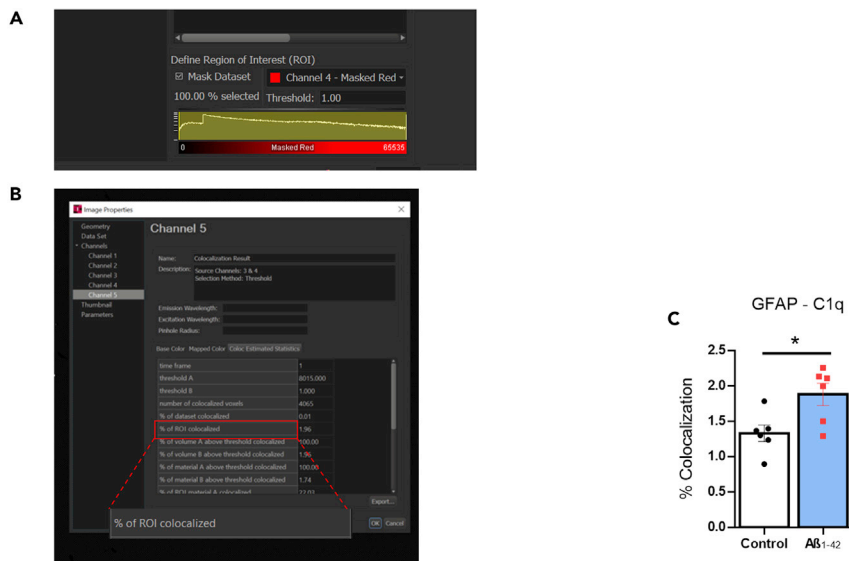
Figure 12. Generation of colocalization statistics

(A) In the "Define Region of Interest (ROI)" box in the lower right corner, select mask dataset and select the masked red channel in the channel box.

(B) The colocalization analysis produces a set of data which define % of colocalization. These values can be exported to a separate "xxx.csv" file. The % of ROI colocalized is of interest to us as it gives us the measurement of synaptic engulfment by the astrocyte.

(C) Representative Dataset showing % colocalization of GFAP and C1q between control and $A\beta_{42}$ treated slices. Data is plotted as mean $\pm$ SEM of six animals per group and is analyzed by unpaired two-tailed t-test. Mean $\pm$ SEM of each group: Control (1.332 $\pm$ 0.1169) and $A\beta_{42}$ (1.884 $\pm$ 0.1597). *p < 0.05, significantly different from control group.

process. Amyloid beta oligomers are known to negatively affect the spine density and synapses. C1q, a complementary protein tag, serves as a destruction signal for the neuronal synapses to be engulfed by the glial cells. The CA1 area of the hippocampus plays an essential role in memory and learning and inducing of long-term potentiation (LTP). From our previous research, the treatment of hippocampal slice with 50 nM $A\beta_{42}$ causes a significant decline in the CA1-LTP (Rammes et al., 2018). This protocol was developed to understand the molecular distributions in the astrocyte when the slices are incubated with $A\beta_{42}$. The % ROI colocalization is an indirect way of measuring (proportional) the amount of synaptic tag engulfed by the whole volume of the astrocytes. By calculating the % of colocalization in the masked red channel (which represents the astrocytic volume), we are determining the % of the C1q signals which is inside this astrocytic volume. This method gives an estimate of the eat-me tags that are inside the astrocytes (proxy to synaptic pruning). This data is correlated with the % of PSD95 (postsynaptic marker) inside the astrocytic volume.

QUANTIFICATION AND STATISTICAL ANALYSIS

The % ROI colocalization, in which the astrocyte is the ROI and percent colocalization refers to the percent of astrocyte volume that colocalizes with a molecular species of interest (e.g., C1q), is extracted from the xxx.csv files for different astrocytes. To have a meaningful number of samples to test the significance, we recommend having at least 6 mice per group. It is advised to average the data from at least 15–20 astrocytes (from every mouse brain) to have a well-represented data set. We observed greater degree of % ROI colocalization of both C1q eat-me tag as well as PSD95 inside the astrocytic volume, in the hippocampal slices that were incubated with $A\beta_{42}$ peptide as compared to the control. This indicates an increased synaptic pruning activity in the presence of $A\beta_{42}$ oligomers which correlates with the previous studies in different Alzheimer's models (Sellgren et al., 2019; Brucato and Benjamin, 2020). The statistical analysis can be performed by common programs such as Graph Pad Prism or SPSS. The statistical test to be used for analysis greatly depends
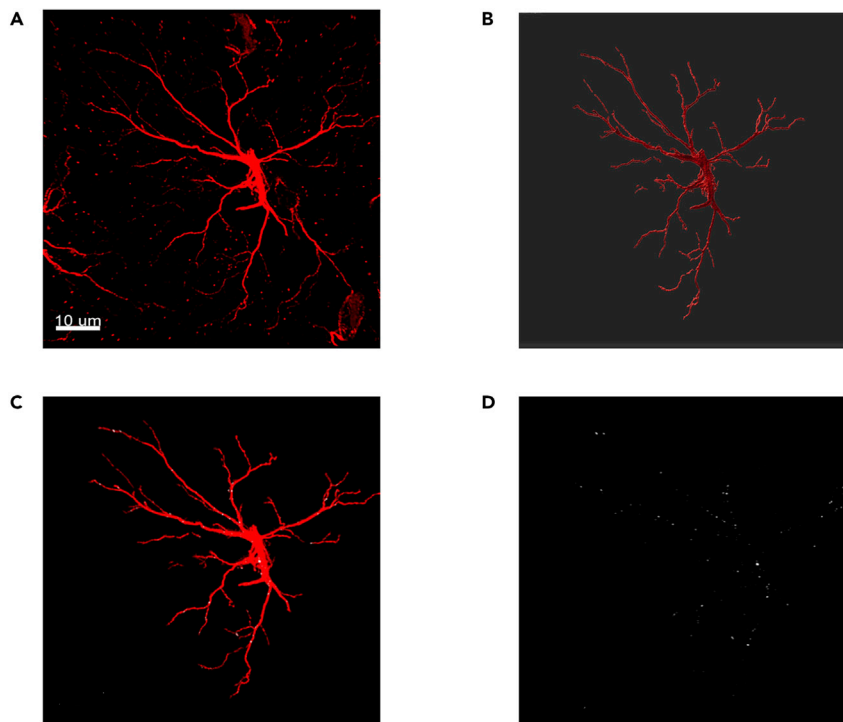
Figure 13. Volumetric rendering and synaptic engulfment of the astrocyte
(A) Original processed image of the astrocyte.
(B) Volumetric 3D rendered image.
(C) Astrocyte (Red) with colocalisation of C1q in white.
(D) Colocalization shown in white.

on the type of experiment and number of groups. If the control group were to be compared with an experimental group only, it would be advisable to use the two-tailed unpaired T-test. An example of representative colocalization dataset is shown in Figure 12C.

LIMITATIONS

The protocol described here and the rationale behind utilizing this protocol applies without any bias to animals of age group 6–8 weeks. Older animals, however, due to the age parameter have more reactive astrocytes as compared to the younger ones. Therefore, it is difficult to know whether the astrocytic activity is due to the treatment with amyloid beta oligomers or due to the age. The quality of the tissue and the protocol used for collecting the samples greatly impacts the quality of rendering as well as the colocalization study. The protocol described in this paper best applies to free floating slices. If the same is applied to frozen slices, the 3D volume reconstruction would be inaccurate. The quantification of intensity of the fluorophores is a relative parameter, and it should always be normalized with respect to the negative control, to obtain a reliable analysis of colocalization. While GFAP is considered to be more appropriate for staining the astrocytes in the hippocampus, corpus callosum and cerebral peduncle (Zhang et al., 2019), one of the recent study suggests GFAP doesn't recapitulate all the morphological processes of the astrocyte (Reeves et al., 2011). While this doesn't greatly impact the colocalization analysis, the researchers performing morphometric analysis of astrocytes should keep additional markers to correlate their datasets.

TROUBLESHOOTING
Problem 1
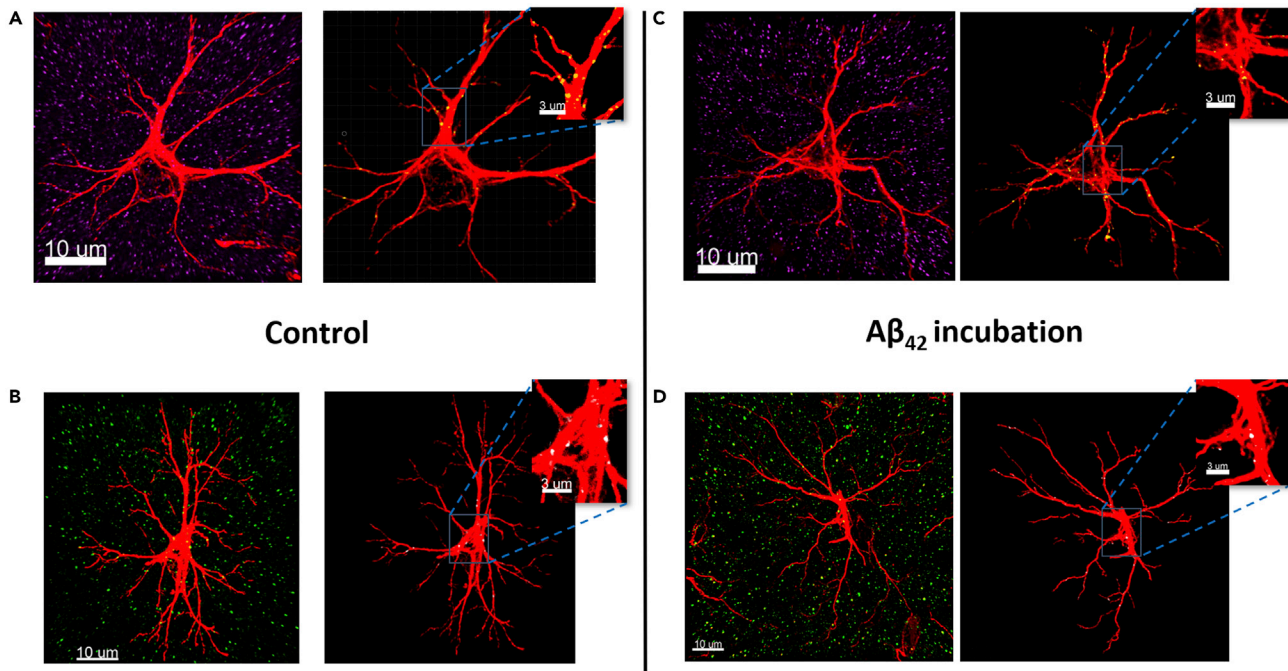The brain section breaks while cutting in the cryotome/there are cracks in the sections collected (step 9).

Figure 14. Representative images showing synaptic engulfment of post synaptic marker PSD95 and complementary tag C1q in control and Aβ$_{42}$ incubated slices

(A) Individual high-resolution astrocyte (shown in red) and post synaptic marker PSD95 (shown in magenta) in control slices. The colocalization points are shown in yellow inside the astrocyte.

(B) Astrocyte (shown in red) and the "eat-me signal" C1q protein (shown in green) in control. The colocalized points between astrocyte and C1q are represented in white.

(C) Individual high-resolution astrocyte (shown in red) and post synaptic marker PSD95 (shown in magenta) in Aβ$_{42}$ incubated slices. The colocalization points are shown in yellow inside the astrocyte.

(D) Astrocyte (shown in red) and the "eat-me signal" C1q protein (shown in green). The colocalized points between astrocyte and C1q are represented in white in Aβ$_{42}$ incubated slices. *Scale Bar 10 μm. Scale Bar for zoomed in section (inset) 3 μm.*

Potential solution

The brain slice if not kept flat, will result in unequal thickness of sections. This might result in breaking of sections or having folds in the sections s. In such cases, there are chances that obtaining the complete hippocampus becomes difficult. An easier way is to put the slices horizontally on the solidified OCT mounting medium and softly pressing it by using a brush. The section (30 μm) needs to be flat for efficient penetration of antibodies. However, if the slices are flat and there are still cracks in the sections, consider changing the tissue-holder temperature and knife temperature.

Problem 2

Poor GFAP signals/astrocytic processes are unclear/fuzzy (step 31).

Potential solution

Low tissue quality as well as the antibody concentration greatly affects the astrocyte (GFAP) signals. Also, it is essential to note that the protocol described in this paper suits best for the free-floating sections. We have observed that when the same protocol is repeated for the frozen section, the astrocytic processes are not clear, and the branching complexity usually cannot be inferred from such data sets. We do have a separate protocol for accessing the colocalization in frozen sections. Briefly, instead of using PFA as a fixative agent we use chilled acetone: isopropanol (1:1) as a fixative agent. The fixation is done by inserting the slides containing brain sections inside the acetone: isopropanol solution for about 25 min. This is followed by the permeabilization with 0.3% triton-X in PBS for 20 min followed by washing three times with 1× PBS and then blocking with 10% NGS at RT for an hour. This is followed by antibody incubation as described in the current protocol.

Problem 3
Poor PSD95 signal obtained from the confocal microscope (step 31).

Potential solution
Initially we started out with incubation of primary antibody of PSD95 overnight (16–20 h) at 4°C, however the signal obtained was sparse and unclear/fuzzy. We recommend therefore to increase the incubation with the primary antibody for PSD95 for 2 days.

Problem 4
The staining of astrocyte and synaptic marker is not bright enough (step 32).

Potential solution
While there could be a lot of parameters impacting the staining, starting from the incubation of slice to the immunofluorescence protocol. We particularly advise to be careful with 2 parameters if the immunofluorescence protocol was performed correctly. First, the incubation period of slices with the amyloid beta peptide needs to be correctly monitored. If the slices are overlapping or there are bubbles in the chamber holding the slice, the health of the slices are greatly hampered. Second, the sections obtained from the cryotome should be flat and without any cracks.

Problem 5
While performing 3D reconstruction of the astrocyte, there are small unnecessary signals away from the main astrocyte under construction (step 41).

Potential solution
There is a twofold way to solve this issue. In step 4 of the volumetric rendering of the astrocyte, extra signals outside the main astrocyte can be controlled by setting a volume filter as described in step 40. This can be used on a trial and error basis to exclude unnecessary background signals. However, if there are still unspecific signals after the 3D rendering of the astrocyte, they can be removed manually by selecting "Circle Selection Mode" on the right side of the Imaris panel. Select the background signal by clicking CTRL and selecting the signals using the right cursors. After all the unnecessary signals are selected, they can be deleted by clicking "pencil" like icon (Edit) on the lower left side and then selecting the delete option.

Problem 6
There is ambiguity regarding the processes whether from a single astrocyte or more than 1 astrocytes (step 41).

Potential solution
There is always a possibility that there are processes from other astrocytes in the same frame. The easiest way to detect or possibly have a clue whether the processes belong to the same astrocyte, is to have a NucBlueTM (nuclear) staining. If the processes from other astrocytes are detected on the same frame as the astrocyte under the consideration, they need to be manually removed as suggested in the solution to Problem 4.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gerhard Rammes (g.rammes@tum.de).

Materials availability
The study did not generate any new reagents.

Data and code availability

The study did not generate/analyze new code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101687.

AUTHOR CONTRIBUTIONS

Conceptualization, A.K.P.; Methodology, A.K.P. and Q.S.; Investigation, A.K.P., K.T., and Q.S.; Writing – Original Draft, A.K.P.; Writing - Review & Editing - A.K.P., K.T., Q.S., and G.R.; Supervision, G.R.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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