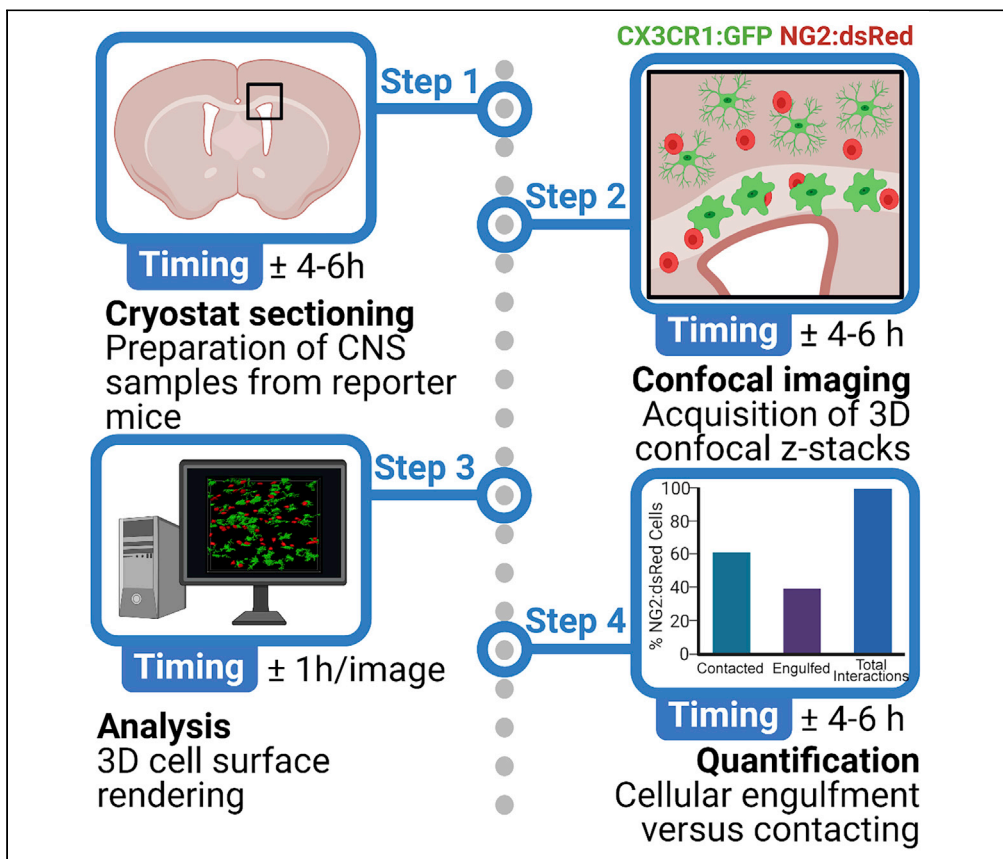


Protocol

Quantification of microglial contact and engulfment of oligodendrocyte progenitor cells in the rodent brain



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HIGHLIGHTS

Create 3D reconstructed images that identify interactions between microglia and OPCs

Evaluate cellular interactions using 3D surface renders and identify internalization

Quantify percent internalization and distinguish cellular contact from engulfment

Microglia have emerged as essential regulators of neurodevelopment by phagocytosing synapses. Recently, we showed that microglia engulf viable oligodendrocyte progenitor cells (OPCs) during development to facilitate myelination. Here, we describe a protocol to quantify microglial engulfment of whole cells using 3D confocal microscopy to differentiate microglial contact. This protocol can be applied to assess whole-cell engulfment in a variety of contexts and cell types.

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Protocol

Quantification of microglial contact and engulfment of oligodendrocyte progenitor cells in the rodent brain

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SUMMARY

Microglia have emerged as essential regulators of neurodevelopment by phagocytosing synapses. Recently, we showed that microglia engulf viable oligodendrocyte progenitor cells (OPCs) during development to facilitate myelination. Here, we describe a protocol to quantify microglial engulfment of whole cells using 3D confocal microscopy to differentiate microglial contact. This protocol can be applied to assess whole-cell engulfment in a variety of contexts and cell types.

For complete details on the use and execution of this protocol, please refer to Nemes-Baran et al. (2020).

BEFORE YOU BEGIN

Microglia are the resident immune cells of the central nervous system. Recent work has highlighted that microglia do more than remove damaged or dying cells during pathological conditions. In fact, microglia are important for regulating homeostasis within the developing and adult brain by phagocytosing synapses as well as neural precursor cells (Schafer et al., 2012, Paolicelli et al., 2011, Cunningham et al., 2013). We recently demonstrated that microglia engulf oligodendrocyte progenitor cells (OPCs) as an important mechanism for cerebral white matter development (Nemes-Baran et al., 2020). This protocol provides detailed methods to study this new and interesting role of microglial engulfment of whole cells. To study the engulfment process, methods for identifying these cells are necessary before image acquisition and analysis can begin. While cells can be labeled using immunofluorescent staining, reporter mice are commercially available which provide robust and specific expression of green fluorescent protein (GFP) under the CX3CR1 promoter (The Jackson Laboratory, Bar Harbor, ME; Jax 005582) on microglia in the brain and red fluorescent protein (dsRed.T1) under the NG2 promoter (The Jackson Laboratory, Bar Harbor, ME; Jax 008241) on oligodendrocyte progenitor cells in the brain (Figure 1). Transgenic mice are also advantageous for ex vivo live imaging studies.

Breeding transgenic reporter mice

⌚ Timing: 20–30 days

1. Pair-house breeding mice (one homozygous CX3CR1:GFP mouse and one hemizygous NG2:dsRed mouse) at least four weeks prior to the planned date for tissue harvesting of pups (Figure 1A). All offspring will be heterozygous for the CX3CR1:GFP gene and express GFP within microglia in the brain. However, approximately 50% of the litter will express the dsRed gene, likely requiring multiple breeding pairs to yield the desired sample size (Figure 1B).



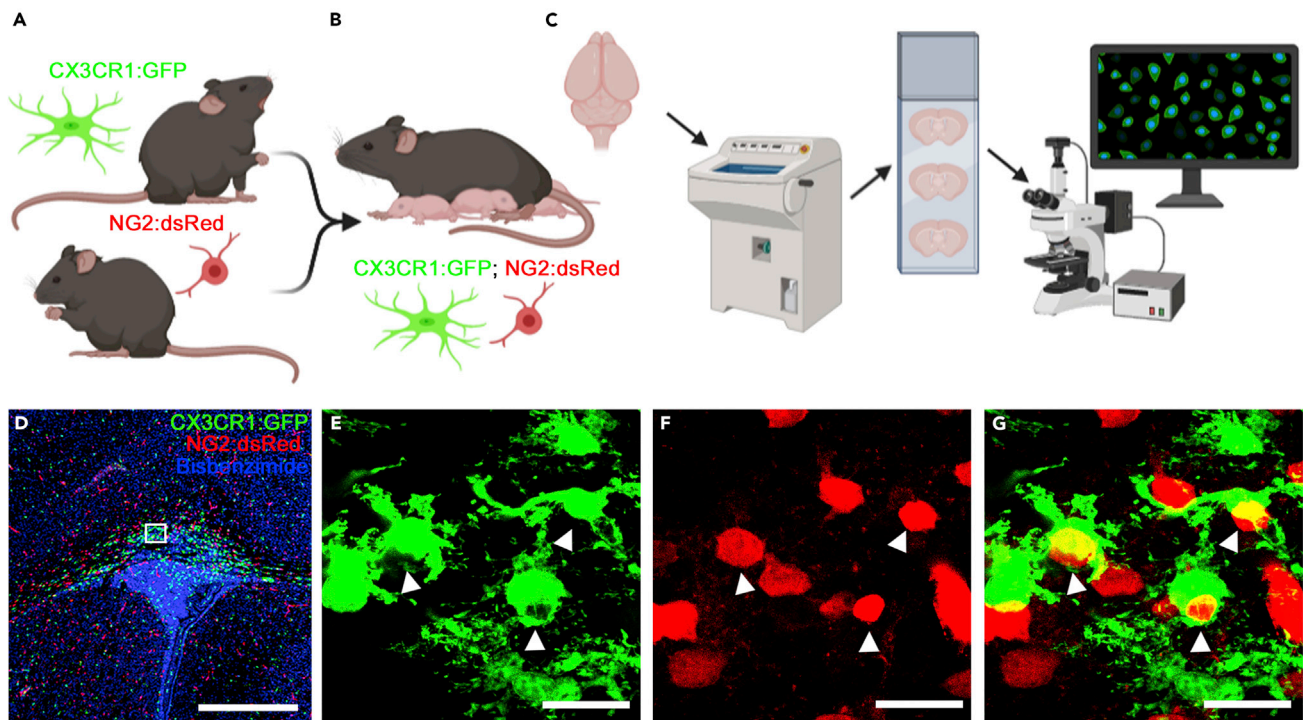


Figure 1. Labeling microglia and OPCs within the developing mouse corpus callosum

(A) CX3CR1:GFP reporter mice crossed with NG2:dsRed reporter mice.

(B) Pups with fluorescently labeled microglia (green) and OPCs (red).

(C) Pups are perfused at postnatal day 7 (P7) and brains are sectioned on a cryostat at 15 μm then mounted on slides to be imaged on a confocal microscope. Illustration was created with [BioRender.com](https://www.biorender.com).

(D–G) (D) Corpus callosum microglia with a confocal z-stack location outlined by a white box (magnified in E–G). Scale bar, 500 μm . Corpus callosum microglia (E) have an amoeboid morphology and (F) engulf OPCs (merged in G, engulfment denoted by arrow heads). Scale bars, 20 μm .

- Genotype mice for the CX3CR1:GFP gene and the dsRed gene using protocols available from Jackson Laboratories (The Jackson Laboratory, Bar Harbor, ME; Jax 005582 and Jax 008241).
- At postnatal day 7 (P7), proceed with brain harvesting of CX3CR1:GFP, NG2:dsRed pups ([Figure 1C](#)). Other time points may be explored such as embryonic or early postnatal development, but this age has been identified as the peak time of microglial engulfment of OPCs within the corpus callosum ([Nemes-Baran et al., 2020](#)).

△ CRITICAL: Transgenic mice should always be characterized before use in experiments. NG2:dsRed mice should be immunostained for an oligodendrocyte marker such as Olig2 to determine reporter specificity and recombination efficiency. Since pericytes also express NG2, it is important to exclude them from the analysis as mentioned throughout this protocol (by volume, sphericity and location on blood vessels). CX3CR1:GFP mice should be immunostained for a microglia marker such as Iba-1 to determine reporter specificity and recombination efficiency as previously described ([Nemes-Baran et al., 2020](#)).

Alternatives: Cells can be labeled using immunofluorescent staining for conventional markers of microglia and OPCs, or other cell types of interest without use of transgenic reporter mice.

Note: Please adhere to institutional animal use regulations and guidelines for the humane treatment of animals.

Transcardiac perfusion and tissue preparation

⌚ Timing: 3 days

4. Prepare 1× phosphate buffered saline solution (PBS) at a pH of 7.4.
 - a. A stock solution of 10× PBS can be prepared by dissolving the following reagents in 800 mL of distilled water. Stock solutions can be stored between 20°C–25°C for up to 12 months. Make new stock solution if a precipitate is observed.

Reagent	Final concentration	Amount
NaCl	1.37 M	80 g
KCl	27 mM	2 g
Na ₂ HPO ₄	100 mM	17.8 g
KH ₂ PO ₄	18 mM	2.4 g

Note: After all ingredients are fully dissolved, adjust final volume to 1000 mL with distilled water.

- b. Dilute 10× PBS into 1× PBS stored between 20°C–25°C for up to 12 months. Make a new solution if precipitate is observed.
 - i. 10 mL 10× PBS
 - ii. 90 mL distilled water
 - iii. Check the pH directly using a clean pH meter and adjust as necessary to 7.4 using NaOH or HCl. Filter the final solution before use using a 0.2 μm filter.
5. Prepare 4% paraformaldehyde (PFA) in 1× PBS stored at 4°C for up to 7 days, however, it is recommended to make fresh for the best quality histology.
 - a. Dissolve 4 g of PFA powder (Sigma Aldrich, St. Louis, MO; 158127) in 100 mL of 1× PBS on a hot plate at 50°C–60°C with a stir rod within a ventilated fume hood.

Note: The solution will not dissolve immediately, and larger volumes may take longer. Adjusting the pH by adding NaOH dropwise can facilitate solubilization.

- b. Once fully dissolved, cool 4% PFA solution on ice.
 - c. Check the pH and adjust as necessary to 7.0 using NaOH or HCl. Filter the final solution using a 0.2 μm filter before use.

⚠ **CRITICAL:** Improper perfusion of mice may result in poor tissue quality and impact data analyses. Additionally, inconsistent perfusion procedures can result in variability within samples. Be sure to follow the protocol closely and ensure rodent brains have been perfused and fixed effectively. Harvested brains should be free of blood with a white appearance and feel firm to the touch. PFA solution should be made fresh for high-quality histology. For additional guidance on transcardiac perfusion of mice, review recommended protocol (Gage et al., 2012).

Alternatives: Live imaging of *ex vivo* brain slices as we have previously described (Nemes-Baran et al., 2020) or dissociated cells can be used instead of perfusion-fixed brain tissue. For live imaging in *ex vivo* slices, follow appropriate protocols in lieu of PFA-fixation steps listed here. Acquire 3D confocal z-stacks of these preparations, and analyze the data as described below in the Image Preparation section and following steps.

6. Set up necropsy station with anesthesia system, dissection tools and perfusion pump.
 - a. Anesthesia system (Gage et al., 2012) should consist of isoflurane vaporizer (VetEquip, Livermore, CA) with induction chamber, or use of injectable anesthetic drugs at appropriate

dosage for a surgical plane of anesthesia. Be sure to have extra anesthesia to use as needed to maintain a deep state of anesthesia.

- b. Dissection tools include sharp scissors, forceps, 2–3 hemostats or dissection clamps, bone rongeurs or strong curved forceps and spatula (Fine Science Tools, Foster City, CA). Tools should be clean and organized upon starting the procedure.
 - c. The perfusion pump (Fisher Scientific, Hampton, NH) should be primed with fluid to avoid air bubbles. To do this, place the input tubing into the 1× PBS solution and attach the output tubing to a 19-gauge needle (any larger gauge needle will work). Turn on the perfusion pump and allow 1× PBS to flow through the tubing until it starts to come out of the needle. Stop the pump and prepare for the perfusion. To collect waste at the end of the perfusion, animals should be perfused over a screen or grate placed on top of a container during the procedure.
7. Anesthetize the P7 mouse by placing it in the isoflurane induction chamber with 5% isoflurane concentration, or with the appropriate dosage of injectable anesthetic drug for a deep state of anesthesia. Wait for the animal to appear anesthetized. Test the plane of anesthesia using forceps to pinch the tail, toes and abdomen of the mouse. If the mouse is unresponsive, move it to the necropsy area over the waste collection container and place its nose into the nose cone for continuous isoflurane anesthesia at 2.5% concentration, adjusting as necessary to maintain a deep state of anesthesia but maintaining heartbeat. At any time that an animal is no longer in a deep state of anesthesia, stop the procedure and administer additional anesthetic and wait for the animal to no longer respond to toe, tail or abdominal pinching.
 8. Once the mouse is in a deep state of anesthesia, make a long lateral incision through the abdominal wall just below the rib cage to completely expose the cavity.
 9. Make an incision through the diaphragm, taking care not to puncture the heart. Cut the diaphragm to expose the thoracic cavity. Cut the sternum to the top of the rib cage and cut along the top of the ribs to provide space to access the heart. Be careful not to cut the heart. Use the forceps or dissection clamps to hold the ribs out of the way and create space to access the heart for the transcardiac perfusion.
 10. Insert the needle into the left posterior end of the ventricle of the heart and hold it in place or clamp it using hemostats or dissection clamps.
 11. Cut a small hole in the right atrium to allow blood to flow out during the perfusion process.
 12. Start the pump at a speed of 10 mL/min and allow 10 mL of ~4°C 1× PBS to perfuse the mouse. Blood should be observed flowing out of the right atrium and the liver will begin to clear from a deep red color to a pink color.
 13. After 10 mL of 1× PBS has been perfused, stop the pump and switch the fluid to the 4% PFA solution at ~4°C. Continue to perfuse the mouse at the same flow rate until 10 mL of 4% PFA has perfused the body and it becomes rigid.
 14. Remove the needle and clamps from the body and decapitate the mouse.
 15. To harvest the brain, use small, sharp scissors (preferably curved). Begin by making a midline incision in the skin starting at the neck and cutting up to the nose to reveal the skull. Expose the skull by removing any muscle or connective tissues. Slide the scissors into the foramen magnum of the skull being careful not to damage the brain by keeping the tips of the scissors pointed away from the brain. Cut along the midline of the skull up to the frontal bones, keeping the tips of the scissors pointed up and away from the brain, sliding across the inner skull. Make two additional cuts from the midline laterally in the frontal bones. Use bone rongeurs to carefully peel the skull away from the brain. Continue until the brain is fully exposed and can be carefully removed from the skull with a spatula.
 16. Post-fix the brain for 24 h in 4% PFA in a specimen cup at 4°C. If performing immunofluorescent staining in lieu of reporter mice, refer to technical information regarding the impact of post-fixation on antigenicity. Antigen retrieval processes or a shorter post-fixation period may be necessary for some antibodies. See [troubleshooting](#) for additional information on immunostaining.
 17. Rinse the brain in 1× PBS three times before placing it in a 30% sucrose solution for 48 h, or until the brain sinks to the bottom of the specimen cup.

- a. 30% sucrose solution can be made with 150 g of sucrose (Sigma Aldrich, St. Louis, MO; S0389) dissolved in 500 mL of 1 × PBS.
- b. If not sectioning immediately, follow the next step for embedding the brain and store it at -80°C until ready for sectioning.

Histology

⌚ Timing: 1–3 days

18. Embed brains in a solution of 1 part 30% sucrose solution and 2 parts O.C.T. compound (Fisher HealthCare, Waltham, MA; 23-730-571) in a well-labeled brain embedding mold (Fisher Scientific, Hampton, NH; 1830) taking care to avoid bubbles. Flash freeze the embedded brain using liquid nitrogen, or 2-methylbutane chilled with dry ice. Store flash-frozen embedded brains in molds at -80°C until ready to section.
19. Remove embedded frozen brains from embedding mold and section using a cryostat (Leica Biosystems, Nussloch, Germany). Depending on the structure of interest, it may be best to section the brain on a coronal, sagittal or axial plane (Figure 1C). Determine this prior to sectioning the brain. Section brain into 15 μm thick sections and mount directly to a glass microscopy slide (Fisher Scientific, Hampton, NH; 22-037-246).
20. Allow slides to air-dry 1–12 h between 20°C – 25°C while protected from light.
21. Wash slides for 5 min three times in 1 × PBS between 20°C – 25°C . If reporter mice were used, continue to coverslip slides with bisbenzimidazole (step 10). If reporter mice were not used, follow the steps below to stain the tissue with markers against microglia and OPCs, or other cell types of interest.

⚠ **CRITICAL:** For immunofluorescent staining, determine if antibodies require cell permeabilization with Triton X-100 (Fisher Scientific, Hampton, NH; 85111) as recommended by the antibody manufacturer and add Triton X-100 to the washes if necessary. Antibodies generated against epitopes on the surface of cells do not require Triton X-100, as permeability to access the target protein epitope is not required. Expression patterns between immunostained tissue and transgenic reporter mice will vary, therefore it is important to use the same method of labeling cells throughout the experiment. Furthermore, immunofluorescent staining will need to be optimized for each antibody and efficiency of recombination will need to be evaluated for transgenic mice. The mice described in this protocol are commercially available.
22. Block tissue using 5% serum corresponding to the host of the secondary antibody with 0.3% Triton X-100 for 1 h between 20°C – 25°C . For example, with goat anti-rabbit secondary antibody, use goat serum for blocking buffer.
23. Add primary antibody to the tissue for 4 h between 20°C – 25°C or 12–16 h at 4°C . As previously published, we used anti-Iba-1 to identify microglia and CD68 to specifically localize cells within the lysosome of microglia (Nemes-Baran et al., 2020). Additionally, we used antibodies generated against NG2 and PDGF receptor α to target OPCs (Nemes-Baran et al., 2020). Use the dilution recommended by the antibody manufacturer mixed in blocking buffer. If using more than one primary antibody, be sure to use different host species when staining. Refer to the troubleshooting section for additional details on immunostaining.
24. Wash slides for 5 min three times in 1 × PBS between 20°C – 25°C .
25. Apply secondary antibody to the tissue for 45 min between 20°C – 25°C while protected from light. Use the dilution recommended by the antibody manufacturer mixed in blocking buffer.
26. Wash slides for 5 min three times in 1 × PBS between 20°C – 25°C while protected from light.
27. Coverslip slides using Fluoromount-G (Southern Biotech, Birmingham, AL; 0100-01) containing bisbenzimidazole (1:1000; Invitrogen, Carlsbad, CA, H3569).
28. Allow slides to dry between 20°C – 25°C for 24 h while protected from light.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Iba-1 (1:750)	Wako	Cat# 019-19741
Rat anti-PDGFR α (1:400)	BD Bioscience	Cat# 558774
Rabbit anti-NG2 (1:200 free-floating)	Millipore	Cat# ab5320
Rabbit anti-Olig2 (1:200)	Millipore	Cat# ab9610
Alexa Fluor 488 goat anti-rabbit IgG (1:1000)	Invitrogen	Cat# A11034
Alexa Fluor 647 goat anti-rat (1:1000)	Invitrogen	Cat# A21247
Chemicals, peptides, and recombinant proteins		
Sodium chloride (NaCl)	Sigma-Aldrich	Cat #71376
Potassium chloride (KCl)	Sigma-Aldrich	Cat #P9541
Sodium phosphate dibasic dihydrate (Na ₂ HPO ₄ ·2H ₂ O)	Sigma-Aldrich	Cat #71643
Potassium phosphate monobasic (KH ₂ PO ₄)	Sigma-Aldrich	Cat #P9791
Paraformaldehyde (PFA)	Sigma-Aldrich	Cat #158127
Sucrose	Sigma-Aldrich	Cat # S0389
O.C.T. Compound	Fisher Healthcare	Cat #23-730-571
Bisbenzimidazole dye	Invitrogen	Cat # H3569
Immersion oil	Nikon	n/a
Triton X-100	Sigma-Aldrich	Cat #T8787
Experimental models: organisms/strains		
Mouse: Tg(Cspg4-DsRed.T1)1Akik/J (NG2DsRedBAC), aged 2–8 months, male and female for breeding	Jackson Laboratory	Cat# Jax 008241, RRID: IMSR_JAX:008241
Mouse: B6.129P2(Cg)-Cx3cr1tm1Litt/J (CX3CR1-GFP), aged 2–8 months, male and female for breeding	Jackson Laboratory	Cat# Jax 005582, RRID: IMSR_JAX:005582
Software and algorithms		
Imaris software equipped with the Surface-Surface Coloc XTension	Bitplane	https://imaris.oxinst.com/
Microsoft Excel	Microsoft Corporation	https://products.office.com/en/excel
MATLAB	MathWorks	https://www.mathworks.com/
NIS-Elements	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements
Other		
Cryostat	Leica Biosystems	n/a
Fluorescent Confocal Microscope with 40 \times oil immersion objective lens	Nikon	n/a
Fluoromount-G	SouthernBiotech	Cat #0100-01
Glass coverslips	Fisher Scientific	Cat #125485MP
Sterile Vacuum Filters	Fisher Scientific	Cat #566-0020
Isoflurane vaporizer system for small animal anesthesia	VetEquip	http://vetequip.com
Brain embedding mold	Thermo Scientific	Cat #1830
Glass microscopy slides	Fisher Scientific	Cat #22-037-246
Peristaltic perfusion pump	Fisher Scientific	Cat # 13-876-1
Sharp scissors	Fine Science Tools	Cat #14060-09
Forceps	Fine Science Tools	Cat #11051-10
2–3 Hemostats	Fine Science Tools	Cat #13008-12
Bone rongeur	Fine Science Tools	Cat #16000-14
Spatula	Fine Science Tools	Cat #10099-15

STEP-BY-STEP METHOD DETAILS

Confocal microscopy

⌚ Timing: 1–3 days (~2 h per animal)

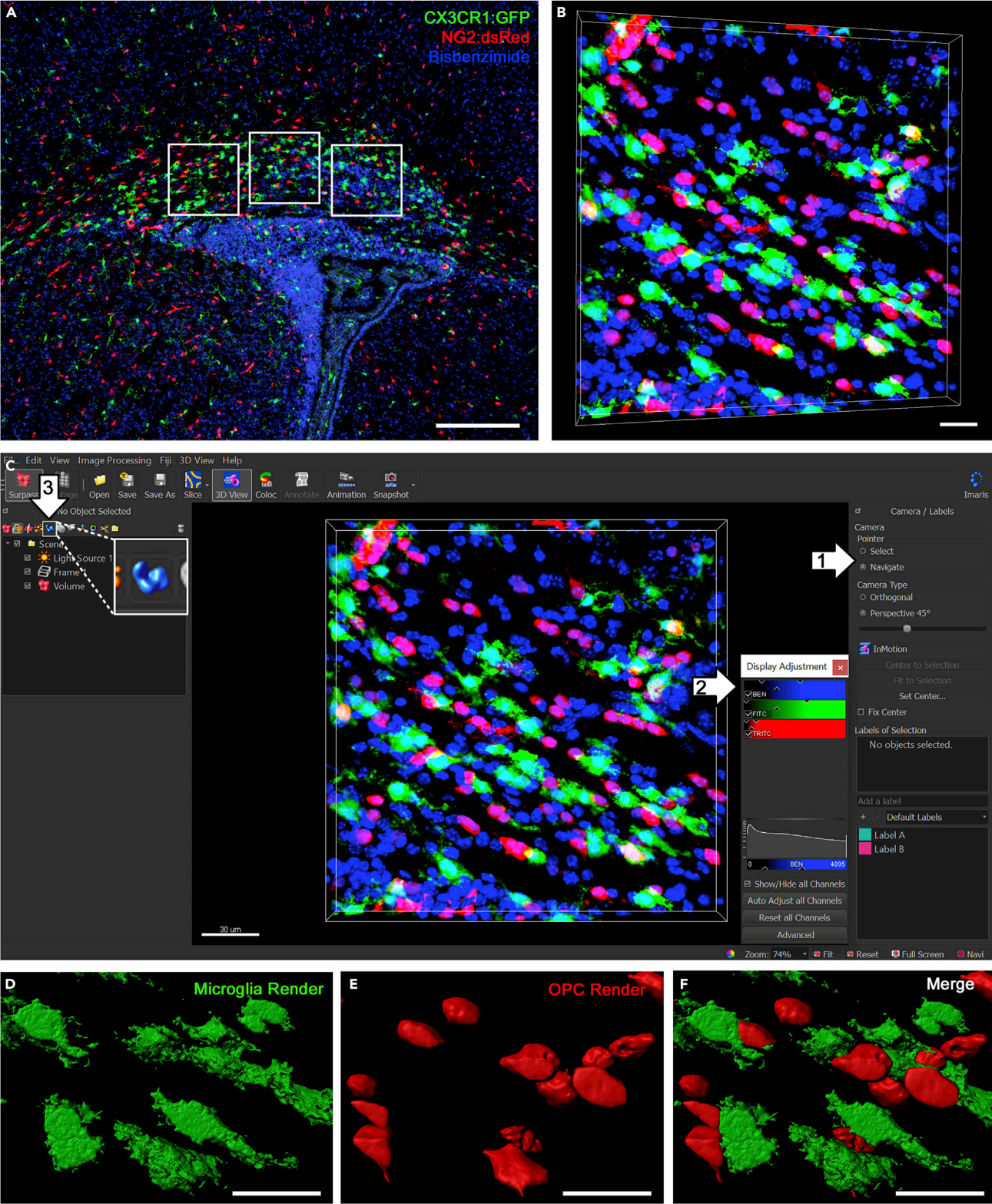


Figure 2. Observing microglial interactions with OPCs in 3D

(A) The locations in the corpus callosum where confocal z-stacks were acquired are illustrated by 3 white boxes. Scale bar, 200 μm .
 (B) Representative 3D reconstructed z-stack from the corpus callosum of CX3CR1:GFP;NG2:dsRed mouse stained with bisbenzamide, a marker of cell nuclei. The image file for this z-stack is provided as part of the practice tutorial for this protocol. Scale bar, 20 μm .
 (C–F) (C) Screen view of Imaris software with the provided sample image file in 3D view. The navigation controls (white arrow 1) can be used to rotate the image (navigate) or select surface rendered objects (select). The brightness of the image can be adjusted (white arrow 2) with the display adjustment tab where individual channels can also be turned on or off using the checkboxes. Surface renders of each channel can be created (white arrow 3) within the “Scene” window on the left side of the screen by clicking the “Add New Surfaces” icon on the top of the window, which appears as a blue 3D bulbous shape, shown in the box of this image. Scale bar, 30 μm . Representative surface renders of (D) microglia, (E) OPCs, and (F) merge of both surfaces together. See [Methods video S1](#) for this process. Scale bars, 20 μm .

This step of the protocol describes how to acquire a high-resolution confocal z-stack for analysis of microglial engulfment of OPCs in the corpus callosum of the P7 mouse brain. Samples should express fluorophores on microglia with CX3CR1:GFP transgenic reporter mice, or be fluorescently labeled by immunofluorescent staining methods described above. Likewise, samples should express fluorophores on OPCs with NG2:dsRed transgenic reporter mice or be fluorescently labeled using immunofluorescent staining methods described above.

1. Place dry slide on the stage of the confocal microscope, such as the Nikon C2 confocal microscope with NIS-Elements software (Nikon, Melville, NY).
2. Locate the area of interest, such as the corpus callosum at the level of the lateral ventricles in a coronal section to identify large, amoeboid microglia ([Figures 1D–1G](#)).
3. Use a high-powered objective, such as a Plan Fluor 40 \times /1.30 NA oil immersion objective, to focus on one region within the area of interest ([Figure 2A](#), white boxes). Set the laser power and gain for each wavelength to optimize the image without overexposure.

Note: The laser power and gain will be different based on the specimen and microscope set up. Be sure to optimize this in a non-essential region of the tissue before imaging the experimental region to avoid photobleaching.

4. Set the top and bottom of the z-stack based on the clarity of the cells in the image. The z-stack should be 15 μm thick. Set the step size to 0.3 μm . To avoid photobleaching, test the microscope settings on a section that is not needed for the experiment. Keep the laser power settings low and adjust the gain to increase the brightness. Since each area will only be imaged one time, the proper microscope settings should not create significant variability in the fluorescence signals between image stacks. If fluorescence intensity is low, it may be an issue with tissue quality from storage or staining technique. See the [troubleshooting](#) section for more details. Acquire the z-stack in the appropriate color channels necessary to capture the image. It is recommended to image 3 regions of the corpus callosum as illustrated by white boxes in [Figure 2A](#). A representative z-stack is shown in [Figure 2B](#). At least 2–3 brain sections are suggested for a total of 6–9 z-stacks per mouse.

△ CRITICAL: To ensure proper sampling, start imaging on one end of the area of interest working across the area to avoid imaging the same region more than once. Make sure that all image stacks are the same size across x, y, and z planes.

Alternatives: Live cells or *ex vivo* slices can be imaged using time-lapsed confocal microscopy. These files can be analyzed using the methods described below.

▮▮▮ Pause point: Once the images have been acquired, the data analysis can begin at any point. It is recommended to keep slides at 4°C for long-term storage.

Image preparation

⌚ **Timing:** ~15 min per image stack

Imaris 9.6 requires a 3 Ghz dual core processor with 8 GB of available ram and graphics support from a 2 GB or stronger graphics card. Depending on the microscopy software used, image stacks may need to be converted into a format that is supported by Imaris. This software is updated regularly and may support different file formats after this publication. Check the updated information to determine the supported image files and current system requirements for the latest version of Imaris: <https://imaris.oxinst.com/>

5. A sample Imaris image file is available in [Data S1](#).
6. Import image stacks into the Imaris software Arena.
 - a. Open Imaris and click the Image button to import an image, or an image folder into the arena. Images can also be dragged directly into the arena to be imported. There should be a status bar on the bottom right corner of the program to provide the status of the file import. Once completed, the images will appear in the arena.
7. Open the file of interest by double-clicking it in the arena. Make sure the image stack is in 3D view by clicking the "3D View" icon from the menu on the top left corner of the window. The sample image file is shown in 3D in [Figures 2B–2F](#). The image can be rotated using the "Navigate" tool ([Figure 2C](#), white arrow 1).
8. Adjust the display of the channels using the "Display Adjustment" window ([Figure 2C](#), white arrow 2). If this window is not appearing, click "Edit" then select "Show Display Adjustment" to show the window. You may also wish to adjust the colors of the image, which is particularly important for colorblind accessibility. This can be done by clicking "Edit" then "Image Properties..." and adjusting the Channels individually for their color.
9. If necessary, images may be processed for noise using the smoothing functions under the "Image Processing" tab at the top of the window.

Note: Use the same image processing methods across all images within a dataset to be consistent. To move around the 3D image, click the "Navigate" circle under the "Pointer" window on the right side of the screen ([Figure 2C](#), white arrow 1). When the pointer is set to navigate, the image can be moved around. Alternatively, the pointer selection can be toggled by pressing the "Esc" key on the keyboard. The "Select" option for the pointer will be used later. It allows objects to be selected instead of moving the 3D image around.

⏸ Pause point: This process can be saved at any time. It is recommended to prepare each image prior to analyzing it, rather than preparing all images and analyzing them afterward. To save the processing, click "File" then select "Store" or "Store As" to save the changes in the Imaris arena.

3D cell surface rendering of microglia

⌚ Timing: ~1 h per image stack

This step will create a surface render, or a mask over each channel corresponding to the different cell types. Microglia in the FITC channel will have a 3D render corresponding to their surfaces, and OPCs in the TRITC channel will have a separate 3D render corresponding to their surfaces. This process is demonstrated in [Methods video S1](#).

10. Create a surface render for microglia in the FITC channel.
 - a. Deselect all channels except FITC in the "Display Adjustment" window to have a clear view of the microglia ([Figure 2C](#), white arrow 2). Adjust the channel as necessary to see a clear boundary of the cell without overexposure.
 - b. Within the "Scene" window on the left side of the screen, click the "Add New Surfaces" icon on the top of the window which appears as a blue 3D bulbous shape ([Figure 2C](#), white arrow 3).

- c. After clicking “Add New Surfaces,” a new line will appear within the “Scene” folder named “Surfaces 1” which can be renamed by double-clicking. Rename it to “Microglia” for better organization.
 - d. With the microglia surface selected, navigate to the menu below with the tab “Create” selected by default. This menu will allow the user to specify parameters for the channel to create a surface render that best overlaps the cells. The parameters can be saved as “Favorites” which will appear in the dropdown menu under “Default.” It is recommended to determine the best parameters before creating a favorite setting, so do not change the “Default” selection initially. For this analysis, ensure that the “Segment a Region of Interest” button is not selected, which should be the default setting. Click the blue arrow button on the bottom to advance to the next step of creating the surface render.
 - e. The next section of this process is labeled “Source Channel” which allows the user to select the channel for surface rendering. For this step, select the FITC channel to create a surface render on microglia. The smoothing should also be adjusted to determine the amount of detail used in the rendering. For microglia, use 0.1 μm smoothing (Figure 2D), which will create a surface render that captures the microglial processes as previously described (Schafer et al., 2012). Test this setting on a few image stacks and determine the best setting to use on all image stacks for the study to remain consistent. Select “Absolute Thresholding” for this protocol. Click the blue arrow to advance to the next step.
 - f. Threshold the image using the histogram by dragging the setting until the image accurately represents the surface of the cells. For this protocol, do not enable the “Split Touching Objects” option. With the “Navigation” pointer selected, check the thresholding by zooming in on the cells and rotating them around to ensure the boundary is accurate. Examples are given for under-representative, representative and over-representative thresholding (Figures 3A–3C, respectively). Thresholding an image will depend on the exposure and brightness of the fluorescence. It is recommended to process all tissue samples at the same time and use the same microscope acquisition settings while acquiring image stacks. Keeping these settings consistent will allow for the same threshold settings to be used on all samples within an experiment. Threshold settings should be determined in a small representative pilot sample and used consistently throughout the experimental image stacks. Thresholding should cover 100% of the cell boundary. Data should always be analyzed in a blinded and unbiased manner. To return to the default view of the image, click the “Reset” button on the bottom right corner of the screen. Once satisfied with the thresholding, click the blue arrow to advance to the next step.
 - g. The next step allows additional filtering of the surface render. For this protocol, leave the default filter for “Number of Voxels” set to 10 which will remove any small particles. This option may be optimized for different studies, but should be kept consistent for all image stacks within a study. Click the green double arrow button to complete the surface render for microglia. There will now be a green 3D surface render on top of the cells (Figure 2D). This surface render can be turned off at any time by clicking the checkbox for “Microglia” under the “Scene” window.
11. Since microglia tend to cluster in tissue, it is important to manually edit the 3D surface render to accurately represent individual cells, using bisbenzimidazole staining of nuclei to determine if objects are cellular. This becomes important for analysis metrics such as determining the number of microglia and their average volume.
 - a. To begin, select the microglia surface render in the “Scene” window. To view the surface render objects individually, click the “Color” button within the bottom menu as shown by a rainbow hexagon. Change the “Color Type” to “Object ID” to view objects in separate colors. The final edited surface render should show each individual cell as a different color, and objects which are not cells (as determined by no nuclear staining) should be deleted. To do this, some objects may need to be merged together in the instance that a cell has been separated in the surface render. This can be done using the “Unify” feature. Other cells may be clustered within one object and need to be separated, which can be done with the

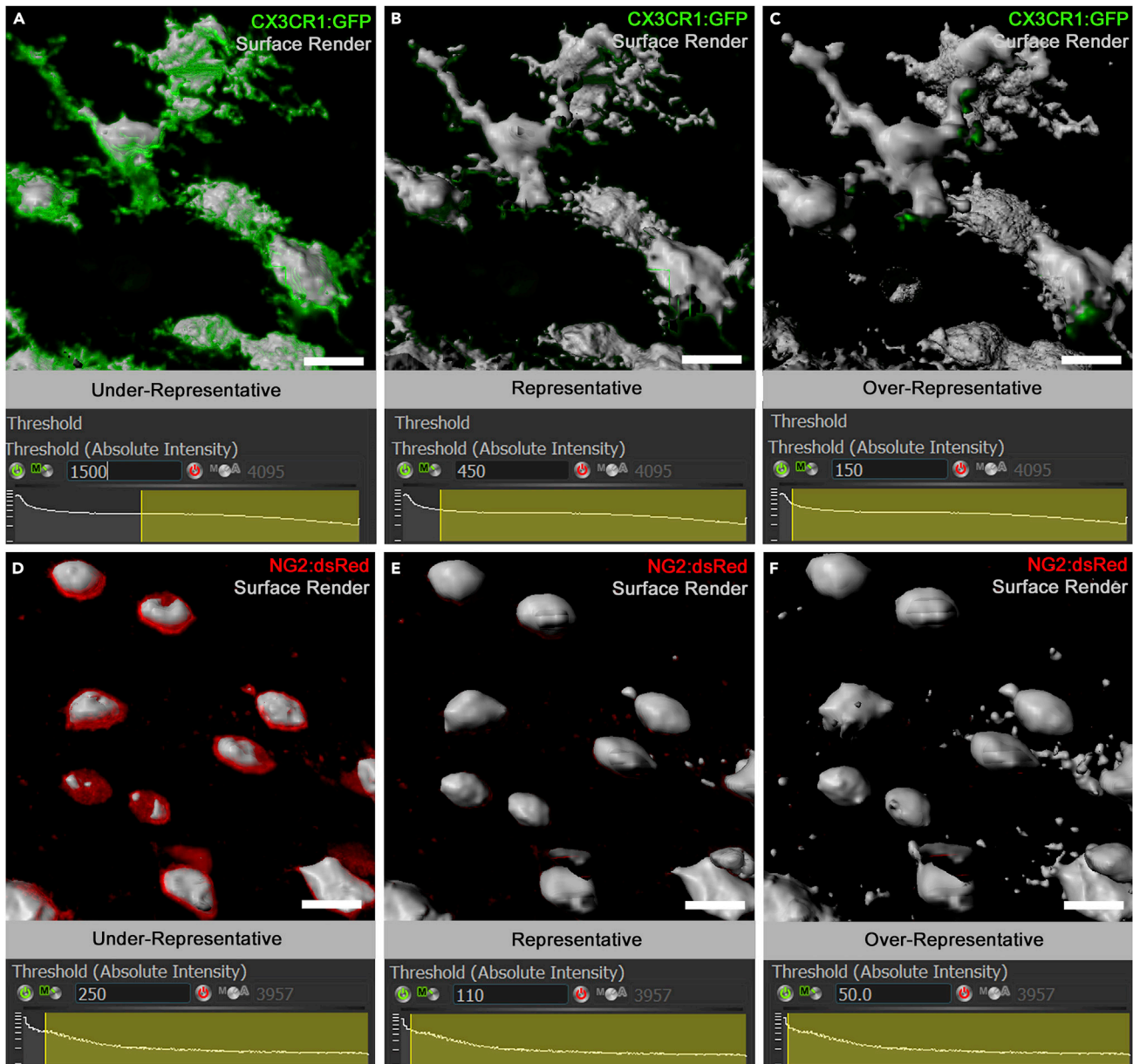


Figure 3. Creating a surface render that accurately represents the cell boundaries

Thresholding images is extremely important to creating an accurate surface render that overlaps 100% of cell boundaries without picking up background noise. These settings are dependent on exposure during acquisition and brightness settings in Imaris. Be sure that all images analyzed are not over- or under-exposed which would result in incorrect surface renders.

(A–C) Examples of thresholding microglia by absolute intensity with an example of an (A) under-representative threshold setting, (B) a representative threshold setting and (C) an over-representative threshold setting.

(D–F) Examples of thresholding OPCs by absolute intensity with an example of an (D) under-representative threshold setting, (E) a representative threshold setting and (F) an over-representative threshold setting. Threshold setting values required to create a representative surface render will vary based on raw fluorescence intensities. It is important to be consistent with image acquisition and processing to ensure low variability of fluorescent intensities between image stacks. Scale bars, 10 μ m.

“Split” feature. To begin editing the surface, select the “Edit” button shown as a pencil icon to open the editing menu.

- b. To edit, it is necessary to move the image around using the “Navigate” feature of the pointer, but also select individual objects using the “Select” feature of the pointer. This can be done

by clicking the buttons to switch between each function of the pointer, or by pressing the “Esc” key to toggle between the two functions. To zoom in and out of an area, scroll the mouse while the pointer is set to “Navigate.” The selection box size can also be changed by scrolling the mouse while the pointer is set to “Select.” Moving from one side of the image to the other in an organized way to review all cells, begin navigating through to merge or separate objects. To move to a new field within the image, right click and drag the image while in “Navigate” mode. It may be helpful to continuously turn the surface render on and off to view the raw image by clicking the checkbox next to the “Microglia” surface within the “Scene” window. Additionally, it is recommended to use a nuclear stain such as bisbenzimidazole in the DAPI channel to identify cells. Turning the channels on and off within the “Display Adjustment” window is helpful for this.

- c. To merge two or more objects, select the first object by clicking on it while the pointer is in “Select” mode, then hold the “Ctrl” key and click any additional objects that need to be merged together. The objects should turn yellow as they are selected. Once all areas needed to be merged are selected and appear yellow, click the “Unify” button under the “Editing” window. The objects should all turn the same color indicating that they are now one object.
- d. To separate two objects, be sure that the “Edit” menu is selected first (this feature will only work in edit mode). Using the nuclear stain to identify individual cells, determine where the object needs to be cut. One limitation of this software is that it can only cut vertical lines. Therefore, using the “Navigate” feature of the pointer, move the object around to orient it in a way that allows the object to be cut in the correct location using a vertical line. Switch the pointer to “Select” mode once the object is in the correct position for the cut and hold down the “Shift” key, then click the object. A line should appear to indicate where it will be cut. If this line is not appropriate, click the “Clear Scissor” button on the editing tab. Switching back to “Navigate” with the pointer, move the object to the correct position and create a new cut in the Select mode of the pointer, holding the “Shift” key and clicking the correct area. Once the line appears in the correct location for the cut, click the “Cut Surface” button under the editing tab. The two objects should turn different colors to indicate that they are cut and now separate objects. In some situations, you may need to cut objects and re-unify them, or unify objects before cutting them to get the correct orientations. At the end, be sure that each cell is labeled a single unique color. Doing this precisely is essential for accurate results.
- e. It may also be necessary to select individual objects that are artifacts and delete them. To do this, be sure that the pointer is set to “Select” and click on the object, then click the “Delete” button under the edit menu for the surface render. It may be helpful to set parameters for objects that should be excluded by volume or sphericity based on the dataset. To check parameters for an individual object before deleting it, click the “Statistics” button shown as a data chart within the bottom menu, followed by selecting “Detailed” to use the drop-down menu to select various parameters such as volume or sphericity. In some cases, it may be necessary to delete all objects below $10 \mu\text{m}^3$ for example, which may be easier to do at the beginning of the editing process if it is not removing any important regions. However, it is recommended to do this again at the end of the editing process if any objects were cut, as they often leave small fragments behind as separate objects. This depends on the images and the amount of artifact which will need to be optimized for the dataset. To do this, sort all of the objects by volume from smallest to largest, click the largest object within the specified range (such as $10 \mu\text{m}^3$) then hold the “Shift” key and click the smallest object within the list. All objects within this volume should be selected (as shown by the yellow color) and can be deleted by clicking the “Edit” tab again and the “Delete” button. All objects that have less than 50% of their cell body within the field or lack a nucleus should also be excluded.

Pause point: The file can be stored at any time to save the edited surface render. The next step is to create a 3D surface render of the OPCs, which are the same basic steps as listed above for microglia.

3D cell surface rendering of OPCs

⌚ Timing: ~1 h per image stack

The steps for creating a surface render for OPCs are almost the same as the steps above for microglia. OPCs in the TRITC channel will now have their own 3D render corresponding to their surfaces. This process is demonstrated in [Methods video S1](#).

12. Create a surface render for OPCs in the TRITC channel.
 - a. To start, deselect the surface render of microglia under the “Scene” window. Also deselect the FITC channel from appearing with the “Display Adjustment” window. Select the TRITC channel instead to show the OPCs.
 - b. Create the surface render by clicking the “Add New Surfaces” icon within the “Scene” window on the left side of the screen which appears as a blue 3D bulbous shape ([Figure 2C](#), white arrow 3).
 - c. After clicking “Add New Surfaces,” a new line will appear within the “Scene” folder named “Surfaces 2” which can be renamed by double-clicking. Rename it to “OPCs” for better organization.
 - d. With the “OPCs” surface selected, navigate to the menu below with the tab “Create” selected by default. Again, leave the “Default” selection in the dropdown menu and make sure that the “Segment a Region of Interest” button is not selected. Click the blue arrow button on the bottom to advance to the next step of creating the surface render.
 - e. In the “Source Channel” step, select the TRITC channel to create a surface render on OPCs. The smoothing setting for OPCs should be 0.4 μm smoothing ([Figure 2E](#)), which corresponds to the cell boundary based on the spherical OPC morphology as we have previously described ([Nemes-Baran et al., 2020](#)). Be sure to use the same settings on all image stacks for the study to remain consistent. Select “Absolute Thresholding” for this protocol. Click the blue arrow to advance to the next step.
 - f. Using the histogram as before, threshold the image until the gray surface accurately represents the surface of the cells. Examples are given for under-representative, representative and over-representative thresholding ([Figures 3D–3F](#), respectively). For this protocol, do not enable the “Split Touching Objects” option. Once satisfied with the thresholding, click the blue arrow to advance to the next step.
 - g. Apply the same filter as before by leaving the default filter for “Number of Voxels” set to 10 which will remove any small particles. This option may be optimized for different studies, but should be kept consistent for all image stacks within a study. Click the green double arrow button to complete the surface render for OPCs. There will now be a red 3D surface render on top of the cells ([Figure 2E](#)). This surface render can be turned off at any time by clicking the checkbox for OPCs under the “Scene” window. To view a merge of both microglia and OPC surface renders, check the “Microglia” surface render under the “Scene” window ([Figures 2F](#) and [4A](#), white box).
13. OPCs should be more spaced out than microglia, but it’s still necessary to manually edit the 3D surface render to accurately represent individual cells. This is especially important for analysis of engulfment, as the equation uses the OPC volume to determine the level of contact. Take the time necessary to ensure all cells are individual objects that colocalize with bisbenzimidazole, a marker of cell nuclei. As we have previously described ([Nemes-Baran et al., 2020](#)), OPC surface rendering also filters out any surfaces with a volume less than $10 \mu\text{m}^3$ and sphericity value lower than 0.49 to exclude pericytes on blood vessels that express the NG2 marker. These criteria were determined based on the size of pericytes on blood vessels as reported ([Peppiatt et al., 2006](#)).
 - a. Be sure that the “OPCs” surface render is selected in the “Scene” window. Click the “Color” button within the menu as shown by a rainbow hexagon. Change the “Color Type” to “Object ID” to view objects in separate colors.

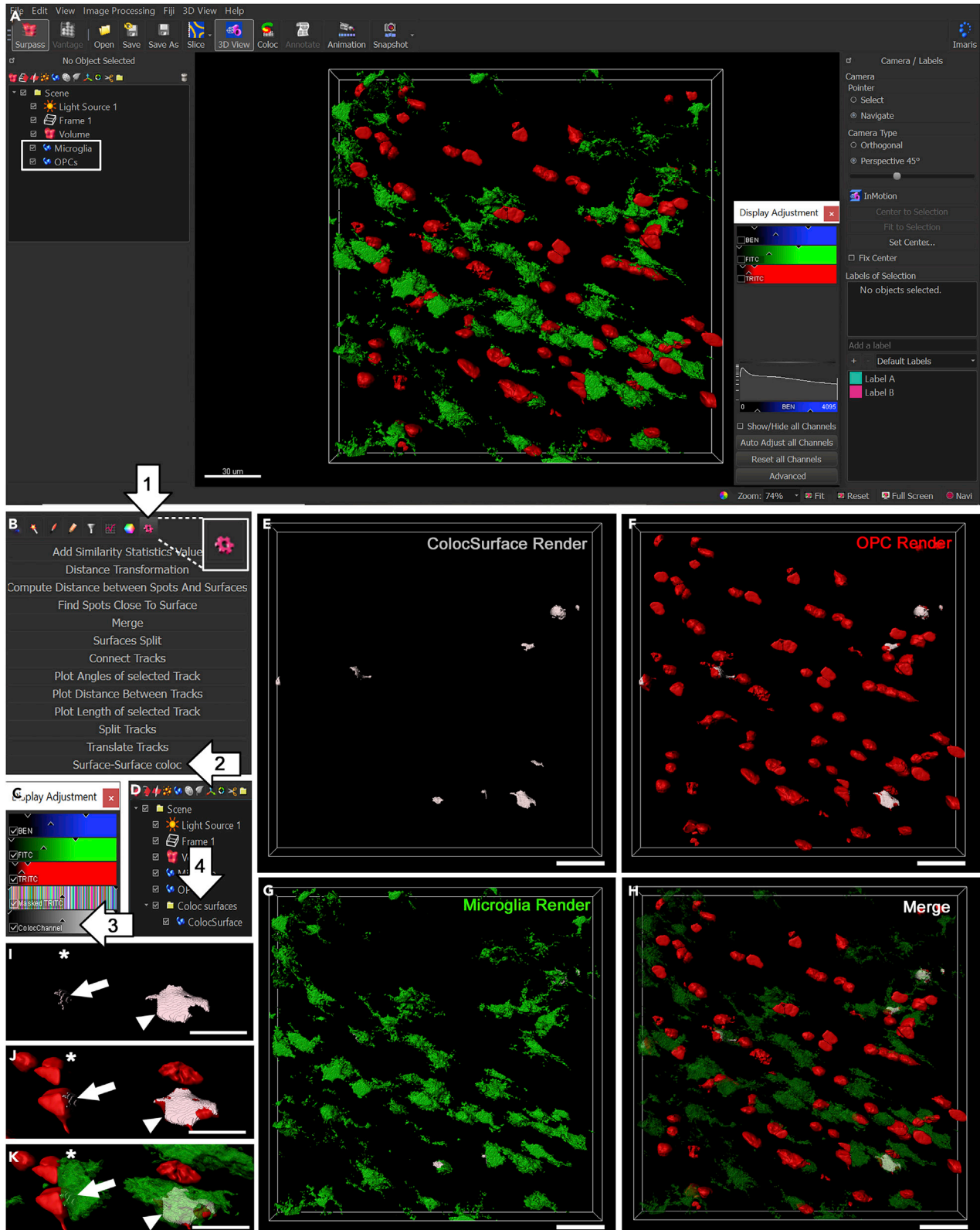


Figure 4. Identifying internalized surfaces by microglia on OPCs using 3D surface renders

(A) Screen view of Imaris software containing the sample image (file provided with this manuscript) with surface renders for microglia (green) and OPCs (red). Surface renders appear in the “Scene” menu as shown by the box on the left side of this image, and each surface can be turned on or off by selecting the checkboxes next to each render. Scale bar, 30 μm .

(B) After surface renders have been created over microglia and OPCs, the internalized surface of OPCs within microglia can be identified with a ColocSurface render. Follow the steps in the protocol to create a Random Color Map on the TRITC channel first. While the OPC render is selected in the “Scene” menu as described in A, click the “Tools” icon in the lower window of the left side of the screen (white arrow 1; shown as a gear, enlarged in this figure). Select “Surface-Surface coloc” (white arrow 2) and follow the steps in the protocol to create a new surface identifying areas on OPCs that are internalized by microglia.

(C) Once a new surface has been created, a new channel will appear (white arrow 3) under the “Display Adjustment” window. Before the ColocSurface render is created, a “Masked TRITC” channel should be generated, as described in the protocol.

(D) Within the “Scene” menu, a new surface (white arrow 4) will appear corresponding to the ColocSurface render. It can be turned on or off using the checkbox.

(E–K) (E) ColocSurface Render, (F) OPC Render, (G) Microglia Render, and (H) Merge from the sample image file that is provided with this manuscript. Scale bars, 30 μm . Region of the sample file showing (I) SurfaceColoc on (J) OPCs not interacting with microglia (asterisk), being contacted by microglia (arrow) and engulfed by microglia (arrowhead) with (K) microglia overlay. See [Methods video S1](#) for this process. Scale bars, 10 μm . Note: the microglia render transparency was reduced in H and K to show internalized regions (gray) on OPCs. This can be done by clicking on the microglia render, selecting the “Colors” tab from the options window on the lower left side of the screen, clicking “Base” for the color type and adjusting the “Transp.” percentage under the RGB tab.

- b. Working from one side to the other as before, begin navigating through the image to merge or separate objects.
- c. As described above, to merge two or more objects, select the first object by clicking on it while the pointer is in “Select” mode, then hold the “Ctrl” key and click any additional objects that need to be merged together. The objects should turn yellow as they are selected. Once all areas needed to be merged are selected and appear yellow, click the “Unify” button under the Editing window. The objects should all turn the same color indicating that they are now one object.
- d. To separate two objects, be sure that the “Edit” menu is selected and determine where the object needs to be cut. Move the object around to orient it in a way that allows the object to be cut in the correct location using a vertical line. Switch the pointer to “Select” mode once the object is in the correct position for the cut and hold down the “Shift” key, then click the object. A line should appear to indicate where it will be cut. If this line is not appropriate, click the “Clear Scissor” button on the editing tab. Switching back to “Navigate” with the pointer, move the object to the correct position and create a new cut in the “Select” mode of the pointer, holding the “Shift” key and clicking the correct area. Once the line appears in the correct location for the cut, click the “Cut Surface” button under the editing tab. The two objects should turn different colors to indicate that they are cut and now separate objects. At the end, be sure that all cells are labeled a different color to indicate that they are each a separate object. Each individual cell should only be one color, indicating that they are single objects. Doing this precisely is essential for accurate results.
- e. If using NG2 as a marker of OPCs such as with the NG2:dsRed transgenic mouse or immunostaining for NG2, blood vessels will need to be excluded from the surface rendering as pericytes also express NG2. Since OPCs are spherical at this stage in development, pericytes and blood vessels can be excluded by volume and sphericity parameters. To exclude them, click the “Statistics” button shown as a data chart within the bottom menu, followed by selecting “Detailed” to use the dropdown menu to select volume. Sort all of the objects by volume from smallest to largest, click the object closest to 10 μm^3 then hold the “Shift” key and click the smallest object within the list to select all objects 10 μm^3 or less. All objects within this volume should be selected (as shown by the yellow color) and can be deleted by clicking the “Edit” tab again and the “Delete” button. Next, sort the objects in the same way by sphericity, which is another option available within the dropdown menu of “Statistics” under “Detailed” view. Sort the objects by sphericity from smallest to largest. Select all objects with a sphericity less than 0.49 and delete them. This should exclude blood vessels and pericytes from the sample, but additional objects may be excluded by the lack of a nucleus as

shown by nuclear markers in the DAPI channel. Objects that have less than 50% of their cell body within the field or lack a nucleus should also be excluded.

Pause point: The file can be stored at any time to save the edited surface renders. Next, a new surface render will be created to identify any OPC surfaces that are internalized within microglia using the Surface-Surface Coloc XTension. Contact Bitplane for support with your Imaris software to ensure that this XTension is installed.

Identify internalized surfaces

Timing: ~15 min per image stack

Now that there are 3D surface renders for microglia and OPCs (Figure 4A), Imaris can determine what volume is internalized using the Surface-Surface Coloc XTension. This will create a new 3D surface render of the internalized material with information available on volume that can be used to identify OPCs that are being engulfed ($\geq 15\%$ of the cell is internalized) or just contacted ($< 15\%$ of the cell is internalized). These categories were based on our previously published observations, showing microglia contacting OPCs but not engulfing OPCs (Nemes-Baran et al., 2020). Therefore by testing analyzed data with other values (10%, 20%, 25%), it was determined that 15% accurately categorizes cell engulfment with no false positives or negatives observed consistently. While analyzing the data, spot-checking can be conducted to ensure that each cell is being appropriately categorized. It is important to remove particles and objects that fit the exclusion criteria described to avoid false categorizations. This process is demonstrated in [Methods video S1](#).

14. Create a Random Color Map on the TRITC channel (or whichever channel is being engulfed) to determine the internalization of this channel.
 - a. With the OPCs surface render selected (or whichever surface is being engulfed), click the "Edit" tab as shown by a pencil icon. Click the "Mask All..." button. Choose the TRITC channel (or whichever channel is being engulfed) and click "Random Color Map." Make sure the "Duplicate channel before applying mask" checkbox is selected. Click "OK" and a new channel should appear in the "Display Adjustment" window that says "Masked TRITC" with a rainbow color.
15. Create a new surface render of the internalized material within microglia.
 - a. Click the "Tools" tab shown by a gear icon within the OPCs surface render menu (Figure 4B, white arrow 1).
 - b. Click the "Surface-Surface coloc" link on the very bottom of the list (Figure 4B, white arrow 2).

Note: If this option is grayed out, check that the MATLAB Runtime MCR software is installed. Click "File," then "Preferences," select "CustomTools." If there is an orange link to download a different MATLAB Runtime MCR software, try downloading it to see if the Surface-Surface Coloc option appears. If it is still not working, contact Bitplane Support for assistance in activating this XTension within the Imaris software installed on the device.

- c. A dialog box will appear. Select the two surfaces to colocalize (Microglia and OPCs) and click continue.
- d. Another dialog box will appear inquiring about using a smoothing feature. It is recommended not to use this smoothing feature for this protocol.
- e. A new channel will appear called "ColocChannel" in a gray gradient color within the "Display Adjustment" window (Figure 4C, white arrow 3).
- f. New objects will also appear which represent the internalized area of OPCs within microglia. They will have their own 3D surface render within the "Scene" window called "ColocSurface" (Figure 4D, white arrow 4). To view individual surface renders, click the checkbox to select each render to view individually (Figures 4E–4G) or merged together (Figure 4H). Viewing

surfaces separately and together can identify regions on the OPCs that have a large volume internalized (Figures 4I–4K).

▮▮ **Pause point:** The file can be stored at any time to save the edited surface renders. One option is to create surface renders on all of the files first, then move on to the data analysis.

Quantification of engulfment

⌚ **Timing:** ~1 h per image stack

After all cells have 3D Surface Renders and the internalized portion of OPCs has its own 3D Surface Render, the data can be analyzed for engulfment. The ColocSurface render represents the voxels labeled in both the OPC and microglia surface volumes that come in contact with each other. This step will use the volume from the ColocSurface render divided by the volume from the OPCs to determine the overall amount of internalization for that cell. This process is demonstrated in [Methods video S2](#).

16. First, obtain the volumes for all internalized surfaces.
 - a. With the ColocSurface render selected, click the Statistics tab as shown by a chart icon ([Figure 5A](#), white box), then click “Detailed” view.
 - b. Sort the objects by volume by selecting Volume in the dropdown menu and sort smallest to largest by clicking “Volume” in the table of data.
 - c. Delete any surface that is less than $1 \mu\text{m}^3$ to avoid any particles that may be present.
 - d. Export the data by clicking the “Export Statistics on Tab Display to File” button that is shown as a single blue disc icon. The data will be saved as a .csv file, and can be opened in Excel and resaved as an Excel Workbook (.xlsx) format at any time.

Note: If the file name is long, has non-alphabetical characters, or the path is within multiple folders, it may not save properly and an error message will appear as “Could not write to file.” In this situation, try to rename the file something simpler or save it to a location with a shorter path.

17. To quantify engulfment, calculate percent internalization by dividing the internalized surface volume (ColocSurface) by the total volume of the OPC ([Figure 5B](#)). Cells can be categorized by the percent internalization, with cells only contacted by microglia having a percent internalization $< 15\%$ ([Figure 5C](#)) and cells that are engulfed by microglia having a percent internalization $\geq 15\%$ ([Figure 5D](#)). To do this in Excel, set up new columns within the Excel sheet to manually enter the volume for the OPC that the ColocSurface corresponds with. Then, set a new column to divide the internalized volume by the total OPC volume to calculate percent internalization. A template Excel sheet ([Data S2](#)) is available with these labels and the calculations described in this protocol. If using the sample image file, export the data then copy and paste it into the provided template.
 - a. Within the exported data file, create a new section of columns to input parameters for the OPC that corresponds to the ColocSurface volume. New columns should include corresponding OPC ID, volume and percent internalization ([Figure 5E](#)).
 - b. Within the Imaris software, organize the ColocSurface render volume data by ID from smallest to largest. This should match the data organization within the data file.
 - c. Click the first object of the ColocSurface render. Its volume should match the volume in the data file for that ID.
 - d. Turn the surface render on for the OPCs to view the cell that this volume corresponds with. Click the OPC to determine its volume and ID. Enter the information within the data sheet to correspond with the appropriate ColocSurface ID.

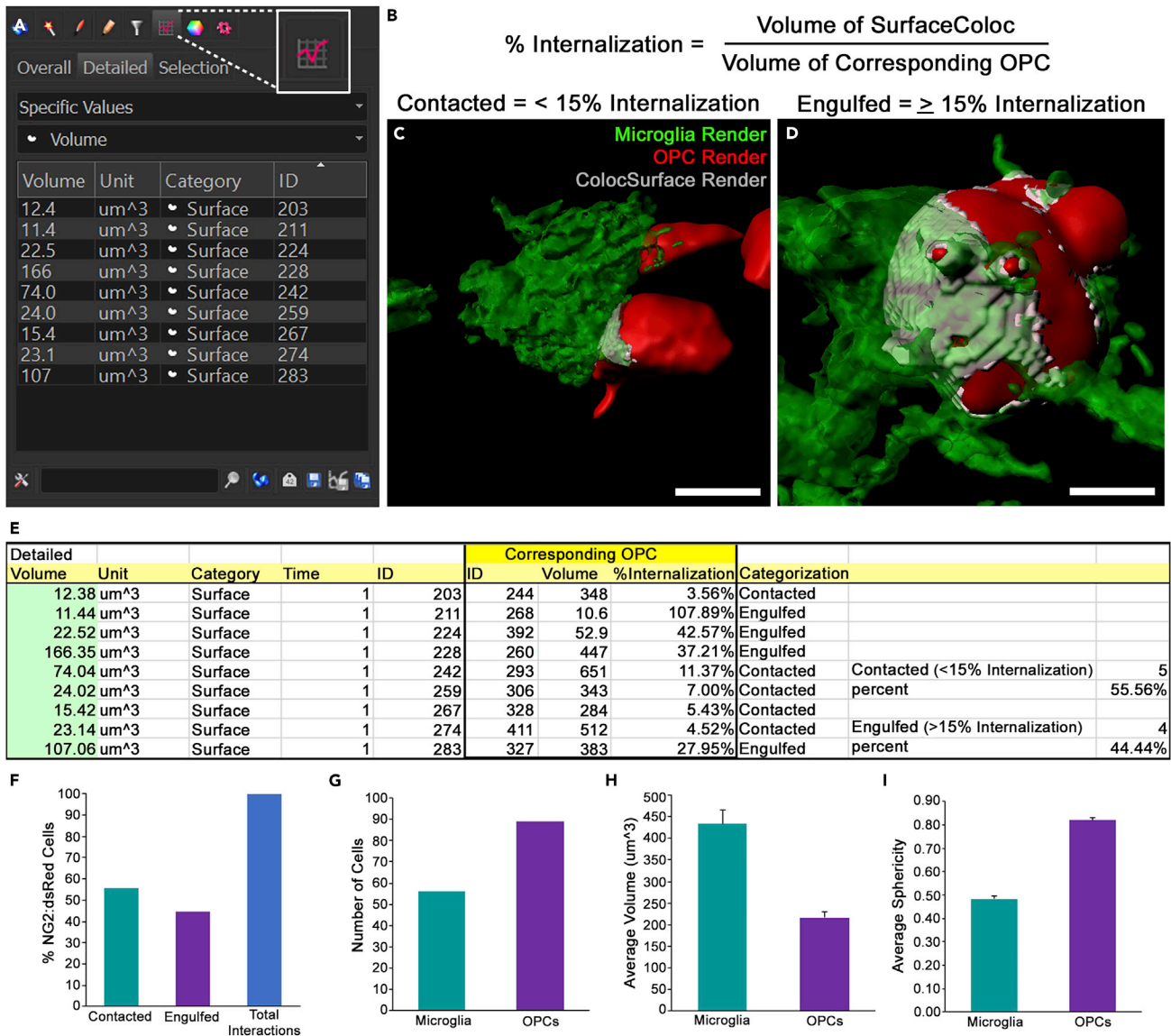


Figure 5. Quantifying microglial engulfment of OPCs

(A) Select the ColocSurface render in the “Scene” menu and click the “Statistics” tab (denoted by a graph icon, enlarged in the image) in the window of the bottom left corner of the screen. Click “Detailed” and select “Volume” from the second drop-down menu to display the volumes of each ColocSurface render. These values can be exported by clicking “Export Statistics on Tab Display to File” (save icon) on the bottom of the window. This process is demonstrated in [Methods video S2](#).

(B) To calculate the OPC internalization within microglia, use the calculation shown to determine percent internalization.

(C) Cells that are only contacted by microglia are categorized as < 15% internalization as shown by the example image.

(D) Cells that are engulfed by microglia are categorized as ≥ 15% internalization as shown by the example image. Scale bars, 5 μm.

(E) A template with these calculations has been provided with this manuscript. Follow the instructions within the protocol and demonstrated in [Methods video S2](#) to calculate percent internalization for each ColocSurface/OPC.

(F) Bar graph showing the percentage of interactions within the sample image file, including contacted (55.56%), engulfed (44.44%), and total interactions (100%).

(G) The number of total microglia (56) and OPCs (89) within the sample image file. This number may vary depending on how the objects were unified, cut, and removed and may slightly differ from these results.

(H) The average volume of all microglia (434.06 ± 30.93) and OPCs (216.68 ± 13.12) within the sample image file.

(I) The average sphericity of all microglia (0.48 ± 0.01) and OPCs (0.82 ± 0.01) within the sample image file. Error bars represent ± SEM. Note: these data can also be represented as normalized to wild-type conditions or by area of the field.

- e. Calculate percent internalization by dividing the ColocSurface volume by the OPC volume. Set the format of the percent internalization column to percentage by right-clicking the column, then "Format Cells..." and select "Percentage," then click "OK." This will now show the percentage of engulfment for this particular OPC.
- f. Continue through each ColocSurface volume and match it with the appropriate OPC surface render. Enter the data as directed in the above steps until the data sheet is complete.
- g. Cellular interactions can now be categorized by OPCs that are contacted by microglia (cells with < 15% internalization) or OPCs that are engulfed by microglia (\geq 15% internalization). These parameters may be altered for different applications.

△ CRITICAL: Be sure to carefully enter the information for the corresponding OPC, as the volume of the internalized material divided by the total volume of the OPC is what determines the percent internalization. If the wrong OPC is selected for an individual ColocSurface volume, the data can be drastically skewed.

Note: In some cases when a cell is completely encapsulated, the percent internalization can be slightly greater than 100% as shown in [Methods video S2](#). This will only occur with complete cell engulfment from slightly overlapping surfaces due to the different smoothing settings used for each cell (0.1 μm for microglia and 0.4 μm for OPCs).

EXPECTED OUTCOMES

Microglial engulfment of OPCs

The role of microglia in the developing nervous system is gaining appreciation in recent years. Recent work has demonstrated their importance in synaptic pruning and engulfment of neural precursor cells ([Schafer et al., 2012](#), [Paolicelli et al., 2011](#), [Cunningham et al., 2013](#)). Interestingly, these processes coincide with the timeline of oligodendrogenesis. We have shown that during a short window of early postnatal development (peak at P7), amoeboid microglia infiltrate the corpus callosum and engulf and phagocytose viable, pre-myelinating OPCs ([Nemes-Baran et al., 2020](#)). However, observation of this process is limited in the 2D plane, and timelapse microscopy studies show that microglia may contact cells without engulfing them. Therefore, it is imperative to analyze whole-cell engulfment using 3D reconstruction methods and quantification to differentiate cell contact from engulfment. After completing the methods described in this protocol using the provided sample image file, the results should be similar to what is presented in [Figure 5](#). The number of ColocSurface Render objects should be 9, with 5 of them categorized as "Contacted" (< 15% internalization) and 4 of them categorized as "Engulfed" (\geq 15% internalization). The data can be presented in various ways, for example, calculate the percentage of NG2:dsRed cells that are contacted (55.56%) compared to engulfed (44.44%) ([Figure 5F](#)). More intricate data analyses can be done with more image files and animals (see note below). After analyzing the sample file, there should be approximately 56 microglia (as counted by number of surface render objects) and 89 OPCs within the field ([Figure 5G](#)). If the numbers are too high, there was likely an issue with thresholding, not unifying objects or excluding objects. If the numbers are too low, there was likely an issue with thresholding, not separating objects or excluding too many objects. See the [troubleshooting](#) section for details. Imaris also provides details on other metrics including object volume and sphericity, which can be useful in understanding differences between control and experimental groups. The sample file provided should result in an average volume near $434.06 \pm 30.93 \mu\text{m}^3$ for microglia and $216.68 \pm 13.12 \mu\text{m}^3$ for OPCs ([Figure 5H](#)). The sphericities of these objects should be around 0.48 ± 0.01 for microglia and 0.82 ± 0.01 for OPCs ([Figure 5I](#)). If the values obtained by analyzing the sample file are significantly different from what is reported here, see the [troubleshooting](#) section to determine the cause.

Note: The sample file provided was selected to offer limited need for filtering particles, pericytes, and blood vessels with less engulfed and contacted cells to facilitate ease of the

practice module. When analyzing raw data from a new study, there will likely be more variability between image stacks. Therefore, it may be best to average the data across all fields per animal and normalize the data by the size of the field of view. If comparing to an experimental group, it may also be useful to normalize the data to the control group.

QUANTIFICATION AND STATISTICAL ANALYSIS

Depending on the research questions, various statistical analyses may be conducted to analyze the data once they have been collected from multiple samples. It is recommended to average the data from 6–9 image stacks per animal and collect data from 3–5 animals per group before beginning to analyze. Once the percent internalization has been determined, the research question should be used to determine the statistical analyses. The data can be presented as incidence of cells contacted and engulfed which can be normalized to the control group or the size of the field. If comparing a control group to an experimental group, it is recommended to analyze the data using a two-tailed unpaired t test. If comparing data across multiple time points (more than 2), it is recommended to compare each time point using a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post hoc testing.

LIMITATIONS

The analyses described in this program cannot be performed without quality tissue and image stacks that have been acquired consistently to limit variability between samples. Additionally, it requires licensed software that must be purchased and access to these programs may be limited to some researchers. Given the short window of time that this process occurs within the corpus callosum, it is important that the brains are harvested at P7 to observe engulfment. Multiple litters are necessary to collect enough samples for meaningful results, and timing of birth must be tightly monitored to ensure the timepoints between litters are similar (within 12 h). Littermate controls should be used for all experiments. Microglia and OPCs should be co-labeled with markers to confirm their identities (Nemes-Baran et al., 2020). Given the reactive nature of microglia, environmental factors may contribute to changes in these results, and researchers should take care in controlling for these confounding factors. By examining microglial morphology subjectively and by objective measures such as volume and sphericity, animals can be compared. Animals with abnormal microglial morphologies should be excluded. Lastly, tissue can be evaluated for the presence of peripheral immune cells.

TROUBLESHOOTING

Problem 1

The signal-to-noise ratio is poor in the sections when viewing under the microscope (step 3).

Potential solution

If using brain sections from transgenic reporter mice, be sure the tissue was prepared according to the protocol and stored properly. Issues with the transcardiac perfusion and post-fixation process can result in poor tissue quality. Ensure that brains were properly perfused and have a whitish appearance and are firm to the touch. If tissue quality is poor, new brains may be necessary with proper fixation procedures. Brain sections that have been stored for greater than 1 year may not appear as bright as fresh sections. Be sure to collect all tissue and acquire image stacks within the same time period for all animals in the study. Otherwise, store embedded brains in the -80°C freezer until all samples have been collected, then begin sectioning and acquiring images as soon as possible to maintain tissue integrity and fluorescence intensity. Also be sure that the laser power on the microscope is turned high enough to see the fluorescent signal without photobleaching it.

If using brain sections that were stained, ensure the staining protocol was followed closely and the brain sections have been properly stored. Brain sections that have been stored for greater than 1

year may not stain as well as fresh sections. Always stain tissue using a positive control (an antibody that has worked well before) and a negative control (tissue stained without the primary antibody).

Before beginning an experiment, always optimize antibody staining using tissue samples that are not needed for the study. Start by testing the antibody with a concentration that has been recommended by the manufacturer or within the literature. Use a higher and lower concentration to determine the concentration necessary for optimal signal to noise ratio (for example, 1:100, 1:250 and 1:500 dilutions). Always stain tissue using antibodies individually before co-labeling together. When co-labeling tissue with two separate antibodies, be sure to use primary antibodies from different host species and secondary antibodies that are conjugated to different fluorophores.

Depending on the antibody, it may require antigen retrieval processing or a shorter post-fixation duration to achieve a high signal to noise ratio. If the signal is still low, consider using an amplification process such as using streptavidin or tyramide signal amplification kits (Fisher Scientific, Hampton, NH; 85111). Reduce background by blocking tissue from nonspecific binding as directed and using the lowest concentrations of antibodies needed to produce a strong signal. Some antigens may also be best stained using free-floating sections instead of slide-mounted sections, and others may be sensitive to long-term storage. Therefore, it is best to stain tissue as soon as possible once all brains have been collected for a study in order to optimize image quality.

Problem 2

There are much fewer objects than presented in the Expected Outcomes section (steps 11e and 13e).

Potential solution

The surface renders may be misrepresentative of cells with too low of a threshold (Figure 3). This may also result in less ColocSurface objects. Alternatively, cells may be clustered together creating one large object instead of several smaller objects. Refer to steps 11 and 13 to ensure that the objects were carefully edited to represent the cells. Use bisbenzimidazole-stained nuclei to determine how many cells are within a cluster. Cut objects that represent multiple cells clustered together. Be aware that one limitation of Imaris software is that the cut tool only makes vertical cuts, and several may be required to separate the cells. The unify tool may also be needed to create the appropriate surface render. Having cells that are clustered be represented by 1 object may also inflate the volume and number for the image, so it's important to ensure that the editing of surface renders was completed with care.

Problem 3

There are much more objects than presented in the Expected Outcomes section (steps 11e and 13e).

Potential solution

Refer to steps 11 and 13 to ensure that the objects were carefully edited to represent the cells. Use bisbenzimidazole-stained nuclei to determine how many cells are within a cluster. Unify objects that are actually part of the same cell. Be sure to follow the protocol to remove all objects that fit the exclusion criteria. For microglia: delete all objects below $10 \mu\text{m}^3$, have less than 50% of their cell body within the field or lack a nucleus. For OPCs: delete all objects below $10 \mu\text{m}^3$, have less than 50% of their cell body within the field, lack a nucleus, or have a sphericity less than 0.49 to exclude blood vessels and pericytes. When using the cut tool to edit, small particles are usually created. Be sure to check these parameters at the end of editing surface renders to ensure no objects remain that should be excluded. Lastly, ensure the thresholding step was not done with overexposure. The threshold should represent the cells in the image. Too high of a threshold with an overrepresentation of the space where a cell is located may also result in artificial ColocSurface objects.

Problem 4

Surface-Surface Coloc button is grayed out, or is not operating when clicked (step 15b).

Potential solution

This suggests that there is an issue with the installation of Imaris software, MATLAB Runtime MCR or the Surface-Surface Coloc XTension. Contact Bitplane for support.

Problem 5

Surface-Surface Coloc renders are not appearing on OPCs (step 15f).

Potential solution

If the software is installed correctly, be sure that the OPCs have been masked first with a Random Color Map as described in step 14. When creating a Random Color Map, be sure that the correct surface render (OPCs) is selected. Also be sure to select the “Duplicate channel before applying mask” checkbox. A new channel should appear when this has been completed titled “Masked TRITC”. See [Methods video S1](#) for a demonstration of this process. If this does not fix the issue, there may be a problem with the installation of the software, contact Bitplane for support.

Problem 6

The percent internalization calculation is very different from what is observed subjectively (step 17e).

Potential solution

Check the formula used for this calculation to ensure that the correct SurfaceColoc volume is being divided by the correct OPC volume. Also check that this OPC volume is correct for the cell that this SurfaceColoc render is associated with. If the incorrect volumes are used in this equation, the percent internalization will be inaccurate.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tara M. DeSilva, PhD (desilvt@ccf.org).

Materials availability

All mouse lines used for this protocol are commercially available through Jackson Laboratory.

Data and code availability

This publication includes practice datasets as a tutorial for the purposes of learning the described protocol.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.100403>.

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AUTHOR CONTRIBUTIONS

T.M.D. and A.D.N. conceived the study and designed the experiments. A.D.N. completed the engulfment analyses. T.M.D. and A.D.N. drafted the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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