# STAR Protocols

**Protocol**

# Histological quantification of cerebral cavernous malformations in the murine brain



Mouse perfusion and tissue collection
3 hours  Step 1

Vibratome sectioning
2 hours  Step 2

Tissue staining
24 hours  Step 3

Imaging
8 hours  Step 4

Quantification and statistical analysis
1-2 days  Step 5

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**Highlights**

Dissection and
sectioning of mouse
brain with CCM
lesions

Fluorescent labeling
of blood vessels and
confocal imaging

Identification and
analysis of CCM
lesions in brain
sections with Fiji
software

In the study of cerebral cavernous malformations (CCMs), the quantification of lesion burden is the main parameter for evaluation of disease severity and efficacy of drugs. We describe a reliable and cost-effective protocol to evaluate the number and the size of vascular malformations in the murine brain. This approach is based on histology and confocal imaging and can be performed with standard laboratory equipment. We detail the preparation of brain sections followed by image acquisition and analysis.

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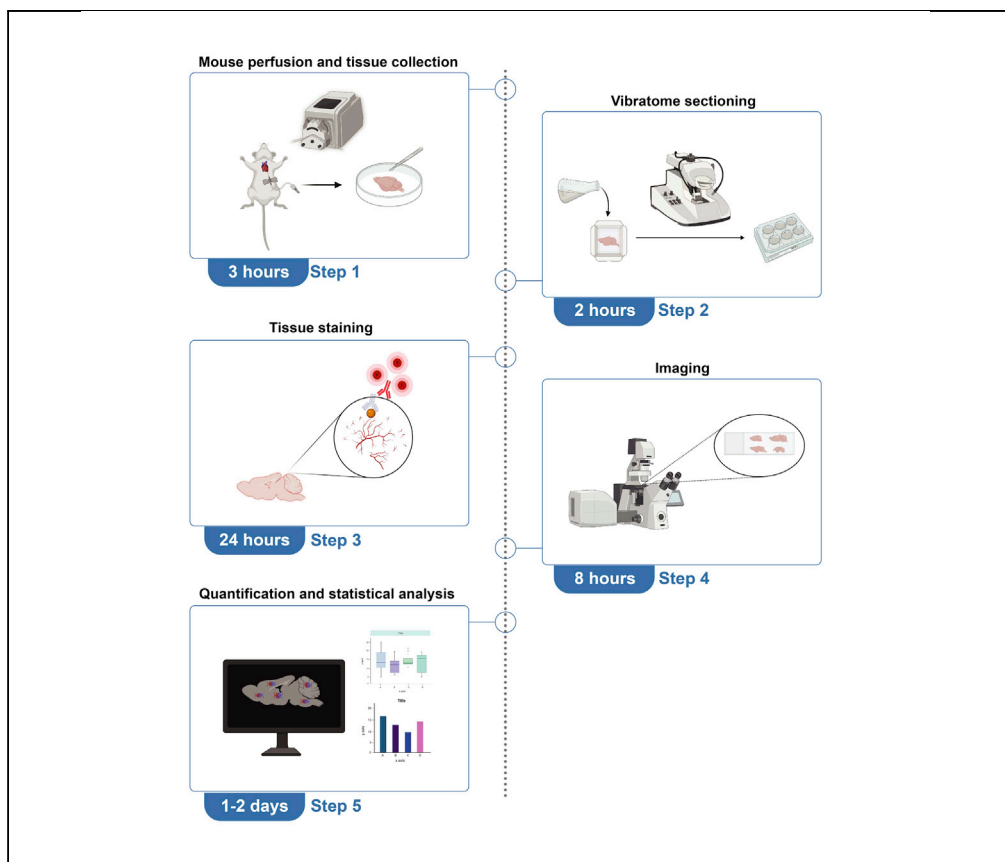
**Protocol**

# Histological quantification of cerebral cavernous malformations in the murine brain

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## SUMMARY

In the study of cerebral cavernous malformations (CCMs), the quantification of lesion burden is the main parameter for evaluation of disease severity and efficacy of drugs. We describe a reliable and cost-effective protocol to evaluate the number and the size of vascular malformations in the murine brain. This approach is based on histology and confocal imaging and can be performed with standard laboratory equipment. We detail the preparation of brain sections followed by image acquisition and analysis.

For complete details on the use and execution of this protocol, please refer to Maderna et al. (2022).

## BEFORE YOU BEGIN

Cerebral cavernous malformations (CCMs) is a genetic neuro-vascular disease characterized by clusters of capillary–venous malformations that are mainly found in the central nervous system. It is due to loss-of-function mutations in either one of three causative genes named *CCM1*, *CCM2* and *CCM3*. This protocol describes the specific steps to quantify the number and the size of cavernous malformations in the whole brain of adult *Procr*$^{CreERT2-IRES-tdTomato/+}$/*Ccm3*$^{f/f}$ mice. This murine model combines the *Ccm3* gene floxed (*Ccm3*$^{f/f}$) with the inducible form of the Cre recombinase driven by the Procr promoter (Procr$^{CreERT2-IRES-tdTomato/+}$). Here, tamoxifen administration results in *Ccm3* deletion restricted to Procr positive endothelial progenitor cells (Malinverno et al., 2019). These mice develop lesions that resemble the human pathology.

The protocol can be applied to any murine strain that develops vascular malformations (Malinverno et al., 2019) and, with some adjustments to the perfusion step (see Table 1), to animals of any age, from postnatal day 8 (P8) to adult. Moreover, this protocol also works for the spinal cord (Maderna et al., 2022).

All of the procedures with the mice, including breeding and experiments, were performed in agreement with the Institutional Animal Care and Use Committee (IACUC) of FIRC Institute of Molecular Oncology, in compliance with the guidelines established in the Principles of Laboratory Animal Care (Directive 86/609/EEC) and as approved by the Italian Ministry of Health.

Before you can begin with the protocol, you need to breed mice to the desired age and prepare the following solutions.

### 4% paraformaldehyde

⏲ Timing: 2 h

**Table 1. Perfusion parameters for mice of different age**

| Age | Weight | PBS | 4% PFA 4% | Speed mL/min |
|---|---|---|---|---|
| p8 | 5 g | 5 mL | 10 mL | 0.6 mL/min |
| p30 | 10 g | 5 mL | 15 mL | 0.9 mL/min |
| Adult | 20 g | 5 mL | 20 mL | 1.6 mL/min |

1. Warm 900 mL of PBS in a 2000 mL beaker at 56°C.
2. Weight 40 g of paraformaldehyde (PFA).
3. Add PFA to warm PBS and mix the solution on a stirrer.
4. Add 200 µL of 10 M NaOH to increase pH and promote PFA dissolving.
5. Cool the PFA solution to room temperature (RT; usually 20°C–25°C).
6. Adjust the pH at 7.4 with 37% HCl (almost 167 µL).
7. Fill up to 1000 mL with PBS, pH 7.4.
8. Filter PFA through a 0.45 µm filter.

   *Note:* for an optimal perfusion, PFA should be prepared fresh the day of the experiment.

   ⚠ CRITICAL: PFA is hazardous: always wear proper protective equipment and use only in a fume hood. PFA waste needs to be collected and disposed according to institutional regulations.

### 4% low melting agarose

   ⏲ Timing: 1 h

9. Weigh 4 g of low melting agarose in a flask.
10. Fill up to 100 mL with PBS and mix well on the stirrer.
11. Warm the solution until all the agar is completely dissolved.

   *Note:* Low melting agarose can be prepared in advanced and stored at room temperature (RT) for up to 6 months.

   ⚠ CRITICAL: This concentration of agar is hard to dissolve; a possible tip is to mix very well the solution an only in a second moment warm the solution to a temperature over 65.5°C.

### Avertin

   ⏲ Timing: overnight (ON; around 16 h)

Prepare stock and working solutions of Avertin as follow:

12. Prepare a stock solution of Avertin by dissolving 25 g of Tribromoethanol in 15.5 mL of Tert-amyl-alcohol and stirring vigorously until the powder is completely dissolved; this will probably take overnight.
13. Prepare the working solution 20 mg/mL combining 0.5 mL Avertin stock solution and 39.5 mL saline solution.
14. In a Becker sealed with Parafilm and completely wrapped with aluminum foil to exclude light, stir vigorously with a magnet overnight at room temperature (RT).
15. Filter the solution through a 0.45 µm filter.
16. Store the solution at 4°C protected from light.

*Note:* We used avertin as injectable anesthetic, as it was allowed in our IACUC protocol. Any IACUC-approved anesthetic can be used.

⚠ CRITICAL: Avertin degrades and becomes toxic killing the animal. Always store both stock and working solution at 4°C protected from light. Discard Avertin if it turns yellow, or the pH becomes less than 5.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| **Antibodies** | | |
| Armenian hamster anti-PECAM1 monoclonal (1:200) | Merck Millipore | Cat# MAB1398Z, RRID: AB_94207 |
| Goat anti-Armenian hamster Cy3-conjugated (1:400) | Jackson Immuno Research | Cat# 127-165-160, RRID: AB_2338989 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Paraformaldehyde | Sigma-Aldrich | Cat# P6148 |
| Vectashield | Vector Laboratories | Cat# H-1000 |
| 2,2,2-Tribromoethanol | Sigma-Aldrich | Cat# T48402 |
| Tert-amyl alcohol | Sigma-Aldrich | Cat# 8.06193 |
| Dulbecco's Phosphate Buffered (10×) (w/Ca$^{++}$, w/Mg$^{++}$) | Life Technologies | Cat# 14080-089 |
| Triton-X100 | Sigma-Aldrich | 93443-500ML |
| Normal Donkey Serum | Euroclone S.p.A | Cat# 017-000-1210 |
| GellyPhor LM Agarose | Euroclone S.p.A | Cat# EMR911100 |
| Ethylene Glycol | Fluka | https://lab.honeywell.com/shop/ethylene-glycol-03750 |
| Glycerol | Carlo Erba | https://actu-all.com/product/cas-56-81-5-glycerol-rpe-for-analysis-acs-reag-usp-1-l-glass-bottle/ |
| **Experimental models: Organisms/strains** | | |
| Procr$^{CreERT2-IRES-tdTomato/+}$ (Mus musculus; 30 and 90 days old; males and females) | Dr. Yi Ariel Zeng, CAS Center for Excellence in Molecular Cell Science/Shanghai Institute of Biochemistry and Cell Biology | N/A Identification of multipotent mammary stem cells by protein C receptor expression, Wang et al., 2014 https://doi.org/10.1038/nature13851 |
| *Ccm3* floxed (Mus musculus; 30 and 90 days old; males and females) | Prof. Elisabetta Dejana, IFOM | N/A EndMT contributes to the onset and progression of cerebral cavernous malformations, Maddaluno et al., 2013 https://doi.org/10.1038/nature12207 |
| **Software and algorithms** | | |
| Fiji - Imagej | National Institutes of Health (Schindelin et al., 2012) | https://imagej.net/software/fiji/ RRID: SCR_003070 |
| LAS X | Leica Microsystems | RRID: SCR_013673 |
| Prism 9 | GraphPad | RRID: SCR_002798 https://www.graphpad.com/scientific-software/prism/ |
| Excel | Microsoft | RRID: SCR_016137 https://www.microsoft.com/en-gb/ |
| **Other** | | |
| Peristaltic Pump | Gilson | https://www.news-medical.net/MINIPULS-3-Peristaltic-Pump-from-Gilson |
| Wacom Intuos | Wacom | https://www.wacom.com/en-it/products/pen-tablets/wacom-intuos |
| TCS SP8-DLS | Leica Microsystems | RRID: SCR_018169 https://www.leica-microsystems.com/products/confocal-microscopes/p/leica-tcs-sp8-dls/ |
| 12-well plate | Thermo Fisher Scientific | Cat# 1878.2 |
| Conical 50 mL tubes | Thermo Fisher Scientific | Cat# 339625 |
| Tissue dispomolds 15 × 15 × 6 mm | Diapath | Cat# 070821 |
| Superfrost plus slides | Thermo Fisher Scientific | Cat# 10149870 |
| Scissors, straight sharp&sharp | RWD Life Science | Cat# S18001-09 |
| Dissecting scissors, straight blunt&blunt | RWD Life Science | Cat# S13047-14 |

**Continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Micro Spring Scissors, straight sharp&sharp | RWD Life Science | Cat# S11007-12 |
| Ophthalmic forceps, straight | RWD Life Science | Cat# F12005-10 |
| Dissecting forceps | RWD Life Science | Cat# F12003-12 |
| Bovine serum albumin | Sigma-Aldrich | Cat.# A8531 |
| 0.45 μm filter | Thermo Fisher Scientific | Cat.# 295-4545 |

## MATERIALS AND EQUIPMENT

**4% Paraformaldehyde**

| Reagent | Final concentration | Amount |
|---|---|---|
| PFA | 4% | 40 g |
| NaOH 10 M | n/a | 200 μL |
| HCl 37% | n/a | 167 μL |
| PBS | n/a | to 1000 mL |
| Total | n/a | 1000 mL |

4% PFA solution needs to be prepared freshly and filtered on a 0.45 μm filter.

**Avertin stock solution**

| Reagent | Final concentration | Amount |
|---|---|---|
| 2-2-2 Tribromoethanol | 1.6 mg/mL | 25 mg |
| Tert-amyl alcohol | n/a | 15.5 mL |
| Total | n/a | 15.5 mL |

**Avertin working solution**

| Reagent | Final concentration | Amount |
|---|---|---|
| Avertin stock solution | 1.6 mg/mL | 0.5 mL |
| PBS | n/a | 39.5 mL |
| Total | n/a | 40 mL |

Avertin stock and working solutions can be stored 3 months at 4°C in the dark.

**Blocking solution**

| Reagent | Final concentration | Amount |
|---|---|---|
| Triton X-100 | 0.3% | 0.3 mL |
| Normal donkey serum | 5% | 5 mL |
| Bovine serum albumin | 2% | 2 mL |
| PBS | n/a | 92.7 mL |
| Total | n/a | 100 mL |

Blocking solution needs to be prepared freshly.

**Low melting agar embedding buffer for vibratome**

| Reagent | Final concentration | Amount |
|---|---|---|
| Low melting agar | 4% | 4 g |
| ddH2O | n/a | To 100 mL |
| Total | n/a | 100 mL |

Low melting agar can be kept at RT and warmed before using.

**Freezing solution for brain section storage.**

| Reagent | Final concentration | Amount |
|---|---|---|
| PBS | 40% | 40 mL |
| Glycerol | 30% | 30 mL |
| Ethylene glycol | 30% | 30 mL |
| Total | n/a | 100 mL |

Freezing solution can be kept at −20°C for up to 6 months.

## STEP-BY-STEP METHOD DETAILS
### Mouse perfusion and brain tissue collection (part 1)

⏱ Timing: 3 h + post-fixation overnight

This first part describes the perfusion of the mouse and the collection and following post-fixation of the brain.

1. Inject intraperitoneally the mouse with 0.4–0.75 mg/kg of Avertin (Figure 1A).
   a. Pinch the toe to be sure that mouse is deeply anesthetized.
2. After the mouse is in deep anesthesia, prepare the mouse for the perfusion.
   a. Arrange the mouse on its back and pin the four limbs down.
   b. By using forceps and scissors clamp and cut the skin to expose the chest cavity (Figure 1B).
   c. Make an incision in parallel and over the diaphragm, in order to avoid to pierce lung and heart.
   d. Uncover the heart by cutting laterally the ribs with the scissors (Figure 1C).
   e. Insert the perfusion needle in the apex of the left ventricle and cut open the right atrium with a small cut (by using small scissors) (Figure 1D).
3. Perfuse the mouse with a peristaltic pump.
   a. Perfuse firstly with 5 mL PBS at RT to wash out the blood. When blood is fully washed out, mucosae, such as palms and tongue, and internal organs, such as the liver, become pale (Figures 1F and 1G; troubleshooting 1).
   b. Switch to 4% PFA and continue perfusion up to final volume (for the final volume refer to Table 1).
      i. Stop the pump.
      ii. Move the tube from PBS to PFA (Figure 1G).
      iii. Start the pump.
4. Dissect the brain.
   a. Cut the head.
   b. Uncover the skull by removing the skin.
   c. Open the skull.
      i. insert the scissor (the flat part) under the lambda and cut the sagittal and interfrontal suture (Figure 2A).
      ii. lift up the pieces of skull to uncover the brain (Figures 2B and 2C).
      iii. with fine forceps carefully remove the brain from the skull (Figures 2D–2F).
   d. Collect the brain in a 50 mL tube with at least 10 mL 4% PFA.

See troubleshooting 5.

5. Post-fix the brain in at least 10 mL 4% PFA overnight at 4°C.
6. Remove PFA, wash twice with PBS and leave the brain in PBS.
7. (Optional) If you want to analyze also the spinal cord, proceed as follow:
   a. Remove all the internal organs to gain access to the vertebral column.
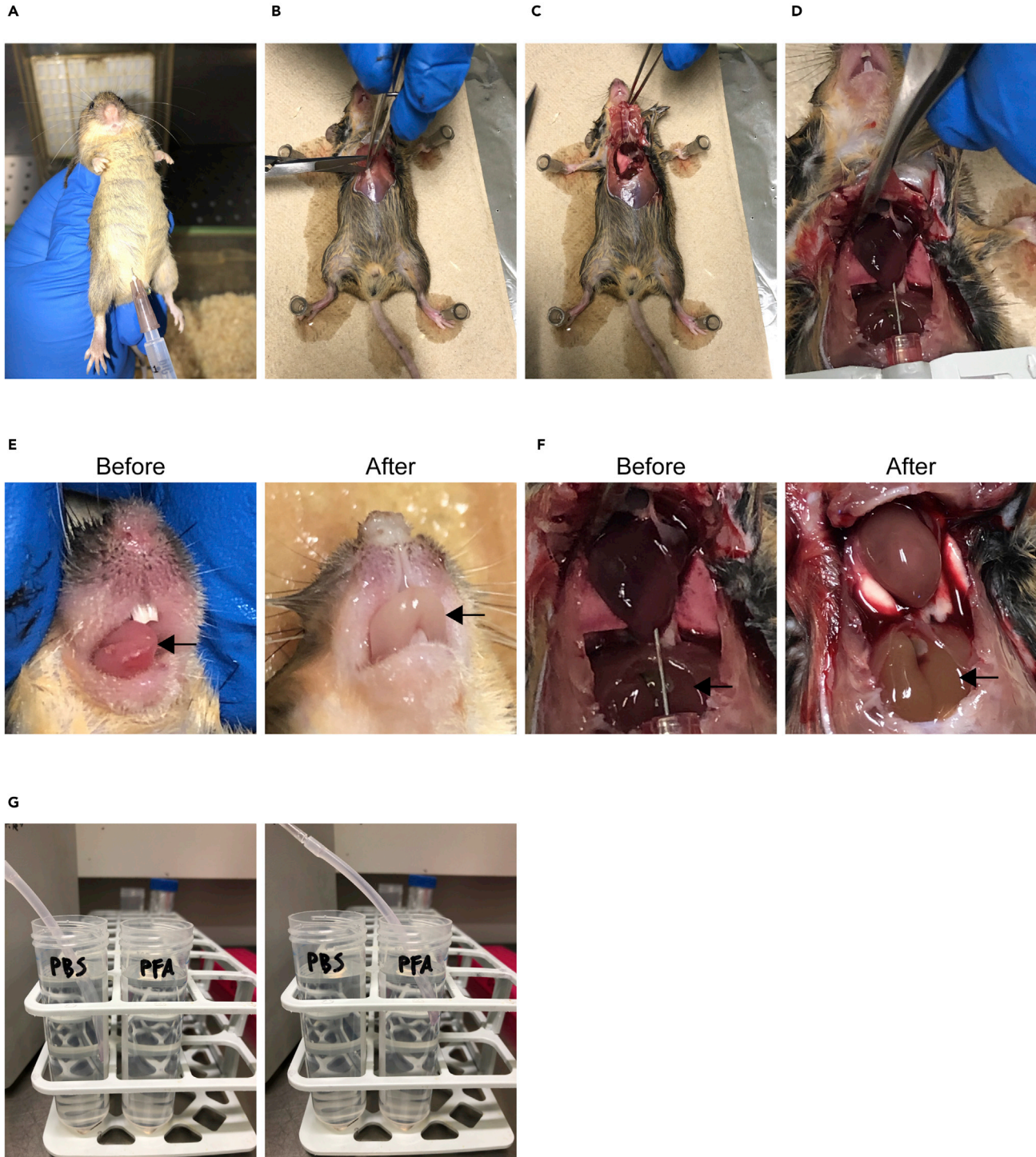   b. Remove the vertebral column by cutting with scissor the ribs and the pelvis.

**Figure 1. Transcardiac perfusion of the mouse**

(A–D) Representative images showing the key steps for transcardiac perfusion of the mouse: (A) intraperitoneal injection of Avertin, (B and C) thoracotomy and (D) needle insertion in the left ventricle.

(E and F) Images showing the whitening of (E) the tongue and (F) the liver.

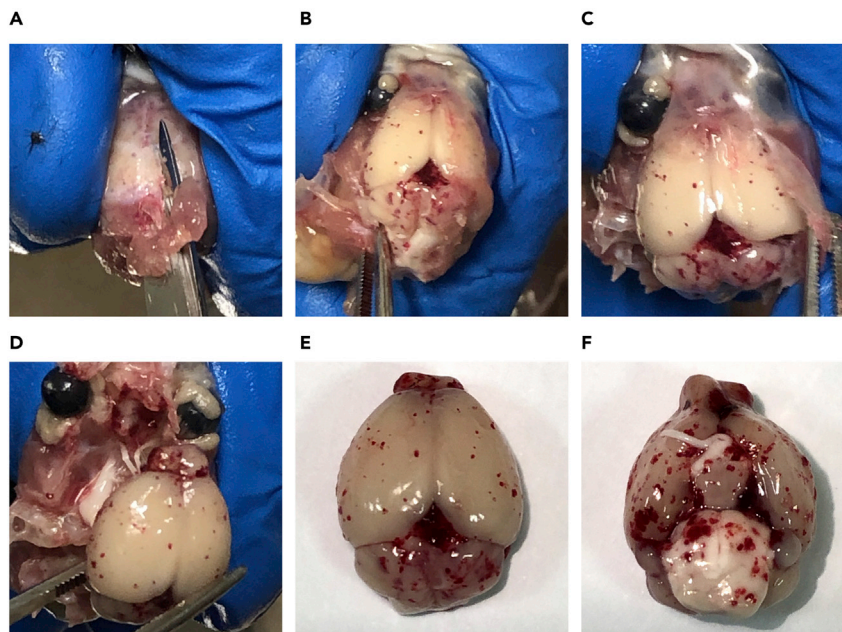(G) Switch between PBS and PFA during perfusion.

**Figure 2. Brain dissection**
(A–D) Key steps of brain mouse brain dissection: (A) incision and (B and C) opening of the skull, (D) removal of the brain.
(E and F) Representative images of the (E) dorsal and (F) ventral side of a brain with CCM lesions.

   c. Clean the vertebral column from the skin and other tissues.
   d. Collect the vertebral column in a 50 mL tube with at least 10 mL 4% PFA.
   e. Post-fix the vertebral column in at least 10 mL 4% PFA overnight at 4°C.
   f. Remove PFA, wash twice with PBS.
   g. Gain access to the spinal cord by a ventral rostral-to-caudal laminectomy.
   h. Cut a 200 μL pipette tip to make a 2–3 mm hole, and mount it on 5 mL syringe (without needle).
   i. Carefully flush the spinal cord out in rostral-to-caudal direction and collect it in a 50 mL tube with PBS.
   j. Proceed with the following steps as for the brain.

*Note:* You can stop here and store the tissues for few days at 4°C in PBS, before proceeding to step 8. However, for the best performance of the procedure, it is better to process immediately the tissue.

⚠ CRITICAL: Correct amount of fixative and speed of perfusion are critical to have an optimal fixation. The following table shows the right parameters to be used in relation to age and size of the animal.

## Agarose embedding and vibratome sectioning (part 2)

⏱ Timing: 2–3 h for 10 brains

This second part describes brain embedding and sagittal sectioning.

  8. Prepare the brain for vibratome sectioning.
    a. Dry the brain by rolling it over a paper towel.
    b. Divide the cerebral hemispheres cutting the medial part with a blade.

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   c. Put one hemisphere in a disposable base mold (15 × 15 × 6 mm) with the inner face towards the bottom.

   d. Cover the tissue with warm 4% low melting agarose solution and cool it on ice or at RT.

   e. When the agarose block is solidified, transfer it to the platform of the vibratome and fix it with glue.

9. Cut the whole cerebral hemisphere into 100 μm thick sagittal sections, at speed 1–1.5 mm/s and amplitude 1 mm.

10. Collect the sections in PBS. Put 4/5 sections in each well of a 12-well plate keeping the order of sectioning.

11. Store sections either in PBS at 4°C for 2 or 3 days or for almost 1 month at −20°C in freezing solution (40% PBS, 30% Glycerol, 30% Ethylene glycol).

*Note:* Long term storage of the tissue may result in bad staining and imaging.

### Primary and secondary antibody staining (part 3)

⏱ Timing: 24 h

This part describes the staining procedure.

12. From the 12-well plate where the sections were collected, select one section from each well and put them in a new well of a 12-well plate. In this way, the selected sections will be around 4–500 μm far away from each other and representative of the whole hemisphere.

13. Incubate sections in 1 mL blocking solution for 1 h at RT.

14. Incubate sections with primary antibody anti-PECAM1, diluted 1:200 in 0.5 mL fresh blocking solution, at 4°C overnight.

15. The next day, wash 3 times with 1 mL PBS at RT for 10′.

16. Incubate sections with goat anti-Hamster-CY3 diluted 1:200 in PBS at RT for 4 h.

17. Remove solution and wash 3 times with 1 mL PBS for 10′.

18. Post fix the stained sections with 4% PFA for 5′ at RT.

*Note:* This step fixes primary and secondary antibodies to the antigen, thus prolonging the stability of the stain.

19. Wash 3 times with 1 mL PBS for 10′.

20. Move brain sections in a petri dish filled with PBS-T (PBS + 0.01% triton-X100).

21. Using a fine brush, mount the sections on a superfrost glass slide.

22. Let the sections dry at RT for 20 min before mounting with Vectashield solution.

23. Seal the slides with nail polish and leave overnight at 4°C.

### Confocal acquisition (part 4)

⏱ Timing: 8 h for 10 brains

This part describes the confocal acquisition of brain sections. The following settings refer to a Leica TCS SP8-DLS confocal microscope equipped with LAS-X plus Navigator software.

Different systems may have other functions to navigate throughout the slide, such as the Mark&Find or the Tiling tool.

24. Switch on the software and the laser.

25. First scan with the navigator system the whole slide with a 2.5× objective to visualize all the brain sections mounted.
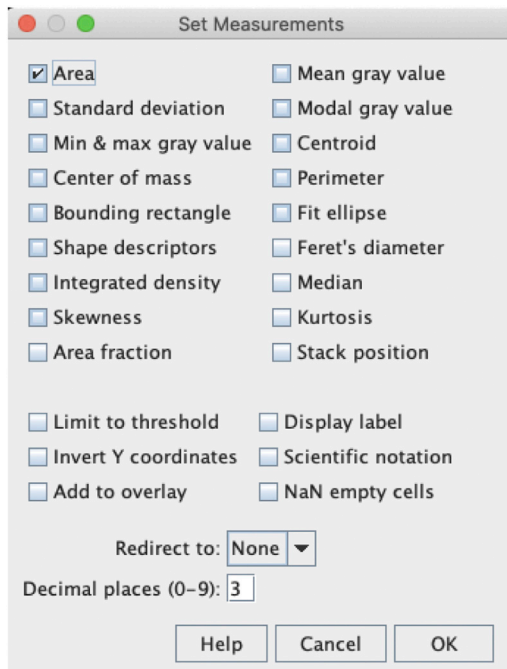
**Figure 3. Screenshot of the "Set Measurements" tool of Fiji software**
Before starting with the analysis, open the "Set Measurements" tool and select "Area".

26. Define a region of interest (ROI) around each single section.
27. Set the 10× objective and redefine the Z-stack of each section, using a 10 μm z-step.
28. Acquire zoom 1×, bidirectional scanning and resolution set at 1024 × 1024 pixels, frequency 400 Hz.
29. Z-stack Images are merged and ready to be saved and processed as ".lif" file.

See troubleshooting 2 and 3 to fix eventual problems with the staining.

**Imaging analysis (part 5)**

&#9673; Timing: 1–2 days for 10 brains

30. Open the Fiji software.
31. Open "set measurement" and select "Area", as shown in Figure 3.
32. Open the image with the Fiji software.
33. Perform Z-projection of the section with max intensity.
34. Make sure the image is calibrated and dimensions expressed in μm. If not, refer to troubleshooting 4 to calibrate the image.
35. Open the ROI MANAGER tool.
36. Draw the outline of the whole section as shown in Figure 4, and add it to the ROI Manager. This will give you the area of the entire section, also referred to as "Analyzed area".
37. Draw the outline of each single lesion and add it to the ROI MANAGER (Figure 5A). CCM lesions can be easily identify and distinguished by normal vessels. In the brain, the majority of vasculature is composed of capillaries and small vessels which usually have a lumen of around 3–7 μm and 15–40 μm, respectively (Figure 5B). On the contrary, lesions appear as enlarged vessels with a "bubble" shape and frequently form multi-lumen clusters (Figure 5C). Lesion diameter can easily exceed 300–500 μm.
38. Measure the area of all ROIs with the "Measure all" tool of the ROI MANAGER (Figure 5).
39. Copy and paste the measurements from the "Results" window to an Excel file (see Figure 6).
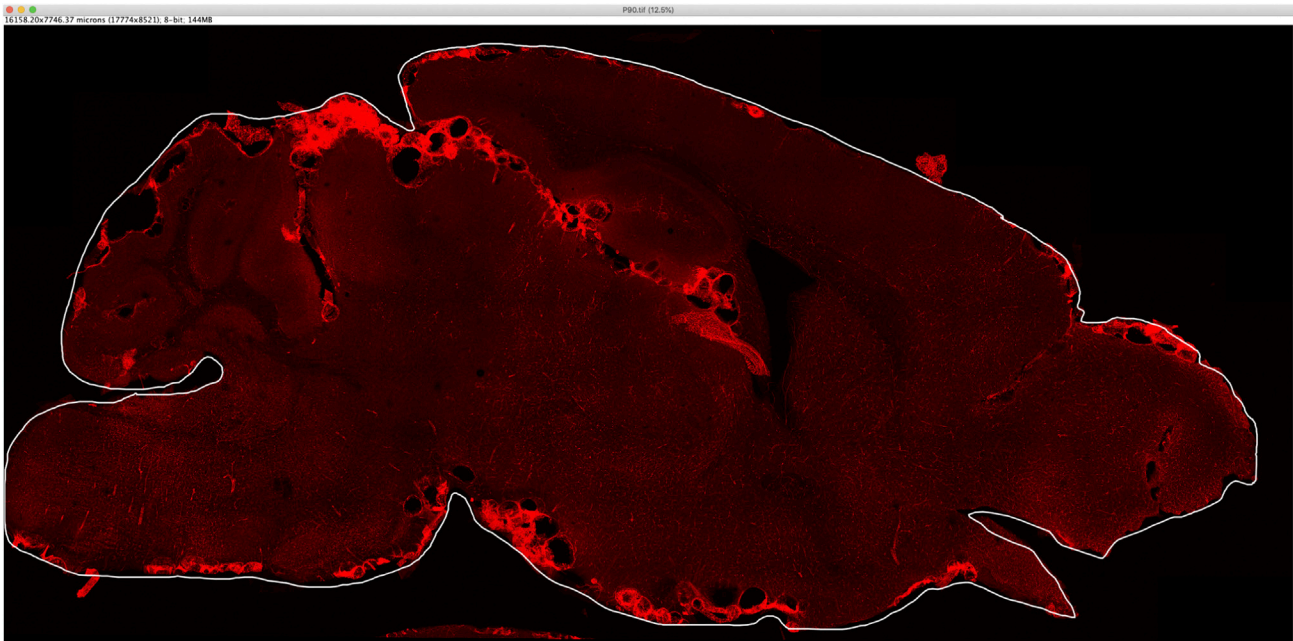
**Figure 4. Representative screenshot of brain section outline**
Representative screenshot of a sagittal brain section during quantification process. White line represents the outline of the section.

40. Convert the "Analyzed area" from $\mu m^2$ to $mm^2$ (cells D5:D7 in the Excel table shown in Figure 6).
41. The "Lesioned area" is obtained by summing the area of all the single lesions in the section (cells F5:F7 in the Excel table shown in Figure 6).
42. The "Number" of lesions is obtained by counting the lesions of the section (cells F1:F3 in the Excel table shown in Figure 6).
43. Finally, both "Lesioned area" and "Number" of lesions are divided for the "Analyzed area $mm^2$" (cells H5:H7 and H1:3 in the Excel table shown in Figure 6, respectively).
44. Repeat steps 32–43 for each section.
45. The average of the values for each single section represents the value for a single mouse.

**EXPECTED OUTCOMES**

The outcome of this protocol is a reliable and consistent quantification of lesion burden in the mouse brain. The two parameters quantified are a) the number of lesions/$mm^2$, and b) the lesioned area expressed as $\mu m^2/mm^2$. Figure 7 shows a representative comparison of lesion burden of $Procr$-$^{CreERT2-IRES-tdTomato/+}/Ccm3^{f/f}$ mice analyzed at 1 and 3 months of age, with relative quantification and analysis. Both lesioned area and number of lesions increases with age as apparent from macroscopic image of the whole brain (Figures 7A and 7D) and confirmed by the histological quantification (Figures 7G and 7H). For a more comprehensive time course of lesion development in this model see (Maderna et al., 2022).

Besides the age of the animal, lesion burden is influenced by many variables which include a) the mouse model used, b) the *CCM* gene deleted and c) pharmacological treatments.

For instance, a different murine model, where the *Ccm3* floxed gene is combined to the Cre recombinase driven by the pan-endothelial promoter of VE-Cadherin (*Cdh5*(PAC)-CreERT2/*Ccm3*$^{f/f}$), has a faster and more aggressive phenotype: lesions start appearing after 8 days and become huge after 1 month (Malinverno et al., 2019).
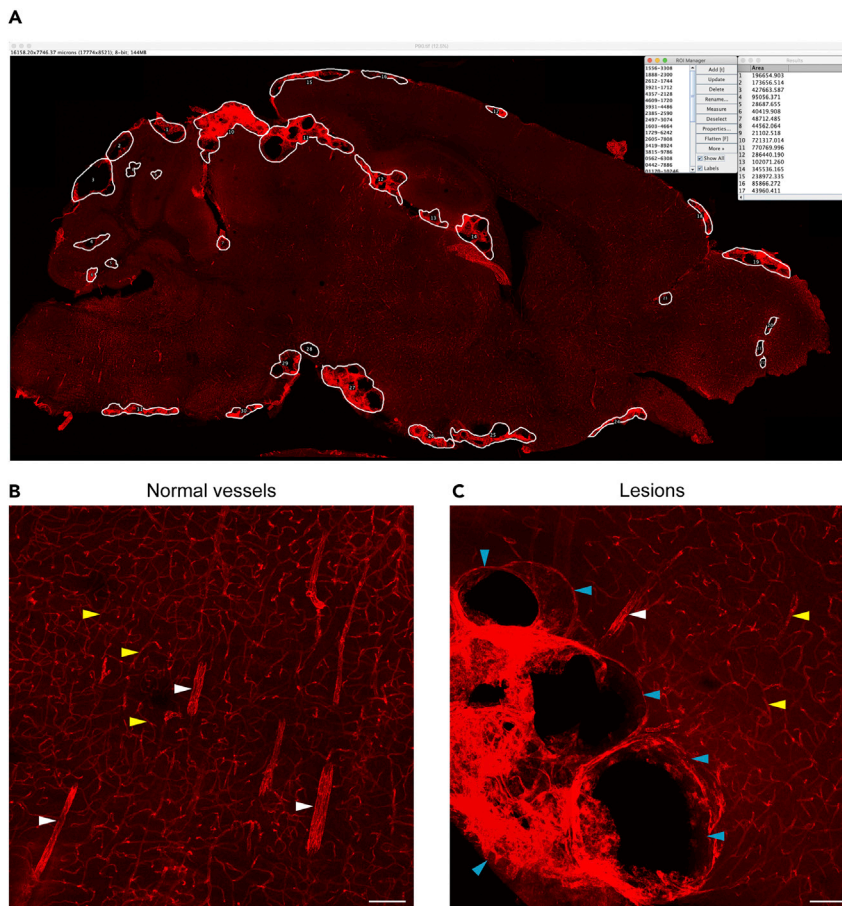
**Figure 5. Representative screenshot of lesion outline**

(A) Representative screenshot of a sagittal brain section during quantification process. White line represents the outline of single lesions.

(B and C) Magnification showing (B) normal vessels and (C) CCM lesions stained by PECAM1. Arrowheads: yellow = normal capillaries, white = normal larger vessels, blue = lesions. Scale bar = 100 μm.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification and statistical analysis should be performed by a blinded operator to avoid any biases. For each brain, select at least 4–5 sections which should not be adjacent in order to be representative of the whole organ. All the lesions of each section must be quantified in order to have a precise value of both the lesioned area and the number of lesions. For the statistical analysis, the following pipeline should be followed:

1. Identify outliers (Optional). Apply the ROUT method with Q set to 1%, and remove eventual outliers from subsequent analysis.
2. Check for normal (Gaussian) distribution of datasets by applying an appropriate method such as the Shapiro-Wilk normality test.
3. Apply the appropriate statistical test to assess statistical significance. Parametric and non-parametric tests should be applied to normally and non-normally distributed datasets respectively.
4. Comparison between two groups should be analyzed by unpaired two-tailed Student's t-test, in case of normal distribution, or by the non-parametric Mann-Whitney test. Comparison among three or more groups should be analyzed by one-way ANOVA test followed by Tukey's post

**Figure 6. Example of lesion quantification and analysis**
Representative Excel sheet showing the analysis process. Calculations are shown with blue font. Final values for Lesioned area and Number of lesions are shown in the box.

hoc test for multiple comparison, in case of normal distribution, or by the Kruskal-Wallis test followed by Dunn's post-hoc test.

## LIMITATIONS

The method described here allows a robust and reliable quantification of CCM lesion number and area in the murine brain. After tissue sectioning and confocal acquisition, z-stacks are projected on a single image. Therefore, this method provides a 2D view of CCM lesions. However, if all the samples are analyzed with the very same parameters the values of lesioned area and number of lesions can be reliably compared among experiments performed at different times.
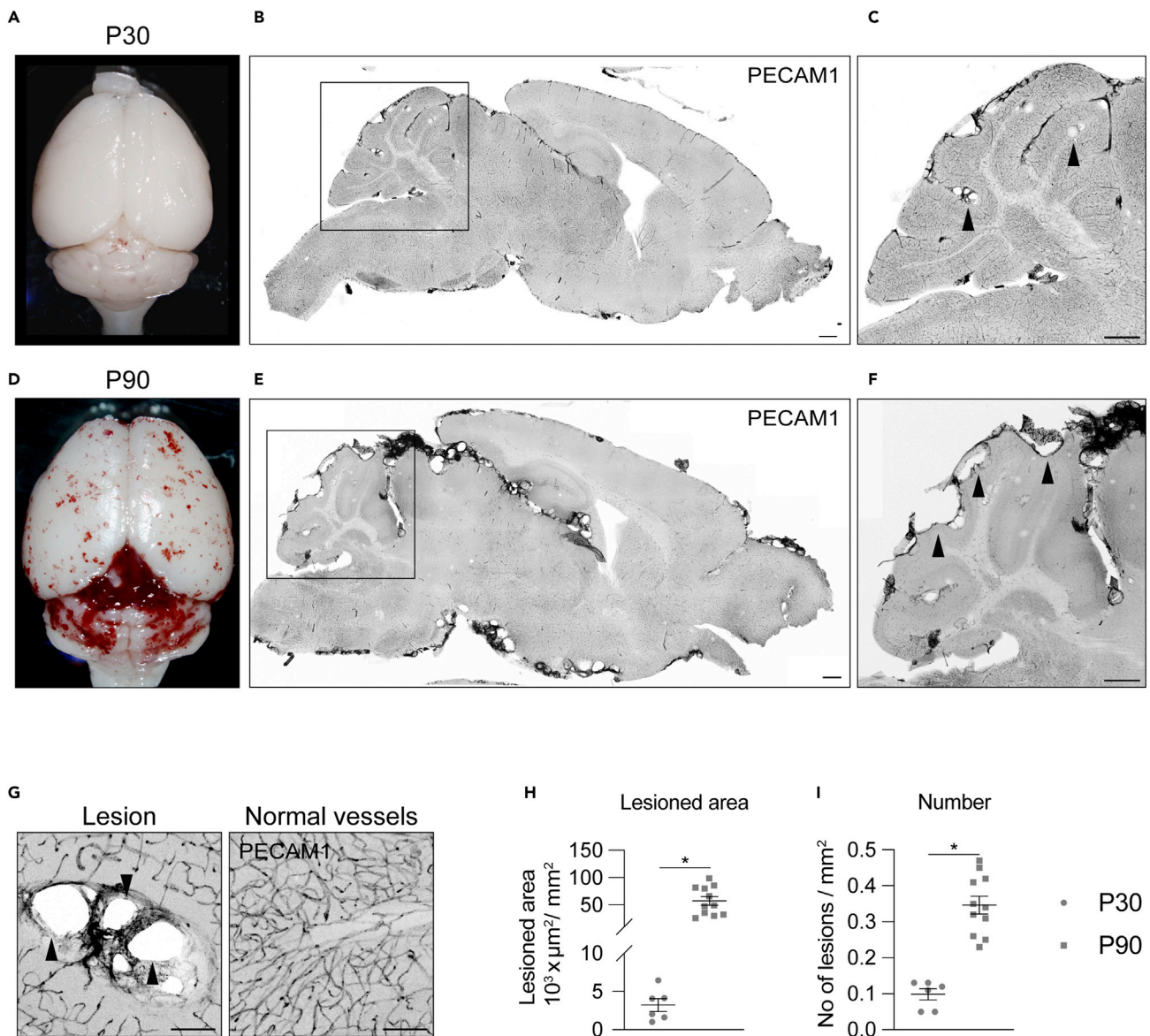
**Figure 7. Expected outcomes**

(A–F) Analysis of lesion burden in 30 (A–C) and 90 (D–F) days old mice. Representative images of (A and D) whole brain and (B, C, E, and F) sagittal sections stained for blood vessels (PECAM1). Scale bar = 500 μm.

(G) Representative magnifications showing lesions and normal vessels stained for PECAM1. Scale bar = 100 μm.

(H) Quantification of lesioned area. Data are means ± SE; each dot represents an animal; * p < 0.001 (Student's t test). Black arrowheads point to lesions.

(I) Quantification of number of lesions. Data are means ± SE; each dot represents an animal; * p < 0.001 (Mann-Whitney test).

On the contrary, a big advantage of this method is the very high spatial resolution which can detect, theoretically, lesions of any size.

## TROUBLESHOOTING

### Problem 1

Unsuccessful perfusion (step 3) leads to incomplete blood removal and tissue fixation. This results in poor immunostaining and bad imaging and therefore compromised and unreliable analysis.

**Potential solution**

After insertion of the perfusion needle, evaluate the success of perfusion by checking that palms, tongue and liver become pale. Whitening of mucosae and abdominal organs means that blood is being removed and that perfusion is working properly (see also Figures 1E and 1F). After switching to fixative check that abdominal organs, such as the liver become hard. If blood has been removed, but the organs are not stiff, consider to increase the perfusion volume.

**Problem 2**

Immunostaining is not uniform throughout the section; in particular the signal is bright at the border and becomes faint in the middle of the section (steps 26–29).

**Potential solution**

This is usually due to non-homogenous tissue fixation as result of incomplete perfusion. Refer to "problem 1" to improve perfusion efficiency.

**Problem 3**

The signal to noise ratio is low (steps 26–29).

**Potential solution**

This is usually due to two related problems:

- Low specific signal.
- High background.

Low signal can be improved using new batch of primary antibody and/or increasing its final concentration. However, do not use the primary antibody too much concentrated, as this can, on the other hand, increase the background.

High background could be due to autofluorescence, usually when the tissue is old. Always consider to process the tissues as soon as possible after post-fixation.

High background can also be due to unspecific binding of the primary antibody. Try to decrease the concentration of the antibody and to increase the concentration of normal donkey serum in the blocking solution.

**Problem 4**

The image opened is not calibrated, and dimensions are only expressed in pixel (step 34). In this case, the "measure" function of Fiji will return the value of lesions' area in pixel, and not in μm.

**Potential solution**

The image must be properly calibrated before starting the analysis.

- Check in the upper left corner of the image window that dimensions are expressed in μm with the corresponding number of pixels in brackets (Figure 8A). If dimensions are expressed only in pixels, the imaged must be calibrated.
- First retriever from the meta data of the ".lif" file the correct pixel dimension. For instance, the picture in Figure 6, acquired with 10× objective and zoom 1×, has a pixel size of 0.909017 μm.
- Open the "Properties…" plugin under the "Image" menu.
- Insert the pixel width and height expressed in μm, and the unit as shown in Figure 8B.
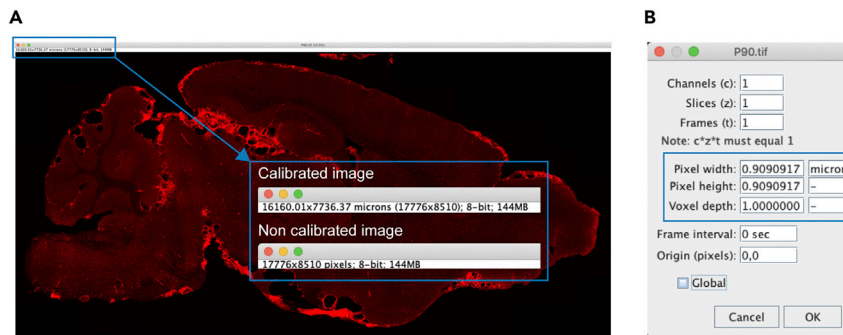
The image is now calibrated.

**Figure 8. Image calibration**
(A) Example of a confocal image with focus on the top left corner. In calibrated images, dimensions are expressed as μm, while in non-calibrated images only in pixels.
(B) Example of the "Properties…" window with the pixel size specified (blue box).

### Problem 5
The brain does not have CCM lesions (step 4).

### Potential solution
In almost hundred animals analyzed, this murine model of CCM had a penetrance of 100%, with a very low variability in terms of severity of lesion burden. In adult animals, lesions should be clearly visible by eye in dissected brain. If this is not the case, we recommend to take a biopsy (tail or ear) to check that the genotype is positive for the Cre recombinase. If the animal expresses the Cre, therefore a problem with tamoxifen solution, or with its administration, should be taken into consideration.

### RESOURCE AVAILABILITY

#### Lead contact
Matteo Malinverno, email: matteo.malinverno@ifom.eu.

#### Materials availability
This study did not generate new unique reagents.

#### Data and code availability
This study did not generate any unique datasets or code.

### ACKNOWLEDGMENTS

### AUTHOR CONTRIBUTIONS

M.M. and C.M. conceived the project, designed and performed the experiments, and collected and analyzed the data. M.M. and C.M. wrote the manuscript that has been revised by E.D. M.M. and E.D. raised funds.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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