The embryonic mouse hindbrain model to study sprouting angiogenesis *in vivo*

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Abstract

Blood vessel growth is a fundamental process for organ development and wound healing but is also associated with ischemic diseases and cancer. The growth of new blood vessels from preexisting vasculature, termed sprouting angiogenesis, is the predominant mode of blood vessel growth in central nervous system vascularisation and pathological vessel growth. Accordingly, studying the molecular and cellular mechanisms of angiogenesis holds the promise to find novel therapeutic targets to stimulate new vessel formation in ischemic tissues or inhibit pathological vessel growth in disease. The embryonic mouse hindbrain provides an excellent model to study sprouting angiogenesis *in vivo* by histochemical or fluorescent wholemount immunolabelling, thus allowing high-resolution image capture of nascent vasculature and subsequent quantification of relevant angiogenic parameters. This chapter describes how to use the mouse embryonic hindbrain as a model to study physiological angiogenesis, including detailed protocols for hindbrain dissection, wholemount staining, and angiogenic parameters analysis.

1 Introduction

The growth of new blood vessels is a fundamental process required for organogenesis during development [1]. After birth, new blood vessel growth is also required during the female reproductive cycle as well as for wound healing and exercise-induced muscle growth. New blood vessel growth, however, is also associated with different diseases such as cancer and neovascular eye diseases, where it has been linked to disease progression [2]. The main mechanism driving new blood vessel growth in these conditions is termed sprouting angiogenesis; this process involves the formation of tip-cell led migratory sprouts that extend from pre-existing vessels in response to growth factor gradients and extracellular matrix cues [3]. Therefore, elucidating the molecular and cellular mechanisms of sprouting angiogenesis is crucial for identifying new therapeutic targets to stimulate new vessel formation in ischemic tissues or to inhibit pathological vessel growth in disease. This chapter describes briefly how the mouse embryonic hindbrain is vascularised during normal development and how it can be used as a model to study physiological sprouting angiogenesis. Specifically, we provide step-by-step protocols for tissue dissection, sample staining and subsequent imaging, and quantitative analysis.

1.1 Mouse hindbrain vascularisation

The mammalian brain is vascularised during embryogenesis by sprouting angiogenesis in a stereotypical and precise spatiotemporal manner to form a well-defined vascular network [4; 5]. At embryonic day (E) 9.5 in the mouse, vessels sprout from a pre-formed perineural vascular plexus into the brain parenchyma, then extending perpendicularly as so-called radial vessels towards the ventricular zone (**Fig. 1a**) [6]. This process occurs in response to vascular endothelial growth factor A (VEGF-A), which is secreted by neural progenitor cells [7]. By around E10.0, the radial vessels reach the subventricular zone and turn at near right angles to grow laterally (**Fig.1a**) [6]. From E10.5 onwards, these lateral vessels continue to sprout but also begin to anastomose, thereby giving rise to a honeycomb-like vascular network known as subventricular vascular plexus (SVP), which nurtures the neural progenitor zone and regulates neural progenitor proliferation in the hindbrain (**Fig. 1a**) [8; 9]. At E11.5,

most of the vascular anastomosis takes place, facilitated by the help of tissue-resident macrophages termed microglia, so that the SVP is largely complete by E12.5 [10]. Throughout this period, the hindbrain vasculature undergoes remodelling and maturation, which involves the recruitment of perivascular cells, initially pericytes, and subsequently also astrocytes to ensure the acquisition of especial vascular barrier properties that characterise most of the vessels in the central nervous system, known as the brain blood-brain barrier [11]. Owing to this unique spatiotemporal process, and because the roof-plate can be opened for hindbrain flatmounting, the mouse embryonic hindbrain has become an instrumental and accessible model system for studying the molecular and cellular mechanisms of sprouting angiogenic *in vivo*.

1.2 Advantages and disadvantages of the hindbrain model

The embryonic mouse hindbrain offers several advantages as a model system to study sprouting angiogenesis. The first is that hindbrain vascularisation occurs early during development and thus allows the study of blood vessel growth and patterning in genetically modified mice that show embryonic lethality, such as NRP1 knockout mice [12; 13]. This is an important consideration, because such mouse mutants cannot be examined with the other widely used angiogenic organ model, the postnatal mouse retina [14; 15].

A second advantage is that the hindbrain is exquisitely suited for wholemount imaging after flatmounting and labelling of endothelial cells and their interacting cells (**Fig.1b**). Accordingly, the hindbrain vascular plexus in a flatmount shows vessels with a one-dimensional growth pattern (the radial vessels on the pial side) and vessels with a two-dimensional network structure (the SVP vessels on the ventricular side). This geometric architecture of vasculature in the flatmounted hindbrain enables easy quantification of a wide range of vascular parameters, including vessel numbers, vessel diameter, branchpoints, tip cell numbers, and endothelial cell filopodia characteristics, thus making the hindbrain system one of the most reliable tools to characterise vascular patterning (**Figs.** and **3**) [8; 15].

A third advantage is that the preparation of embryonic hindbrains for wholemount imaging is fairly simple and can be adapted for versatile applications. For

example, this method permits staining and subsequent analysis immediately after dissection and fixing, but also much later on, after the hindbrain tissue has been stored. This allows pooling of samples, for example to account for genotype, and thus examining a sufficiently large sample number at the same time to minimise the possibility of intrinsic technical variability and to increase statistical power (see **Subheading 3**).

However, the hindbrain model may not be suitable in some circumstances. For example, genetically modified mice with embryonic lethality before E10.5 cannot be examined with this model, because vessel sprouting in the hindbrain begins only after E9.75. This limitation can be overcome by utilising Cre-Lox technologies, which allows refinement of gene targeting to specific cell types or temporally (reviewed by[16]). Indeed, the ablation of VEGF-A results in early embryonic lethality by E9.5 [17; 18], but its role in the developing brain have been elucidated with a constitutive Cre-Lox approach that inactivates VEGF-A in neural progenitors only (**Fig. 3**) [7]. Another disadvantage of the hindbrain model is its poor suitability for local injections of drugs or viruses for evaluating candidate molecules with the potential of inhibiting or promoting vascularisation during development. Although the *in utero* manipulation of embryos can be achieved in principle, e.g., *via* injection or electroporation after transient, surgical exposure of the embryos, both are extremely challenging techniques that are more suitable to late gestational stages, such as E15.5 embryos [19; 20; 21], and therefore after the time window when angiogenic sprouting in the hindbrain is usually assessed (see **Subheading 3**).

2 Materials

2.1 Sample collection and hindbrain preparation

2.1.1 Equipment

1. Micro-dissecting scissors (*e.g.* Sigma).
2. Watchmaker forceps no. 5 (*e.g.* Dumont).
3. Watchmaker forceps no. 55 (*e.g.* Dumont).
4. Scalpels (no. 10; *e.g.* Swann-Morton).
5. 2.0 mL round-bottom safe-lock tubes (*e.g.* Eppendorf).
6. Plastic cell culture dishes (60 cm diameter; *e.g.* BD Falcon).
7. Plastic Pasteur pipettes (*e.g.* Alpha Laboratories).
8. 50 mL reagent tubes (, *e.g.* Falcon tubes from Corning).
9. Orbital/plate shaker (*e.g.* SciQuip).
10. Tabletop balance (*e.g.* ED Precision balance, Sartorius).
11. Water bath (*e.g.* Stuart SWB15D, Stuart).
12. Stereomicroscope with bright light illumination (*e.g.* Nikon SMZ1270).

2.1.2 Reagents

1. Phosphate buffered saline tablets (PBS, Sigma-Aldrich).
2. Paraformaldehyde (PFA, *e.g.* Sigma-Aldrich).
   CAUTION: It is harmful if inhaled and swallowed. It is irritating to eyes, respiratory system and skin and may cause sensitisation of skin upon contact. Wear protective goggles, clothing and gloves. Use it in a chemical fume hood/fume cabinet.
3. Absolute methanol (*e.g.* Fisher Scientific).
   CAUTION: It is toxic and can cause various serious irreversible effects through inhalation, in contact with skin and if swallowed. It is irritating to eyes and skin. Wear protective goggles, clothing, and gloves.
4. Triton X-100 (*e.g.* Sigma-Aldrich).

CAUTION: It is harmful if ingested and can cause serious damage to the eyes. Wear protective goggles, clothin,g and gloves.

2.1.3 Equipment and reagents/buffers set up

1. Plastic Pasteur pipettes (used to transfer embryos and hindbrains): Slice the bulb of the pipette to create a spoon-shaped opening suitable to transfer embryos. For hindbrain transfer, cutoff the tip with a scalpel or scissors to create an appropriately wide opening.
2. PBS: Dissolve five tablets of PBS in 1 L of distilled water. Store at room temperature indefinitely.
3. PBT: Add 50 µL Triton X-100 to 50 mL PBS in a 50 mL tube. Store at room temperature indefinitely.
4. Formaldehyde solution (4%, w/v): Under a chemical fume hood, weigh 2 g of paraformaldehyde (PFA) powder into a 50 mL tube and add 50 mL of PBS. Dissolve the PFA by heating up at 60°C in a water bath, mixing frequently until the PFA powder has dissolved. Store in aliquots at -20°C for up to 1 year. Thaw at room temperature or at 37°C in a water bath and cool it down on ice prior to use.
5. Methanol gradient: To prepare 50 mL of 25%, 50% or 75% methanol in PBT, mix 12.5, 25 or 37.5 mL of absolute methanol with 37.5, 25 or 12.5 mL of PBT, respectively, in 50 mL tubes. Store at room temperature indefinitely.

2.2 Hindbrain staining, mounting and imaging

2.2.1 Equipment

1. 2.0 mL round-bottom safe-lock tubes (as above).
2. Plastic Pasteur pipettes (as above).
3. Orbital/plate shaker (as above).
4. Microscope glass slides (*e.g.* VWR International).
5. Microscope glass coverslips (18x18 mm; *e.g.* from VWR International).

6. Black electric tape (*e.g.* SCOTCH33, 3M).
7. Stereomicroscope with bright light illumination (as above) equipped with a digital camera (*e.g.* Nikon DS-Fi3) and data acquisition software (*e.g.* NIS-Elements Microscope Imaging software, Nikon).
8. Confocal laser scanning microscope (*e.g.* LSM710, Zeiss) with associated data acquisition software (*e.g.* ZEN Microscope Imaging software, Zeiss)

2.2.2 Reagents

1. PBS (as above).
2. Triton X-100 (as above).
3. Absolute methanol (as above).
4. Hydrogen peroxide (*e.g.* Sigma-Aldrich).

CAUTION: It is harmful if ingested and can cause serious damage to the eyes. Wear protective goggles, clothing, and gloves.

5. Heat inactivated normal goat serum (NGS, Sigma-Aldrich).
6. Heat inactivated normal rabbit serum (NRS, Sigma-Aldrich).
7. Ready-to-use serum-free protein block solution (Dako).
8. Biotinylated lectin from *Bandeiraea simplicifolia* BS-I isolectin B4 (IB4; Sigma-Aldrich).
9. Rat anti-mouse PECAM1 monoclonal antibody (CD31, also known as MEC13.3; BD Pharmingen).
10. Rat anti-mouse endomucin monoclonal antibody (V.7C7; Santa Cruz Biotechnology).
11. HRP-tagged streptavidin (Dako).
12. HRP-tagged rabbit anti-rat secondary antibody (Dako).
13. Diaminobenzidine and urea hydrogen peroxide tablets (Sigma-Aldrich).
14. AlexaFluor488-conjugated goat anti-rat secondary antibody (Invitrogen).
15. AlexaFluor488-conjugated streptavidin (Invitrogen).
16. PFA (as above).
17. SlowFade Antifade reagent (*e.g.* Life Technologies).

2.1.3 Equipment and reagents/buffers set up

1. Plastic Pasteur pipettes (used to transfer hindbrains from round-bottom tubes to microscope slides as previously described).
2. Orbital/plate shaker in a cold room or cold cabinet, because antibody incubations should be performed in tubes that are gently agitated at 4°C.
3. PBS (as previously described).
4. PBT (as previously described) *(see Note 1)*
5. Methanol gradient (to rehydrate hindbrains that were fixed and stored, as previously described).
6. Blocking buffer (10%, v/v): For 1 mL, mix 100 µL of serum with 900 µL of PBT.
7. Diaminobenzidine solution: Dissolve 1 DAB tablet in 5 mL of distilled water at room temperature. Adjust tablet amount to final volume required. Protect from light. Prepare fresh for each immunostaining experiment.
8. Diaminobenzidine and urea/hydrogen peroxide solution: Dissolve 1 DAB and 1 urea hydrogen peroxide tablet in 5 mL of distilled water at room temperature. Adjust tablet amount to final volume required. Protect from light. Prepare fresh for each immunostaining experiment.
9. Formaldehyde solution (4%, w/v) to post-fix wholemount stained hindbrains, as previously described.

3 Methods

3. 1 *Day 1*: Dissection of hindbrains from mouse embryos

1. Euthanise a timed-mated pregnant female mouse using an ethically approved procedure, *e.g*. cervical dislocation, at the intended gestational stage (see Note 2). Dissect both uterine horns containing the embryos using sharp scissors.

Transfer the excised uterus into a 6 cm plastic dish containing ice-cold PBS and keep it on ice until moving to step 2.

2. Perform all dissections using a stereomicroscope. Using watchmaker forceps (number 5), peel-off the uterine muscle wall, open the yolk sac to expose the embryo, and release carefully each embryo by cutting the umbilical cord (see Note 3).

3. Transfer each embryo into a clean plastic dish containing ice-cold PBS (**Fig.1c, *i***)

4. Using sharp scissors or a scalpel, slice off the embryo head (**Fig. 1c, *ii***) (see Note 4).

5. Position the head dorsal side-up and identify the fourth ventricle and the thin epithelial covering known as the roof-plate (**Fig. 1c, *iii***).

6. Pierce the roof-plate using watchmaker forceps (number 5 or 55) and peel-off the tissue, moving rostrally along the midline over the midbrain and then caudally over the posterior hindbrain and spinal cord (**Fig. 1c, *iv***).

7. Using watchmaker forceps (number 5 or 55), carefully peel away any remaining head mesenchyme and meninges attached to the pial side of the hindbrain. Remove the midbrain and spinal cord tissue (**Fig. 1c, *v***) (see Note 5).

8. The hindbrain should now be exposed as an open-book preparation (**Figure 1C*, vi***)

9. Transfer hindbrains to 2 mL round-bottom safe-lock tubes using a plastic Pasteur pipette with a wide-bore tip, aspirate the PBS and fix for 2 h at 4°C with gentle-agitation in freshly made (or thawed from frozen aliquots) cold 4% formaldehyde solution in PBS.

10. Rinse fixed hindbrains three times with PBS and proceed with wholemount immunolabelling.

    PAUSE STEP: Fixed hindbrains can be stored at 4°C in PBS if immunolabelling will begin within 2-3 days. Alternatively, replace PBS with an increasing methanol gradient (e.g. 25%, 50% and 75% absolute methanol in PBS; for 5 minutes each at room temperature) before transferring to absolute methanol for

longer storage at -20°C. NOTE: For some antibody stainings, e.g. to detect PECAM1, avoid fixation for longer than 2 h or methanol storage [15].

3. 2 *Days 2-3*: Hindbrain wholemount immunolabelling

3.2.1. Hindbrain Wholemount Immunolabeling (*e.g.*, isolectin B4, PECAM1 or endomucin)

1. If hindbrain was stored in methanol, replace absolute methanol with a decreasing methanol gradient (e.g. 75%, 50% and 25% absolute methanol in 0.1% PBT for 5 minutes each at room temperature) before transferring to PBT alone (see Note 6).
2. Place the hindbrains into new 2.0 mL round-bottom safe-lock tubes before proceeding with either histochemical or fluorescent wholemount immunostaining.

3. 2. 2. Hindbrain wholemount histochemical staining

1. Quench endogenous peroxidase activity by incubating in 1% hydrogen peroxide in PBS for 30 minutes at room temperature with gentle agitation.
2. Wash samples in PBT two times for 5 minutes each at room temperature.
3. Incubate in blocking solution for 30 minutes at room temperature with gentle agitation. For biotinylated isolectin B4 (IB4), PECAM1 or endomucin, use 10% heat-inactivated normal goat or rabbit serum in PBT as blocking solution (see Note 7).
4. Incubate overnight at 4°C with gentle agitation in blocking solution containing IB4 or primary antibody at the appropriate dilution (*e.g.* use biotinylated IB4 at 1:100, rat anti-mouse PECAM1, at 1:200 or rat anti-mouse endomucin 1:400).
5. Wash thoroughly the hindbrains five times for 20-30 minutes each in PBT at room temperature.
6. Incubate overnight at 4°C with gentle agitation in blocking solution containing secondary antibodies, diluted as recommended by the manufacturer (typical range 1:200 to 1:500). For biotinylated IB4 detection, use HRP-tagged

streptavidin, for PECAM1 or endomucin detection, use HRP-tagged anti-rat antibodies.

7. Wash the hindbrains as described in step 5.
8. Incubate the hindbrains in a solution of diaminobenzidine for 30 minutes at room temperature with gentle-agitation, and then in a solution of diaminobenzidine and urea/hydrogen peroxide for ~ 3-5 minutes or until colour develops (see Note 8).
9. To stop the reaction, rinse the hindbrains in distilled water, wash twice in PBS for 5 minutes each and then post-fix in 4% formaldehyde for 5 minutes at room temperature for long term preservation of antibody binding. Briefly rinse twice in PBS.

   PAUSE STEP: Samples can either be processed for imaging or can be stored at 4°C in PBS for up to one week before imaging without loss of signal.

3. 2. 3. Hindbrain wholemount fluorescent staining

1. Incubate in blocking solution for 30 minutes with gentle agitation at room temperature. For biotinylated IB4, PECAM1 or endomucin labelling, use 10% heat-inactivated normal goat or rabbit serum in PBT as blocking solution.
2. Incubate for overnight at 4°C with gentle agitation in blocking solution containing IB4 or primary antibody at the appropriate dilution (*e.g.* use biotinylated IB4 at 1:100, rat anti-mouse PECAM1 at 1:200 or rat anti-mouse endomucin at 1:400).
3. Wash thoroughly the hindbrains five times for 20-30 minutes each in PBT at room temperature.
4. Incubate overnight at 4°C with gentle-agitation in blocking solution containing fluorophore-tagged streptavidin to detect biotinylated IB4, *e.g*. AlexaFluor488 streptavidin. For PECAM1 or endomucin detection, use fluorophore-tagged anti-rat antibodies, *e.g*. AlexaFluor488 goat anti rat. If combining IB4 and antibody staining, use complementary fluorophores, *e.g*. AlexaFluor488 and AlexaFluor647 (see Note 9).
5. Wash the hindbrains as described in step 3.

6. Post-fix in 4% formaldehyde for 5 minutes at room temperature for long term preservation of antibody binding. Briefly rinse twice in PBS (see Note 10).

3. 3 *Day 4*: Hindbrain mounting and image acquisition and analysis

1. Cover a glass microscope slide with two layers of black electrical tape and use a scalpel to cut a rectangular well big enough to hold one hindbrain (**Fig. 2a*, i-ii***).

2. Transfer each hindbrain into the well using a plastic Pasteur pipette with a wide-bore tip (or cut-off the very end of a small-bore tip), place the hindbrain ventricular side facing up, and then remove excess PBS. Add an appropriate aqueous-based mounting media reagent (*e.g*. SlowFade Antifade reagent) and cover the well slowly with a glass coverslip to seal it (**Figure 2A*, iii-iv***) (see Notes 11 and 12).

3. Imaging of histochemical stained hindbrains can be done using a stereomicroscope with bright light illumination and an appropriate zoom range (**Fig. 2b-d**). For fluorescent stained hindbrains, use an epifluorescent or confocal laser-scanning microscope equipped with lenses suitable for aqueous-based mounted slides and optical filters appropriate for the fluorophores used for immunolabelling (**Figs. 3 and 4**).

4. Histochemical images for the embryonic hindbrains can be acquired at low magnification (*e.g.* zoom 0.8x) to visualise the superficial vascular plexus (SVP) on the ventricular side as a whole. Turning the slide upside down allows visualising the radial vessels entering the brain from the pial side (**Fig. 2b**). At least 2 randomly selected areas in the center of each hindbrain half should be acquired at higher magnification (*e.g.* zoom 8x) to allow manual quantification of the number vessel branchpoints (junctions) or radial vessels in the ventricular or pial side, respectively (**Fig. 2c,d**). The values obtained for these areas should be averaged for each hindbrain to obtain the value for that hindbrain to decrease technical error. Additionally, these angiogenic parameters can be automated identified utilising freely available software *e.g.* AngioTool [22] or Angiogenesis Analyser [23], macros in Image J platform (**Fig. 2e,f**).

5. Fluorescence hindbrain images are best documented with maximum intensity projections of z-stacks scans acquired with a laser scanning confocal microscope (**Figs. 3 and 4**) (see Note 13). High magnification images (*e.g.* acquired with 40x or 63x lenses) are required to visualise individual cells and their features, such as endothelial tip cell cells and their filopodia extensions (**Fig. 3a**), or to observe endothelial cell interactions with other hindbrain cell types such as pericytes and macrophages (**Fig. 3b,c**) or endothelial cell-lineage tracing (**Fig. 4**). Freely available (see above) or commercially available software packages (*e.g.* IMARIS from Bitplane or Volocity from Improvision) may be used for semi-automated and automated quantification of angiogenic parameters, including filopodia numbers, length and branchpoints (**Fig.3a, *iii***).

4 Notes

1. *Use PBS for non-permeabilised tissue staining, e.g. for visualisation of extracellular rather than cytoplasmic epitopes, and PBT for permeabilisation of the tissue to detect both cell surface and cytoplasmic epitopes.*

2. The success of tissue wholemount immunolabelling procedures usually relies on good antibody penetration. This could be a limitation at later gestational stages, thus alternative methods and protocols for hindbrain processing have been developed, *e.g.* staining of vibratome or cryostat preparations, and are elsewhere described [15; 24].

3. *A small piece of yolk can be kept for genomic DNA extraction and subsequent PCR-based genotyping of genetically modified mice*.

4. *A small piece of embryonic tissue (e.g. 2 mm of tail snip) can be kept for genomic DNA extraction and subsequent PCR-based genotyping of genetically modified mice, instead of using yolk sac tissue.*

5. *This step should be followed from E11.5 onwards, when the meningeal tissue is consolidated, as it increases antibody penetration into the hindbrain neuroepithelium. For E9.5 or E10.5, the hindbrain neuroepithelium is thin and fragile, therefore avoid this step so not to damage the hindbrain tissue. Consider using alternative methods for hindbrain analysis at these early gestational stages, e.g. vibratome or cryostat preparations, are elsewhere described* [15; 24].

6. *The graded series of methanol ensures gently hindbrain rehydration to prevent tissue damage*.
7. *Use serum from the host species that the secondary antibodies were raised in. For primary antibodies raised in goat, incubate in serum-free protein blocking solution (e.g. 5% bovine serum albumin in PBS or a suitable commercial alternative such a Dako serum free protein block). This will reduce non-specific staining often observed when using primary antibodies raised in goat.*

8. *Samples need to be light-protected, as diaminobenzidine and urea/hydrogen peroxide solutions are photosensitive. Is critical to observe the samples periodically under a stereomicroscope to avoid overdeveloping the reaction.*

9. *Samples need to be light-protected to prevent photobleaching. When including primary antibodies raised in goat in the protocol, use anti-goat Fab fragments of secondary antibodies to reduce non-specific staining*.

10. The described protocol can be adapted to the multi-labelling of endothelial cells, vessel-associated cells and surrounding extracellular matrix in the hindbrain as desired. This is achieved by combining primary antibodies raised in different species, or using such antibodies together with IB4 (*e.g*. IB4 and NG2 or IB4 and F4/80, see **Fig. 3**). For this purpose, apply the primary antibodies, or IB4 and the primary antibodies, together in the blocking solution; after washing, apply the appropriate secondary antibodies (and streptavidin in the case of IB4) together in the blocking solution. Additional antibodies specific for:

    Endothelial cells (EC):
    goat anti-mouse VEGFR2. Dilution 1:200, R&D Systems.

    Various cell types, including EC:
    goat anti-mouse/rat NRP1. Dilution 1:200, R&D Systems.

    Pericytes:
    rabbit anti-mouse NG2 chondroitin sulfate proteoglycan. Dilution 1:200., Millipore.

    Macrophage:
    rat anti-mouse F4/80. Dilution 1:400, Bio-Rad.

    Basement membrane: rabbit anti-mouse collagen IV. Dilution 1:400, Bio-Rad.

11. Trapping of air bubbles in mounted samples can occur when placing the coverslip over the sample. Lower the coverslip carefully (*e.g*. applying a right to left motion) onto the slide to avoid trapping air bubbles; and if bubbles persist, remove the coverslip carefully and repeat the mounting procedure.

12. If the hindbrain was fluorescently labelled, store the slide at 4°C in the dark until image acquisition to avoid photobleaching of the fluorophores.

13. If fluorescent speckles are seen, the reason could be that the secondary antibodies have formed precipitates. To avoid this problem, always centrifuge the fluorescently labelled antibody solutions in a refrigerated benchtop centrifuge at top speed and carefully remove the supernatant without disturbing any precipitates.

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Figure Legends

Figure 1. Mouse hindbrain vascularisation and dissection.

a. Schematic illustration of vascular development in the embryonic hindbrain. Vessel sprouting takes place in a temporally and spatially well-established sequence of events from E9.5 onwards in the mouse.

Abbreviations: *E*, embryonic day; *PVP*, perineural vascular plexus; *rv*, radial vessel; *SVP*, subventricular vascular plexus.

b. Brightfield stereomicroscope images (zoom 8x) of selected areas from the pial and ventricular sides of a flatmounted E12.5 wild-type mouse hindbrain, histochemically stained for the endothelial cell marker IB4. Scale bar: 50 µm.

c. Step-by-step hindbrain dissection from a wild-type mouse embryo at E12.5 (insets *i* to *vi*). Scale bars: 1 mm (*i-ii*) and 0.5 mm (*iii-vi*).

Figure 2. Analysis of flatmounted, vessel-stained mouse hindbrains.

a. Schematic presentation of the mounting procedure for a wholemount stained hindbrain onto a microscope glass slide (insets *i* to *iv*).

b. Brightfield stereomicroscope images (zoom 1.8x) of an entire E12.5 wild-type mouse hindbrain, histochemically immunostained for the capillary/venous endothelial cell marker endomucin (EMCN). Scale bar: 0.5 mm. The stippled boxes indicate the areas shown in (**C**).

c,**d**. Brightfield stereomicroscope images (zoom 8x) of the SVP in E11.5 (**c**) and E12.5 (**d**) wild-type mouse hindbrains, stained for EMCN. Scale bars: 100 µm.

e. Representative image of the SVP in an EMCN-stained, flatmounted E12.5 hindbrain (zoom 8x), including rendering with the AngioTool macro in Image J. The vessel outlines (yellow), the vessel 'skeletons' (red) and vascular branching points (blue dots) are shown, as used for automated quantification of vascular parameters. Scale bar: 100 µm.

f. Quantification of vessel branchpoints in the wild-type SVP, measured as junctions with AngioTool at E11.5 and E12.5. Analysis performed on 8 individual images of each hindbrain to illustrate the technical variation between different fields. The average is the representative value for the hindbrain. Data are shown as mean ± SE.

Fig. 3. Endothelial cell features and interaction with surrounding cell types.

a. Maximum intensity projection of a confocal z-stack from an E11.5 wild-type hindbrain that is fluorescently stained for IB4. (*i*) SVP, imaged at 40x; the dotted box in (*i*) is shown at higher magnification, imaged at 63x; in a 3D orthogonal view in (*ii*) to visualise endothelial cell filopodial bursts (examples are indicated with arrows). The dotted box in (*ii*) is shown in (*iii*) after processing with the Filament tracer module in IMARIS to analyse *e.g*. filopodia numbers (green dots) and lengths (purple segments). Scale bars: 20 μm (*i-ii*); and 5 μm (*iii*).

b. Maximum intensity projection of a confocal z-stack from (*i*) an E12.5 wild-type hindbrain, stained for IB4 and NG2 to identify SVP endothelium and pericytes, respectively. The single channel for NG2 is shown in (*ii*). A higher magnification of the area indicated with a dotted square in (*i*) is shown in *(iii)* as a 3D surface view, generated with IMARIS, to show that pericytes cover the endothelial surface. Scale bars: 50 μm (*i-ii*); and 5 μm (*iii*).

c. Maximum intensity projection of a confocal z-stack from (*i*) from an E12.5 wild-type hindbrain, stained for IB4 and ADGRE1 (F4/80 antigen) to identify SVP endothelium and monocyte/macrophages, respectively; (*ii*) shows only the ADGRE1 channel. A higher magnification of the area indicated with a dotted square in (*i*) is shown in (*iii*) as a 3D surface view generated with IMARIS software to analyse endothelial cell-macrophage interactions. As reported [10], tissue macrophages interact with neighboring endothelial tip cell (*arrowhead*). Scale bars: 50 μm (*i-ii*); and 5 μm (*iii*).

Fig. 4. Cre-mediated labelling of endothelium in embryonic hindbrain vessels.

Littermate E12.5 hindbrains from a cross of mice carrying *Tie2-Cre* [25] and *Rosa26*^{tdTom} [26] to activate vascular endothelial tdTomato expression, shown in maximum intensity projections of confocal z-stacks scans after fluorescent staining for IB4 and RFP to identify endothelial cells and Cre-dependent tdTomato, respectively. *Tie2-Cre*;*Rosa26*^{tdTom} mice express Cre recombinase in endothelial cells under the control of the *Tie2* promoter, and Cre-mediated recombination allows expression of the fluorescent tdTomato reporter due to removal of a floxed stop codon (*e.g.[12],* [13]). Hindbrains from embryos lacking Cre do not activate the tdTomato reporter. The overlay and individual IB4 and RFP channels are shown. Scale bars: 50 μm.

Figure 1

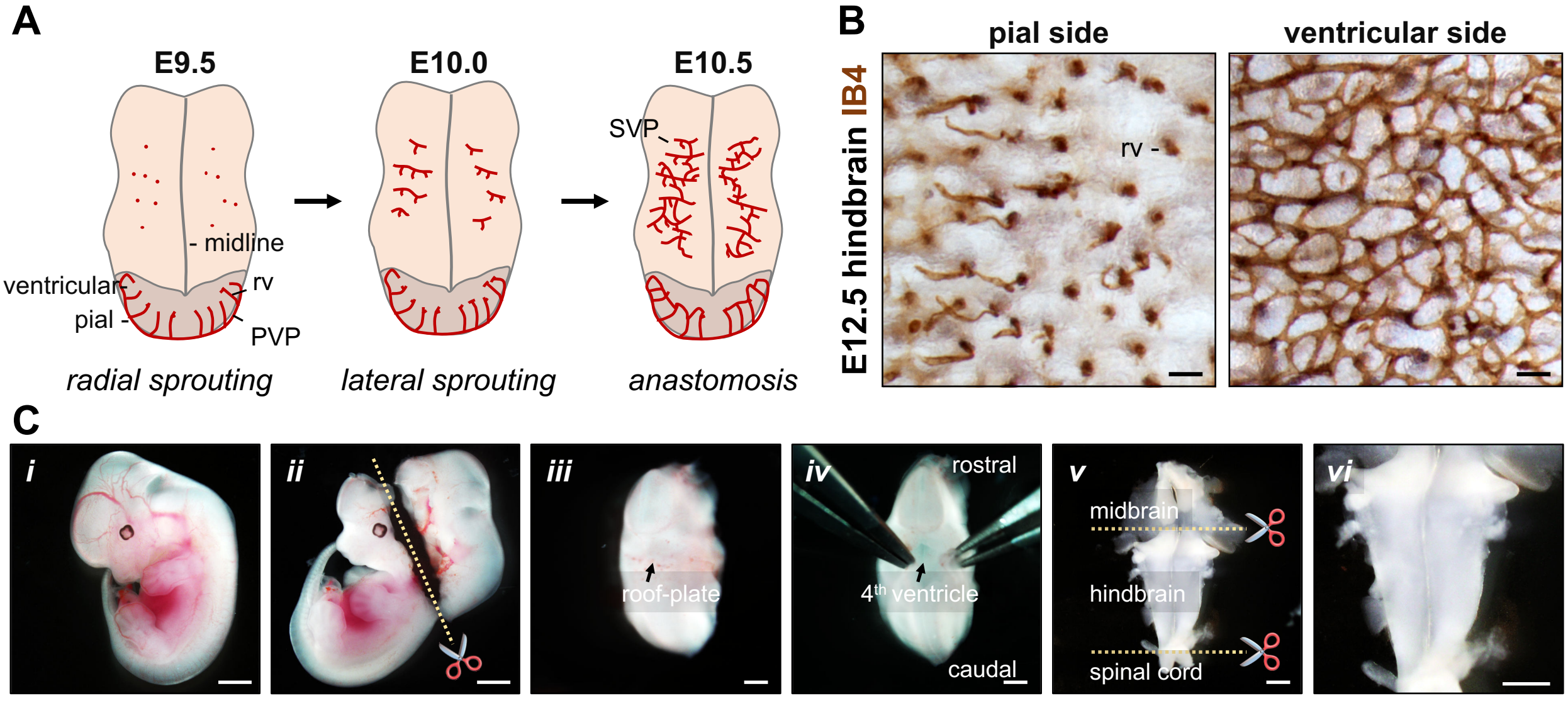
A
E9.5
E10.0
E10.5
- midline
ventricular -
rv
pial -
PVP
SVP
radial sprouting
lateral sprouting
anastomosis
B
pial side
ventricular side
E12.5 hindbrain IB4
rv -
C
i
ii
iii
roof-plate
iv
rostral
4th ventricle
caudal
v
midbrain
hindbrain
spinal cord
vi

Figure 2

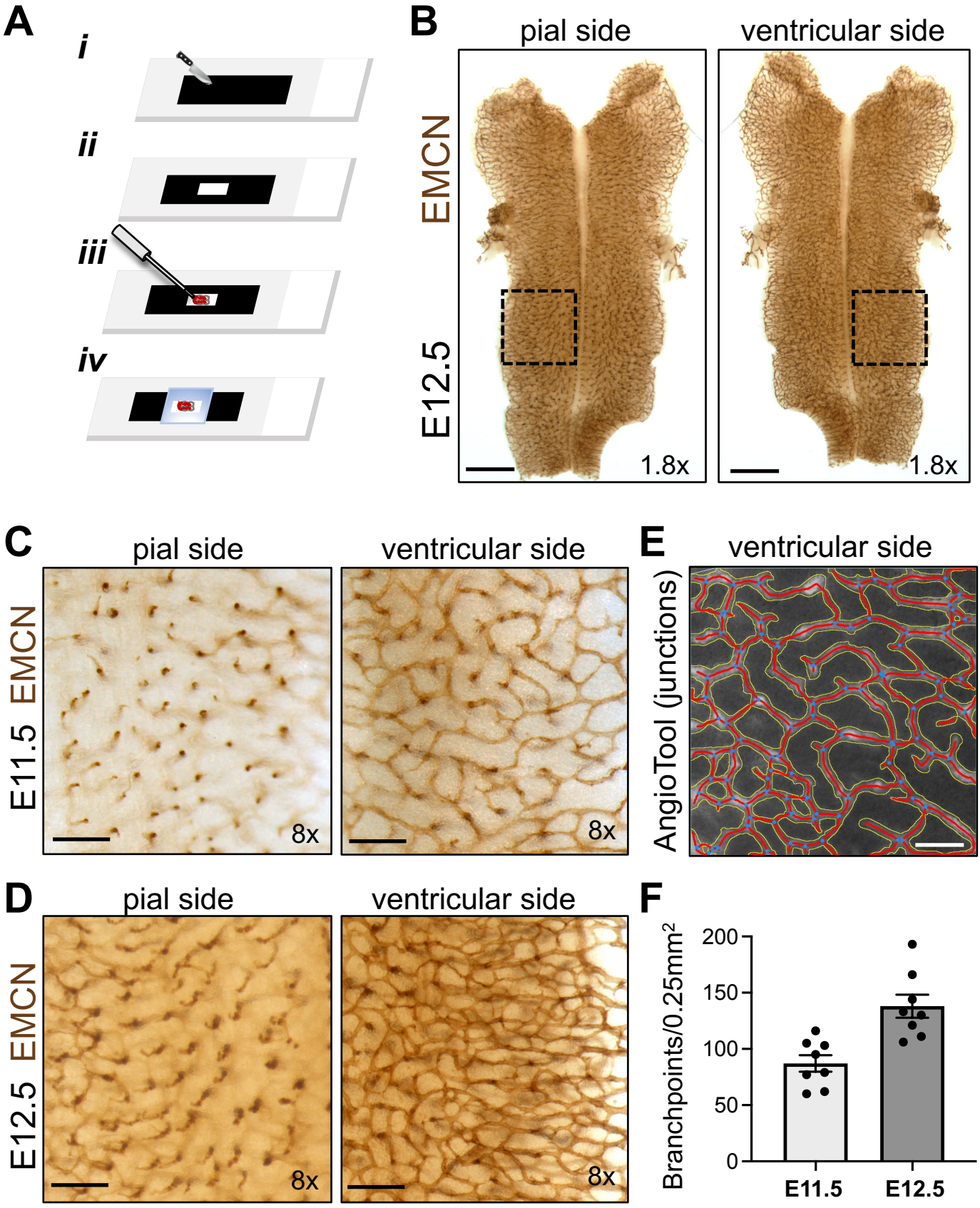
A
i
ii
iii
iv
B
pial side
ventricular side
EMCN
E12.5
1.8x
1.8x
C
pial side
ventricular side
E11.5 EMCN
8x
8x
D
pial side
ventricular side
E12.5 EMCN
8x
8x
E
ventricular side
AngioTool (junctions)
F
Branchpoints/0.25mm²
200
150
100
50
0
E11.5
E12.5

Figure 3

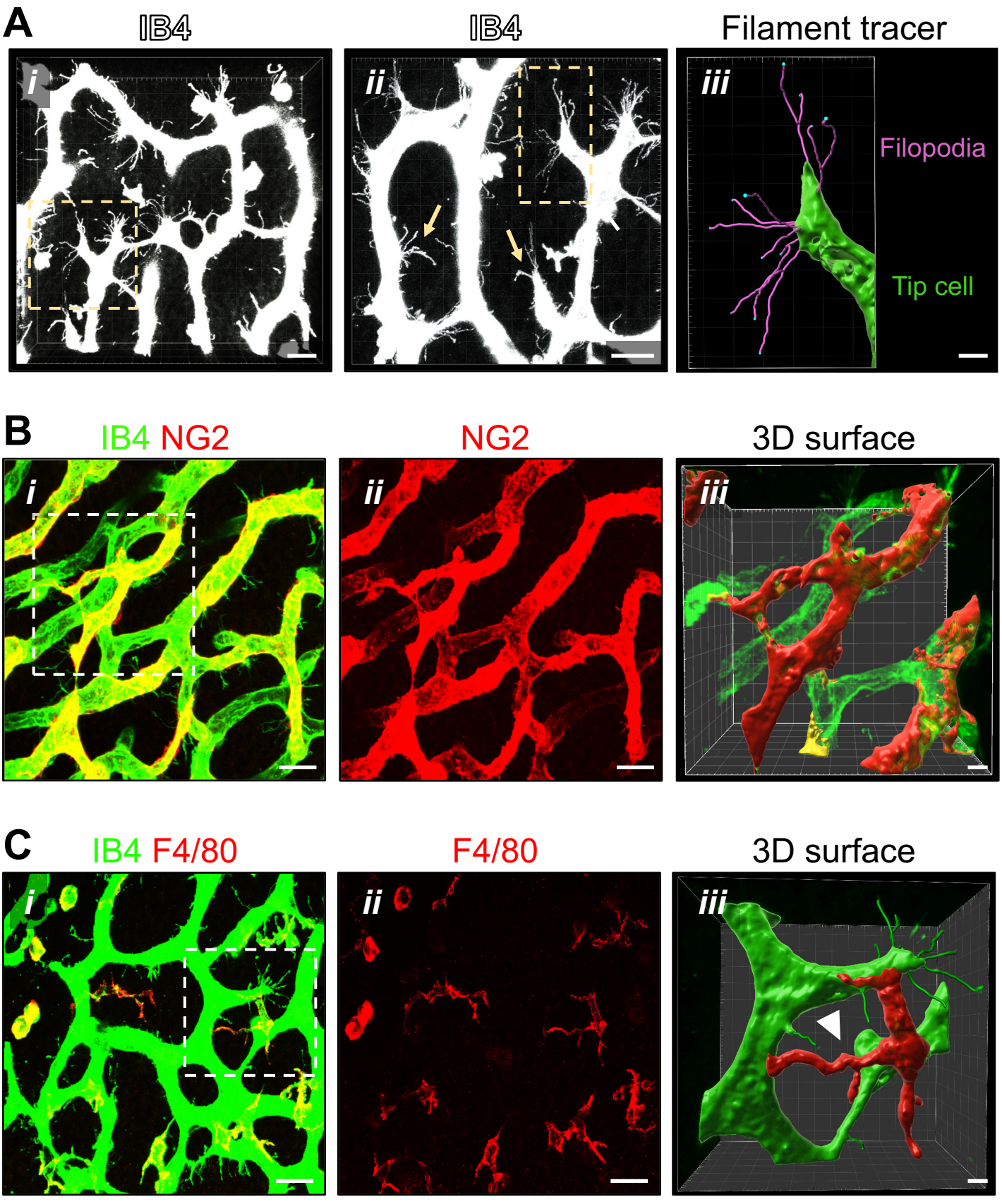
A
IB4
IB4
Filament tracer
i
ii
iii
Filopodia
Tip cell
B
IB4 NG2
NG2
3D surface
i
ii
iii
C
IB4 F4/80
F4/80
3D surface
i
ii
iii

Figure 4