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TITLE:

Visualization and Analysis of Pharyngeal Arch Arteries using Whole-mount Immunohistochemistry and 3D Reconstruction

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Endothelial cells, pharyngeal arch artery development, whole-mount immunofluorescence, tissue clearing, confocal microscopy, 3D reconstruction

SUMMARY:

Here, we describe a protocol to visualize and analyze the pharyngeal arch arteries 3, 4, and 6 of mouse embryos using whole-mount immunofluorescence, tissue clearing, confocal microscopy, and 3D reconstruction.

ABSTRACT:

Improper formation or remodeling of the pharyngeal arch arteries (PAAs) 3, 4, and 6 contribute to some of the most severe forms of congenital heart disease. To study the formation of PAAs, we developed a protocol using whole-mount immunofluorescence coupled with benzyl alcohol/benzyl benzoate (BABB) tissue clearing, and confocal microscopy. This allows for the visualization of the pharyngeal arch endothelium at a fine cellular resolution as well as the 3D connectivity of the vasculature. Using software, we have established a protocol to quantify the number of endothelial cells (ECs) in PAAs, as well as the number of ECs within the vascular plexus surrounding the PAAs within pharyngeal arches 3, 4, and 6. When applied to the whole embryo, this methodology provides a comprehensive visualization and quantitative analysis of embryonic vasculature.

INTRODUCTION:

During mouse embryogenesis, pharyngeal arch arteries (PAAs) arise as symmetrical, bi-lateral pairs of arteries that connect the heart with the dorsal aortae[1]. As the embryo develops, the first and second pairs of PAAs regress, while the 3rd, 4th, and 6th PAAs undergo a series of asymmetrical remodeling events to form the aortic arch arteries[2].

The PAAs 3, 4 and 6 develop via vasculogenesis, which is the de novo formation of blood vessels[3]. Defects in the formation or remodeling of these arch arteries give rise to various congenital heart defects, such as those seen in patients with DiGeorge Syndrome[4,5]. Therefore, understanding mechanisms that regulate the development of PAAs can lead to a better understanding of congenital heart disease (CHD) etiology.

Current approaches for visualizing and analyzing PAA development include immunofluorescence of tissue sections, vascular casts, India ink injection, high resolution episcopic microscopy, and/or whole-mount immunohistochemistry[1,4–7]. Herein, we describe a protocol combining whole-mount immunofluorescence, confocal microscopy and 3D image rendering in order to gather, analyze, and quantify volumetric data, vascular connectivity and cell identity. Further, we detail a method to compartmentalize and quantify the numbers of ECs in each pharyngeal arch as a means to study formation of the pharyngeal arch vascular plexus and its remodeling into the PAAs. While this protocol is designed for analyzing PAA development, it can be used to analyze other developing vascular networks.

PROTOCOL:

Animal use and procedures were approved by the Institutional Animal Care and Use Committee at Rutgers University.

1. Preparation of solutions

1.1. Prepare 1 L of phosphate buffered saline with 0.1% Triton-X-100 (PBST) and filter sterilize. This solution can be stored at room temperature (RT) for at least a year.

1.2. Prepare 600 µL of blocking buffer consisting of 10% of normal donkey serum in PBST. Make this solution fresh each time.

1.3. Prepare 50 mL of the following methanol (MeOH) dilutions in a flow hood: 25% MeOH in deionized water (dH_2O), 50% MeOH in dH_2O, and 75% MeOH in dH_2O. Vortex to mix. Store at RT.

1.4. Prepare 50 mL of the following benzyl alcohol-benzyl benzoate (BABB) solutions in 50 mL conical tubes.

1.4.1. For 100% BABB, add 32 mL of benzyl benzoate to 16 mL of benzyl alcohol (2:1 volume per volume ratio).

1.4.2. For 50% BABB, add 16 mL of benzyl benzoate and 8 mL of benzyl alcohol to 24 mL of MeOH.

1.4.3. Cover conical tubes in aluminum foil to protect from light. These solutions can be stored at RT for up to a year.

CAUTION: BABB is toxic and corrosive. It should be handled and disposed according to MSDS.

2. Embryo dissection and fixation

NOTE: This protocol is suitable for E9.5 and E10.5 mouse embryos (male or female) isolated from any mouse strain. For younger and older embryos, incubations times should be experimentally determined to maximize signal to noise ratio of fluorescence signal.

2.1. Fill one 35 mm and one 60 mm Petri dishes with 1x PBS and place on ice until needed.

2.2. Euthanize a pregnant mouse via CO_2 inhalation. Perform cervical dislocation as a secondary measure of euthanasia.

2.3. Clean the abdominal area of the dam with 70% ethanol. Pinch the abdominal area using forceps and make a V-like incision using surgical scissors starting from the base of the abdominal wall at the midline; continue to open up the thoracic cavity. Lift the abdominal tissue and move the intestines to the side to expose the uterine horns.

2.4. Make a cut at the base of the vaginal canal, and with forceps, pull the uterus away from the dam. Make an additional cut at each ovary to free the uterus. Transfer the uterus into one of the 60 mm Petri dishes containing cold 1x PBS.

2.5. Using straight scissors, cut the uterine wall between each implantation site. Pick up a decidua with a glass pipet and transfer into the 35 mm Petri dish with 1x PBS. Under a dissection microscope, insert straight scissors into the space between the decidua and the uterine wall. Cut and remove the uterine wall.

2.6. With fine forceps, remove the decidua and Reichert's membranes from the embryo by carefully making transverse incisions along the tissue and pulling the tissue away from the yolk sac. Remove yolk sac and amniotic sac by carefully pulling the tissue away from the embryo and making cuts at the allantois and umbilical vein.

NOTE: Yolk sacs can be used for genotyping embryos.

2.7. Transfer each embryo with a glass pipet into individual 2 mL tubes filled with 1 mL of 1x PBS. Label each tube with a unique identifier.

2.8. To fix embryos, carefully remove the 1x PBS and add 4% paraformaldehyde (PFA) solution in 1x PBS. Incubate at 4 °C with gentle agitation overnight.

NOTE: 4% PFA fixation is suitable for the antibodies mentioned in this protocol. However, fixation procedures should be optimized for additional antibodies.

3. Embryo staining

NOTE: In this section, embryos are permeabilized and stained with primary and secondary antibodies. Because PAA development proceeds rapidly, differences in embryonic stage will greatly affect the analysis downstream. Therefore, embryos must be age-matched by carefully counting somites to match control and mutant pairs prior to further manipulations.

3.1. To wash embryo(s), carefully remove 4% PFA and add 1x PBS. Gently invert the tube(s) several times. Place tube(s) right-side up and allow the embryo(s) to sink. Repeat wash 3 times. Place the tube(s) with embryo(s) on ice.

NOTE: (Optional stopping point) Following the washes, embryos can be dehydrated in graded series of MeOH for 30 min per dilution, as in section 1.3, and stored at -20 °C in 100% MeOH for later use for up to 6 months.

3.2. For E10.5 embryos, use a glass pipet to transfer one embryo to a 35 mm Petri dish filled with chilled 1x PBS. Carefully pinch the embryo just above the hind limb with fine forceps and make a transverse cut to remove the posterior half of the embryo. This allows the embryo to lay flat in a sagittal position for step 4.2. Place the embryo back into the 2 mL tube with fresh 1x PBS.

NOTE: A control and mutant embryo can be paired and stained with the same antibody solution in one tube, for steps 3.3 through 3.8.

3.2.1 If staining two embryos together, cut the head off of one embryo above the first pharyngeal arch by pinching with fine forceps to make a transverse cut. This will distinguish embryos of two different genotypes within each tube.

3.3. To permeabilize the embryo(s), pipet out 1x PBS from the tube, being careful not to touch the embryo(s). Add 1 mL of PBST. Place the tube at 4 °C with gentle agitation overnight.

NOTE: (Optional stopping point) Embryos can be kept in PBST solution at 4 °C for several days.

3.4. To prevent non-specific binding of antibodies, first remove PBST from the tube, being careful not to touch the embryo(s). Add 600 µL of blocking buffer solution to the embryo(s). Block the embryo(s) at 4 °C with gentle agitation overnight.

NOTE: Blocking solution needs to be spun at top speed on a benchtop centrifuge immediately before use to remove debris.

3.5. To stain and quantify ECs, use antibodies against VEGFR2 and ERG. Antibody solutions are made in the blocking buffer. Anti-VEGFR2 antibody is diluted 1:200 and ERG antibody is diluted 1:1000.

NOTE: Antibody solutions need to be spun at top speed on a bench-top centrifuge immediately

before use to remove particulates.

3.5.1. To incubate embryo(s) with primary antibodies, remove blocking buffer solution from the tube, being careful not to touch the embryo(s). Add 600 µL of primary antibody solution to each tube. Incubate embryo(s) at 4 °C with gentle agitation for 4-5 days.

3.6. To wash the embryo(s) of the antibody solution, first remove the primary antibody solution from the tube. Wash embryo(s) every hour with 1 mL of PBST at room temperature (RT) with gentle agitation. Wash embryo(s) 4-5 times during the day and then incubate at 4 °C with gentle agitation overnight. Repeat washes the following day.

3.7. Make secondary antibody solutions by diluting anti-goat Alexa Fluor 488 and anti-mouse Alexa Fluor 555 1:300 in blocking buffer. Dilute the stock DAPI 1:1000 in blocking buffer.

NOTE: Antibody solutions must be spun at top speed on a bench-top centrifuge immediately before use to remove particulates. In addition, other Alexa Fluor dyes can be used in lieu of 488 or 555.

3.7.1. To incubate the embryo(s) with secondary antibodies, remove PBST from the tube. Add 600 µL of secondary antibody solution to each tube. Incubate embryo(s) at 4 °C with gentle agitation for 4-5 days.

3.8. To wash the embryo(s) of the antibody solution, first remove the secondary antibody solution from the tube. Wash the embryo(s) every hour with 1 mL of PBST at RT with gentle agitation. Wash embryo(s) 4-5 times during the day and then incubate at 4 °C with gentle agitation overnight. Repeat washes the following day.

4. Embedding embryos in agarose

NOTE: In section 4, the embryo(s) will be embedded in agarose. This embedding process serves two purposes: to properly orient the embryo prior to imaging, and to aid in locating the embryo after it has been cleared in BABB (steps 5.2.2 – 5.3.2).

4.1. Prepare 200 mL of 1% agarose solution by adding 2 g of agarose to 200 mL of dH_2O. Microwave until all agarose is dissolved.

NOTE: Remaining agarose can be stored at 4 °C and reheated for later uses.

4.2. Using a plastic paraffin mold and glass pipet, gently transfer one embryo to the mold. Carefully remove PBST from embryo. Place the embryo in a sagittal position. **Quickly**, add about 0.5 mL of hot agarose to the mold – just enough to cover the embryo and fill the mold. Ensure that no air bubbles surround the embryo.

4.3. Place the mold on ice and cover with aluminum foil until the agarose has solidified.

NOTE: Do not allow the embryo to dry following the removal of PBST. Agarose solution must be warm enough to remain liquid when it is being added to the embryo. Add just enough agarose to cover the embryo, but not too much, otherwise it will be difficult to image. Image depth is determined in part by the working distance of the objective.

5. Dehydration and tissue clearing

NOTE: In this section, embryo(s) are dehydrated using methanol series, then cleared in the organic solvent, BABB, and mounted between two coverslips separated by a rubber spacer; in this protocol Fast Well rubber spacers are used. The Fast Well bumper has a double-sided adhesive surface. The spacer is needed to create a well, in which the embryo will be placed and held between two coverslips.

5.1. Methanol dehydration

5.1.1. Label new 2 mL tubes, one per embryo. Add 1 mL of 25% MeOH per tube.

5.1.2. Using a clean scalpel, gently cut the agarose around the embryo, leaving enough around the embryo so that it can be picked up by forceps. Use fine forceps to gently grab the agarose with the embedded embryo and place it into the labeled tube with 25% MeOH. Do not allow the forceps to touch the embryo.

5.1.3. Incubate embryo(s) at RT with gentle agitation for 1 hour in the dark.

5.1.4. Remove 25% MeOH from the tube, being careful not to touch the embryo. Add 1 mL of 50% MeOH per tube. Incubate at RT with gentle agitation for 1 hour in the dark.

5.1.5. Remove 50% MeOH from the tube, being careful not to touch the embryo. Add 1 mL of 75% MeOH per tube. Incubate at RT with gentle agitation for 1 hour in the dark.

5.1.6. Remove 75% MeOH from the tube, being careful not to touch the embryo(s). Add 1 mL of 100% MeOH per tube. Incubate at RT with gentle agitation for 1 hour in the dark. Repeat 100% MeOH wash twice.

5.2. Clearing with BABB

5.2.1. Remove 100% MeOH from the tube, being careful not to touch the embryo. Add 1 mL of 50% BABB per tube. Incubate at RT with gentle agitation for 1 hour in the dark.

5.2.2. Remove 50% BABB from the tube, being careful not to touch the embryo. Add 1 mL of 100% BABB per tube. Incubate at RT with gentle agitation for 1 hour in the dark. Repeat 100% BABB wash twice.

NOTE: (Optional stopping point) Embryos can remain in 100% BABB in tubes for about a week. Longer storage will cause BABB to dissolve the plastic of tubes.

5.3. Mounting embryos for imaging

5.3.1. Place a Fast Well bumper on a 24 mm x 60 mm #1.5 glass cover slip, by peeling off the plastic adhesive from one side. Make sure there are no air bubbles between the coverslip and bumper by applying gentle pressure on the plastic adhesive atop the rubber bumper. Label the coverslip according to embryo number, genotype, and antibodies used for staining.

NOTE: Any spacer can be placed between the coverslips as long as it is thick enough to prevent crushing or squishing an embryo. We use Fast Well spacers due to their thickness and convenience, which includes adhesive surfaces on either side of the spacer for securing it to coverslips.

5.3.2. Carefully pipet out and discard the 100% BABB from the tube. After visualizing the agarose-embedded embryo in the tube, use fine forceps to pick up the agarose and carefully transfer the embryo onto the coverslip inside the Fast Well – do not allow the forceps to touch the embryo.

5.3.3. Remove the second plastic adhesive from the bumper and place the second coverslip on top. Remove air bubbles by gently pressing on the coverslip. Be careful to not break the glass.

NOTE: Samples can be stored flat in a slide holder in the dark at RT for up to a year if the seal is tight.

6. Acquisition of data

NOTE: In the following steps, the endothelium of pharyngeal arches 3, 4, and 6 will be imaged using confocal microscopy.

6.1. Positioning of slides on microscope stage

6.1.1. To image embryos, use a confocal microscope equipped with a 20x water immersion objective, numerical aperture 0.95, working distance 0.95 mm, and the NIS-Elements AR 5.11.01 64-bit software.

6.1.2. Using wide-field fluorescence, visually locate the pharyngeal arches. Center the field view around the 4^{th} PAA.

6.1.3. If the objective's field of view does not capture the entire pharyngeal arch area, take and stitch a large panel of images with 1% overlap. To prevent movement of the sample during the acquisition of the large image, gently secure the cover slip assembly to the stage using molding clay.

6.2. Setting up acquisition parameters

6.2.1. Set the pinhole size to 1.0.

6.2.2. Under the **ND Acquisition** tab, set the top and bottom limits of imaging using the coarse adjustment. Set Z step size according to software specifications. Determine the thickness that can be imaged by the working distance of the objective and the clarity of the sample.

6.2.3. Due to the thickness of the embryo, adjust the gain throughout the Z-stack. Set the laser intensity and gain at the middle of the Z-stack for each channel (405, 488, and 555) and assign values under the **Z Intensity Correction** tab. Set the same values for the bottom slice.

6.2.4. Scroll through the embryo until fluorescence signals begin to appear dimmer. Increase the gain of each channel until signal intensity appears similar to the previous segment. Assign the new value under the **Z Intensity Correction** tab. Repeat until z-stack is complete. Import settings back to **ND Acquisition**.

6.2.5. Run scan using **Run Z Correction** option.

7. Analysis using the Imaris software

NOTE: In these steps, confocal images will be analyzed using the microscopy image analysis software, Imaris version 9.2.0. During this analysis, we will first select regions of interest to be analyzed by creating surfaces. Next, we will use the **Mask** function to visually separate these regions. Finally, we will use the **Spot** function to quantify the number of ECs within each region of interest.

7.1. Depending on the imaging software used in step 6, convert images to .ims using **Imaris File Converter**.

7.2. Open the .ims files. Set image to **Orthogonal** under **Camera/Labels | Camera Type** panel.

7.3. Locate the PAAs and orient the image for surfacing.

NOTE: When the files are first opened, they will appear as a 3D compilation of all slices imaged. In this step, the PAAs will be located by making the 3D image into a 2D image. The 2D image then allows for the PAAs to be properly oriented for analysis.

7.3.1. Under the **Properties** panel, turn off **Volume**. Under the **Properties** panel, click on **Add New Ortho Slicer**. Set **Slice Orientation** to the **XY Plane**. Use the **Slice Position** to scroll through the image until finding the PAAs.

7.3.2. If the PAAs are not parallel to the top and bottom of the image, freely rotate the image

using the mouse cursor so that PAAs run from left to right across the screen. Under the **Image Processing** drop down menu, select **Free Rotate** and click **OK**.

7.4. Surfacing the 3rd Pharyngeal Arch (Figure 2A, B – B")

NOTE: In these steps, the pharyngeal arches and PAAs will be traced using the **Surface** tool to generate a 'surfaced' region of interest. This will allow for each region of interest to be visually isolated from the surrounding tissue. Herein we describe the steps to surface and analyze the endothelial components of the 3rd pharyngeal arch. Pharyngeal arches 4 and 6 are analyzed similarly.

7.4.1. To surface the endothelium in the entire 3rd pharyngeal arch, click on the **Add New Surface** button located under the **Properties** panel. Double click on **Surface 1** and rename the new surface to "3rd Pharyngeal Arch".

7.4.2. Select **Skip automatic creation, edit manually**. Set Surface Orientation to the YZ Plane (coronal orientation). Use the Slice Position to place the 3rd pharyngeal arch surface plane to where the 3rd PAA and Dorsal Aorta connect.

7.4.3. Rotate the image so that the 3rd pharyngeal arch surface plane is in view. Turn off Ortho Slicer 1.

7.4.4. Under the **Draw | Contour | Mode** tab, select the **Distance Drawing Mode** function. Adjust parameter settings if needed. Maintain consistent surfacing parameters between samples. For this example, **Vertex spacing** is 10 μm.

7.4.5. To begin surfacing, press the **Esc** key and then click on the **Draw** button. Trace the perimeter of the 3rd pharyngeal arch with the mouse cursor. Use the Slice Position to move 10-25 slices. Trace the perimeter of the pharyngeal arch. Repeat until the pharyngeal arch is fully traced.

7.4.6. To generate the surface of the traced region, select the **Create Surface** button in the Properties panel.

7.5. Surfacing the 3rd PAA (Figure 2C – C")

7.5.1. To surface the endothelium of the 3rd PAA, first turn off the surfaced region from step 7.4, by deselecting the 3rd Pharyngeal Arch surface box. Then, click on the **Add New Surface** button again. Double click on **Surface 1** and rename the new surface to "3rd PAA".

7.5.2. Select **Skip automatic creation, edit manually**. Set Surface Orientation to the YZ Plane (coronal orientation). Use the **Slice Position** to place the 3rd PAA surface plane to where the 3rd PAA and Dorsal Aorta connect, then repeat steps 7.4.

7.6. Masking of surfaced structures

NOTE: In the following steps, each surfaced region of interest will be Masked. Masking allows the region of interest to be visually distinct from the rest of the imaged tissue and allows for the quantification of these distinct structures of interest. Below, we describe the steps in Imaris to visualize and analyze the PAA endothelium, as well as the plexus – the smaller vasculature surrounding the PAAs within the pharyngeal arches. In these steps, the 3rd PAA surface will be masked in order to visualize and perform analysis only on the PAAs using the Spot function described in Section 7.7.

7.6.1. Select **Volume** in order to visualize all masked channels. Press the **Esc** key in order to rotate the image and position the image into an XY position.

7.6.2. Under the **Edit** tab, select **Mask Selection** for the 3rd PAA. Select the DAPI channel and click **OK**. Repeat for the remaining channels.

7.6.3. On the keyboard, press **Ctrl + D** to view the Display Adjustments panel. Select each new channel and rename them to make it clear what each channel shows. For example, this will lead to three new channels: "3rd PAA DAPI", "3rd PAA ERG", and "3rd PAA VEGFR2".

NOTE: In steps 7.6.4-7.6.7 we will create channels for pharyngeal arch plexus only.

7.6.4. To visualize the endothelial plexus separately from the PAA, we will first select **Mask Selection** for the 3rd PAA surface. Select the DAPI channel. Uncheck **Select voxels outside surface to** and check **Select voxels inside surface to** buttons, set **Select voxels inside surface to zero**. Click **OK**.

7.6.5. Repeat for the remaining channels. This operation will exclude the region containing the PAA from the new masked channels. Rename channels to make it clear what each channel shows. For example, this will lead to three new channels: "Non-PAA DAPI", "Non-PAA ERG", and "Non-PAA VEGFR2".

7.6.6. To visualize endothelial plexus within the 3rd pharyngeal arch, select **Mask Selection** under the **Edit** tab, for the 3rd Pharyngeal Arch surface. Select the **Non-PAA DAPI** channel. Click **OK**.

7.6.7. Repeat for the remaining Non-PAA channels. Rename channels to make it clear what each channel shows. For example, this will lead to three new channels: "Plexus DAPI", "Plexus ERG", and "Plexus VEGFR2".

7.7. Quantification of EC numbers

NOTE: The expression of ERG marks endothelial nuclei making it convenient to quantify EC numbers. In these steps, number of ECs will be quantified using the Spot function to generate a

spot for each EC marked by ERG expression in the PAA and plexus. In section 7.7, spots will be generated for each ERG-positive cell in the masked PAA, followed by the deselection of spots in ERG-positive, VEGFR2-negative cells.

7.7.1. On the keyboard, press **Ctrl + D** to view the Display Adjustments panel. Turn off all channels except for PAA ERG.

7.7.2. Under the properties tab, click on the **Add New Spots** button. Click on **Spots 1** and rename it to "PAA Total Number of ECs**"**. Click on the **blue arrow** button. For **Source Channel**, select the PAA ERG channel. Adjust the **Estimated XY Diameter** to 4 μm. Proceed to the next panel by clicking on the blue arrow button.

7.7.3. Adjust number of spots seen using the sliding scale, to ensure that each EC nucleus (marked by ERG expression) is represented by one spot. Click on the **green double arrow** button.

7.7.4. Turn off the PAA ERG channel in the Display Adjustment. Turn on the PAA VEGFR2 channel to visualize PAA endothelium.

7.7.5. To accurately quantify the number of ECs, we ensure that each spot expresses both EC markers, ERG and VEGFR2. To do this, select **Surface of Object** under the **Edit** tab **| Add/Delete** panel. Press the **Esc** key and delete any spots that are not VEGFR2 positive, by holding down shift and selecting the spot.

NOTE: In the following step, spots will be generated for each ERG-positive cell in the masked plexus, followed by the deselection of spots in ERG-positive, VEGFR2-negative cells.

7.7.6. Under the properties tab, click on the **Add New Spots** button. Select **Spots 1** and rename to "Plexus Total Number of ECs**"**. Click on the **blue arrow** button. For Source Channel, select the Plexus ERG channel. Adjust the **Estimated XY Diameter** to 4 μm. Repeat steps 7.7.1 – 7.7.5 for Plexus total number of ECs.

7.7.7. Click on the **Statistics tab** of each spot function to determine the total number of ECs in the 3rd PAA and pharyngeal arch plexus.

7.7.8. Repeat steps 7.7.1 – 7.7.6 for the remaining PAAs and pharyngeal arch plexus.

REPRESENTATIVE RESULTS:

The whole-mount immunofluorescence protocol presented here produces clear and clean results, allowing for the 3D reconstruction of pharyngeal arch endothelium, as seen in **Figure 1A**. It is important to incubate embryos for a sufficient amount of time in each antibody solution to ensure complete penetration through the sample, as well as, thoroughly washing embryos post antibody incubation. In **Figure 1B**, large, bright dots appear as a result of particulate in either the antibody or blocking buffer solutions. We have found that centrifuging each solution before use and longer periods of PBST washes after each antibody incubation resolves this problem.

Figure 2 illustrates the process used to surface a pharyngeal arch and a PAA for analysis as described in section 7 of the protocol. Using the masked function, Imaris software allows surfaced regions to be visually separated and analyzed independently.

Figures 3 demonstrates individual masking of different vascular compartments in the pharyngeal arches: the PAA (**Figure 3A, B, C**) and the plexus (**Figure 3A', B', C'**). Masking allows for the analysis and quantification of EC numbers in each structure separately. In **Figures 3C–C'**, the Spot feature is used to quantify the total number of ECs in both the PAA and plexus, by assigning a single spot for each nucleus expressing ERG. It is important to note that the algorithm used for the Spot function is designed to generate a dot for any pixel of a specified size. ERG, which is used here as a marker of EC nuclei, is also expressed in neural crest cells[8]; neural crest cells do not express VEGFR2. **Figure 3D** illustrates an example of an ERG-positive (green), VEGFR2-negative (pink) spot that has been generated by the Imaris Spot function. As a result, it is essential to verify that each dot represents a single EC and is labeled with both ERG and VEGFR2.

Table 1. Overview of whole mount immunofluorescence protocol.
O/N – overnight; RT – room temperature.

Figure Legends

Figure 1. Comparison of clean and dirty images following whole-mount immunofluorescence.
Sagittal views of E10.5 embryo show the use of anti-VEGFR2 antibody (white) to visualize the PAA endothelium. Embryos thoroughly washed with PBST post antibody incubations (**A**) have a higher signal-to-noise ratio and produce a cleaner image, when compared with embryos that are not thoroughly washed (**B**). Arrows in **B** show areas of noise/dirt that has appeared in the image when an embryo is not thoroughly washed or the antibody solution has not been centrifuged.

Figure 2. Surfacing of pharyngeal arch (PA) and PAA.
A 2D sagittal view (**A**) is used to identify the location of the PAAs in the confocal image. A coronal ortho slicer (**A, yellow line**) is place through the PAAs. The pharyngeal arch (**B**) and PAA (**C**) are then surfaced in the coronal orientation using the Distance Drawing tool in Imaris. The Distance Drawing tool, set to 10 μm, is used to trace the perimeter of the 3rd pharyngeal arch (**B**) or the PAA (**C**). Outlines are drawn every 10-25 slices through the entire arch (**B', C'**). Outlines are combined to generate a 3D surface of the pharyngeal arch (**B"**) or the PAA (**C"**).

Figure 3. Quantification of EC numbers in a PAA and a plexus.
3D reconstructions are used to visualize vessel structure and expression of EC markers in a PAA or in an EC plexus separately. Panels **A-A'** show the expression of VEGFR2 in the PAA (**A, yellow**) and in the plexus (**A', pink**). Panel **A"** illustrates a merge of the PAA and plexus VEGFR2 expression. Panels **B-B'** show the expression of ERG in the PAA (**B, red**) and in the plexus (**B', green**). Panel **B"** illustrates the merge of the PAA and plexus. **C – C'.** The Spot function in Imaris is used to quantify the number of ECs in either the PAA or plexus. Each ERG-positive cell in the PAA (**C, red**) or plexus (**C', green**) are assigned a single spot to mark a single EC. The arrow in **C'-D** shows an example ERG-positive, VEGFR2-negative spot in the plexus that has been generated

by the Imaris Spot function. This spot is excluded from quantification.

DISCUSSION:

The ability to visualize the endothelium in mouse embryos in 3D has provided new insights into their development[3]. Here we present a protocol that allows for high-resolution 3D imaging of embryos, visualization of vascular connectivity, and quantitative analyses of PAA formation. This protocol can be employed to see how genetic alterations or environmental insults impact PAA development. The procedure reported here uses antibodies against VEGFR2 and ERG to visualize PAA formation and quantify EC number; however, additional antibodies can be used to visualize and analyze other aspects of arch artery development, such as neural crest recruitment or smooth muscle cell differentiation. If this procedure is to be used at earlier stages of embryogenesis, it is important to note that some antigens (e.g., ERG) detected in this protocol may not yet be expressed. Other nuclear stains such as DAPI or DRAQ5 or lineage labeling with nuclear-tagged tracers can be used to quantify EC number.

There are several critical steps within the protocol: ensuring that 1) embryos do not become desiccated between solution changes; 2) embryos are thoroughly washed after antibody incubations; and 3) that embryos are completely dehydrated with MeOH before tissue clearing with BABB.

Methanol washes prior to tissue clearing serve two purposes: to eliminate fluorescence due to the expression of fluorescent proteins (e.g. the expression of EGFP or tdTomato used for lineage tracing) in the embryo, and to dehydrate the tissue. The elimination of fluorescence from fluorescent proteins allows for the use of any combination of fluorophores for imaging. Antibodies against EGFP and TdTomato (cherry) can be used to visualize expression of these fluorescent proteins. Alternatively, MeOH can be replaced by tetrahydrofuran to preserve the fluorescence of fluorescent proteins[9].

We have found that embryos which have not been properly dehydrated prior to BABB clearing are difficult to image due to light scattering. BABB is a hydrophobic solution that requires complete dehydration in an organic solvent in order to clear the opaque tissue. Complete clearing ensures the ability to obtain images at the deepest possible levels within the embryo[10,11]. In this protocol, we used a 20x water immersion objective, due to its long working distance and availability at the time of our experiments. Oil immersion objectives are better suitable for this protocol, as BABB and oil have closer refractive indices than water and BABB. However, despite the difference in refractive index, water immersion objective used in this protocol provided excellent image quality.

There are a few limitations of this protocol. BABB clearing utilized here is toxic and corrosive[11–13]. BABB dissolves glue and plastics. If samples are not handled properly during imaging, microscope objective lens can be damaged by BABB that may escape from the sample via cracks in the coverslip or a broken seal between the Fast Well bumper and the coverslip. Clearing methods that do not use organic solvents, such as CLARITY, can be used as alternatives[10,11,14]. CLARITY's refractive index matching solution has a refractive index similar to that of water, which

makes it a suitable clearing method if using a water immersion objective. An additional limitation of this protocol is that it can only be performed on non-living tissues, thus preventing its application for live imaging.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

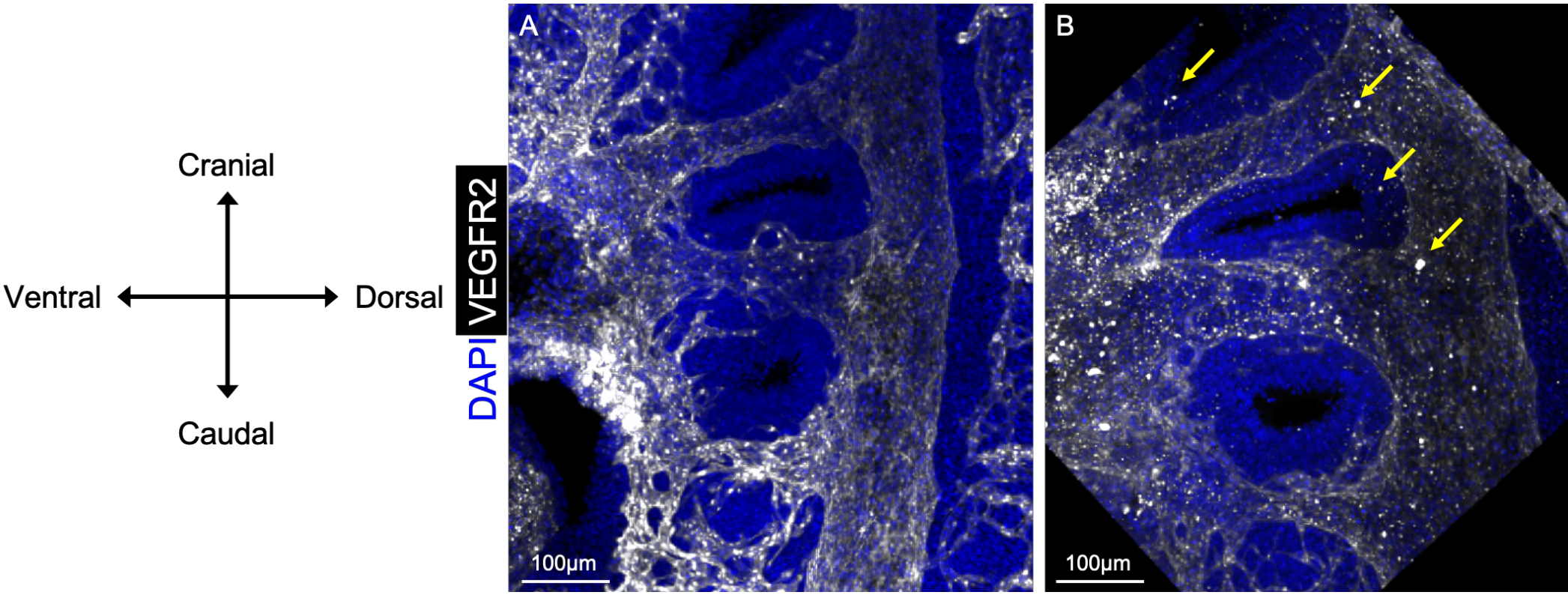


Figure 2

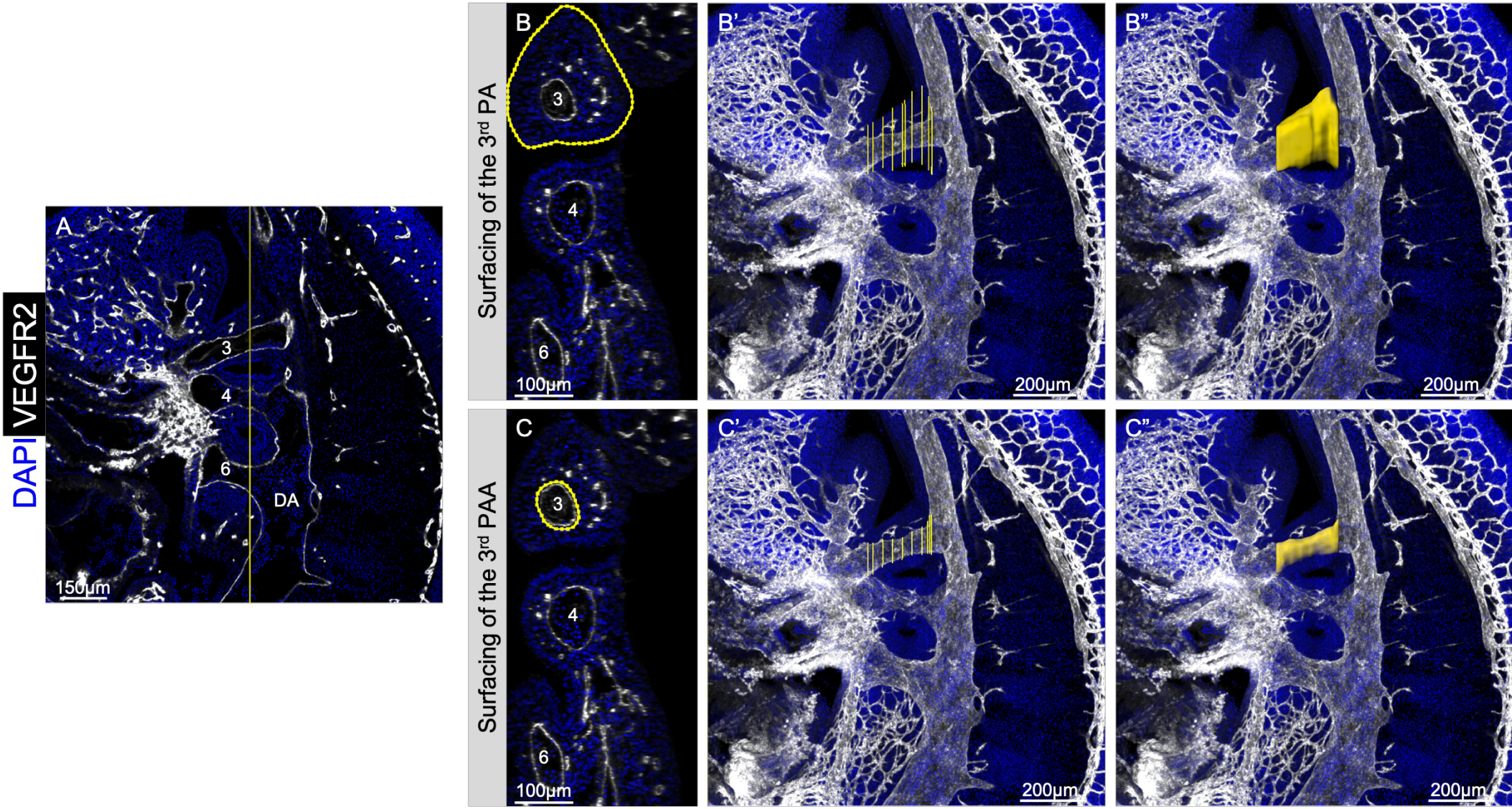
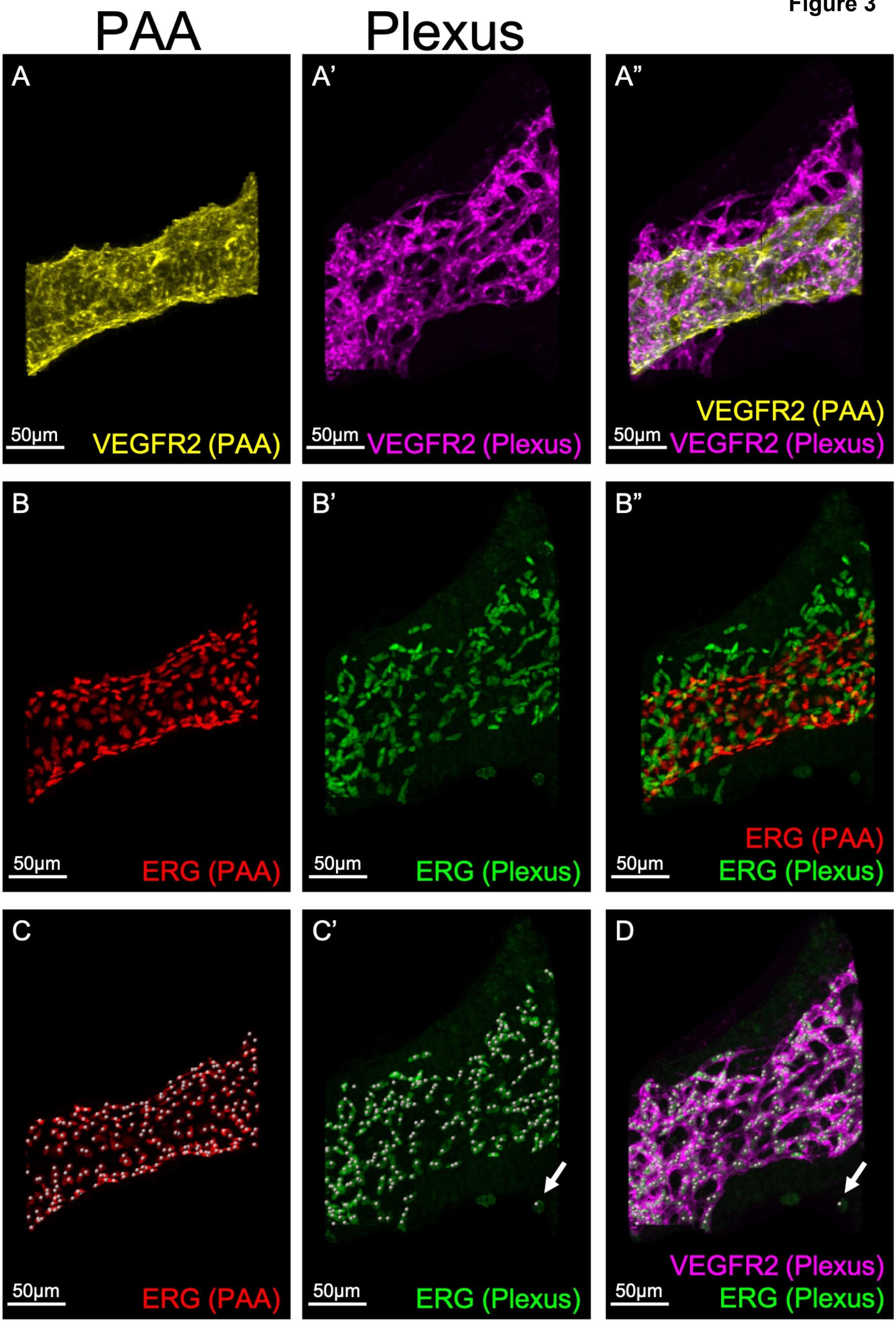


Figure 3



Table

	Step	Time	Temperature
1	PBST Wash/Permeabilization	24 h or O/N	4 °C
2	Blocking Buffer	25 h or O/N	4 °C
3	Primary Antibody	4-5 days	4 °C
4	PBST Wash	4-5 times a day for 2 days	RT (or 4 °C if O/N)
5	Secondary Antibody	4-5 days	4 °C
6	PBST Wash	4-5 times a day for 2 days	RT (or 4 °C if O/N)
7	Embed	N/A	RT
8	Methanol Dehydration and BABB	1 hour per step	RT

Table of Materials

Click here to access/download;Table of Materials;Materials List.xlsx

Name	Company	Catalog Number
10x PBS	MP Biomedicals	PBS10X02
20x water immersion objective	Nikon	MRD77200
Agarose	Bio-Rad Laboratories	1613101
Alexa Fluor 488 anti-goat	Invitrogen	A-11055
Alexa Fluor 555 anti-mouse	Invitrogen	A-31570
Analysis Software	Imaris 9.2.0	
Benzyl Alcohol	Sigma-Aldrich	305197
Benzyl Benzoate	Sigma-Aldrich	8.18701.0100
Cover Slips	VWR	16004-312
DAPI (5 mg/mL stock)	Fisher Scientific	D3571
Eppendorf Tubes (2.0 mL)	Fisher Scientific	05-408-138
Ethanol	VWR	89370-084
Falcon tubes (50 mL)	Corning	352098
Fast wells	Grace Bio Labs	664113
Forceps	Roboz	RS-5015
Goat anti-VEGFR2	R&D Systems, Inc.	AF644
Methanol	VWR	BDH1135-4LP
Microscope	Nikon	A1HD25
Mouse anti-ERG	Abcam	ab214341
Normal Donkey Serum	Sigma-Aldrich	D9663
Paraformaldehyde	Electron Microscopy Sciences	15710
Pasteur pipets	Fisher Scientific	13-678-20D
Petri dishes (35 mm)	Genesee Scientific	32-103
Petri dishes (60 mm)	Genesee Scientific	32-105
Plastic Molds	VWR	18000-128
Scapels	Exelint International Co.	29552
Triton-X-100	Fisher Scientific	BP 151-500

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Dear Nam,

We are submitting the revised manuscript entitled "Visualization and analysis of Pharyngeal Arch Arteries using Whole-mount Immunohistochemistry and 3D Reconstruction." We have made the majority of the changes. However, some changes we could not make since they would render our protocol non-sensical. Our protocol is designed to use Fast Well spacers. If we remove that name, it will make no sense. So we will leave it as is. We added a note that non-commercial spacers could be used instead.

Similarly, if we remove the name Imaris, the procedure that we developed to quantify endothelial cell populations will be non-sensical. I am not aware of any non-commercial or commercial software that can be used here. If you want us to delete the Imaris part, we will have to delete the Analysis sections (section 7) and Figure 3, both of which are essential to the protocol we developed.

Best regards,
Sophie Astrof