Whole-mount immunofluorescence protocol for 3D imaging, reconstruction and quantification of 4th pharyngeal arch artery formation

Elena Ioannou and Christiana Ruhrberg[1]

UCL Institute of Ophthalmology, University College London, 11-43 Bath Street, London EC1V 9EL, UK

[1] Corresponding author:
Phone: +44 7608 4017; FAX: +44 7608 6810; E-mail: c.ruhrberg@ucl.ac.uk

Abstract

The paired pharyngeal arch arteries form during mouse embryonic development to connect the aortic sac of the primitive heart to the paired dorsal aortae. Thereafter, the pharyngeal arch arteries undergo asymmetric remodelling into the aortic arch arteries. The protocol provided here offers detailed instructions and illustrations how to generate and analyse high-resolution confocal microscope images suitable to immunofluorescently visualise endothelial cells in the fourth pharyngeal arch area during artery formation. The protocol allows determining the number of endothelial cells present in the arch, both within the artery and in the vessel plexus around the artery. This protocol is a modification of a published method. Key modifications include a marker to selectively visualise the arterial environment for quantification of pharyngeal arch size, technical adaptations to increase protocol speed and additional illustrations to ease protocol implementation.

Key words: pharyngeal arch arteries, whole-mount immunofluorescence, endothelial cells, tissue clearing, 3D reconstruction, surface rendering

1. Introduction

During mouse embryogenesis, the pharyngeal arch arteries (PAA) form as five pairs of symmetrical structures in a rostro-caudal fashion to connect the aortic sac and the paired dorsal aorta ***(1)***. The arteries take their name from the pharyngeal arches (PA) they form in, and are termed the first, second, third, fourth and sixth pair. In the mouse embryo, the formation of these arteries starts around embryonic day (E) 8.5 and is completed by E10.5. The first and second PAA regress early whereas the third, fourth and sixth undergo asymmetric remodelling to form the aortic arch arteries. The third PAA gives rise to the common carotid arteries. The fourth left PAA contributes to the aortic arch and the fourth right PAA becomes a proximal segment of the subclavian artery. The sixth left PAA becomes the ductus arteriosus and the sixth right PAA regresses ***(2)***. The PAA pairs 3, 4 and 6 form via vasculogenesis, a process of blood vessel formation in the embryo that involves the *de novo* production of endothelial cells and their assembly into a plexus ***(3)***. Failure of PAA formation or remodelling causes congenital heart defects, for example, similar to those seen in patients with DiGeorge Syndrome ***(4, 5)***. Comprehensive knowledge of the mechanisms that regulate PAA development therefore identifies causes congenital heart disease and, in the long run, will improve molecular diagnosis and identify suitable strategies for intervention.

A plethora of approaches have been used to visualise and analyse PAA formation, such as vascular casts, India ink injection, high resolution episcopic microscopy, immunofluorescence of tissue sections and whole mount immunohistochemistry ***(1, 4 – 11)***. Although all these techniques have provided invaluable information on the timing and mechanisms of PAA formation, these approaches do not provide information about how PAA pairs are formed. The protocol below is an adaptation of the technique developed in Sophie Astrof's lab to observe PAA formation ***(3,12)***. This approach involves whole-mount immunofluorescence staining for the vascular endothelial growth factor receptor 2 (VEGFR2, also known as KDR or FLK1) and the nuclear endothelial marker ETS-related gene (ERG) as a cell surface and nuclear endothelial markers, respectively, together with staining for islet 1 and islet 2 (ISL1/2) to mark the PA endoderm and ectoderm, followed by tissue clearing with benzyl benzoate/benzyl alcohol (BABB). Subsequent high-resolution imaging of embryos using laser confocal microscopy and 3D image rendering with appropriate software such as Imaris. This workflow allows the embryonic vasculature of the PAA endothelium to be visualised by surface rendering and quantitatively analysed by counting the number of endothelial cells in both the PAA and the PA vascular plexus surrounding them. This protocol can be adapted for the analysis of other vascular networks.

2. Materials

2.1 Reagents

Prepare all solutions using ultrapure water and analytical grade reagents.

1. **Phosphate-buffered saline (PBS):** to make 1 L of 1 x PBS isotonic solution, add 100 mL of 10 x PBS to 900 mL of water in a glass bottle and mix well. 1 x PBS solution contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4. Store at 4 °C.

2. **Phosphate buffered saline containing 0.1% Triton-X-100 (PBST):** to make 1 L of 0.1 % PBST, prepare 900 mL of 1 x PBS and add 1 mL of Triton-X-100 using a 1 mL syringe, because Triton-X-100 is viscous. Adjust final volume to 1 L with 1 x PBS. Filter-sterilise the final solution prior to using it. Store at 4 °C.

3. **Blocking buffer:** to make 300 µL of 10% (v/v) serum free protein block in PBST, add 30 µL of serum free protein blocking solution (Dako) into a 1 mL reagent tube and add PBST to a final volume of 300 µL. Make this solution fresh each time.

4. **Methanol (MeOH) dilutions:** to make 25 %, 50 %, 75 % (v/v) MeOH in PBS and 100 % MeOH, in a fume hood, use 50 mL disposable tubes and add 12.5 mL of MeOH and PBS to a final volume of 50 mL to make 25 % MeOH/PBS, 25 mL of MeOH and PBS to a final volume of 50 mL to make 50 % MeOH/PBS, 37.5 mL MeOH and PBS to a final volume of 50 mL to make 75 % MeOH/PBS, or 50 ml of MeOH for 100 % MeOH. Vortex to mix well. Store at room temperature (RT). **CAUTION:** MeOH is toxic and flammable, wear gloves.

5. **Benzyl alcohol-benzyl benzoate (BABB):** to make 100 % BABB, use two parts benzyl benzoate and one part benzyl alcohol. In a fume hood, add 32 mL of benzyl benzoate to 16 mL of benzyl alcohol in a 100 mL glass bottle. Cover the glass bottle with aluminium foil to protect it from light. Store at RT. **CAUTION:** BABB is a toxic, corrosive solution. Handle in a fume hood whilst wearing appropriate protective clothing.

6. **Sodium azide (NaN_3):** to make 0.02 % NaN_3, add 2 µL of 10 % NaN_3 stock solution to a final volume of 1 mL water. To make the 10 % stock solution of NaN_3, weigh 10 g of NaN_3 and dissolve in 100 mL of distilled water. Store at RT. **CAUTION:** NaN_3 is toxic, wear gloves.

7. **Formaldehyde (4 %):** to make 100 mL of 4 % formaldehyde, pre-heat 50 mL 1 x PBS at 65 °C. Add 4 g paraformaldehyde in a fume hood and dissolve for 15 minutes at 65 °C using the flow hood. Add 12.5 µL 10 M NaOH to the solution, then adjust the final volume to 100 mL. Swirl the bottle gently to dissolve the PFA. Filter-sterilize the final solution through a 0.45 µm membrane filter to remove any particulate matter and aliquot in 15 mL tubes. Store at -20 °C. **CAUTION:** Formaldehyde is known to be toxic, allergenic and carcinogenic – avoid contact with skin and inhalation (*see* **Note 1**).

8. **Ethanol (70 %):** to make 100 mL of 70 % ethanol (v/v), to 70 mL of absolute ethanol add water to a final volume of 100 mL. Store at RT. **CAUTION:** ethanol is toxic and flammable, wear gloves.

9. **Low-melting agarose (1 %):** to make 200 mL of 1 % low-melting agarose solution, weigh 2 g of low-melting agarose and transfer to a 500 mL glass bottle. Add 200 mL of distilled water. Swirl the bottle to suspend the powder and microwave for 1 minute or until agarose is dissolved – become a clear solution (*see* **Note 2**). Be careful not to overheat the solution, to prevent the agarose from spilling in the microwave. Cool to 55 °C before use. Store the remaining 1 % low-melting agarose at 4 °C.

10. **Fab fragment affinity-purified antibody:** unconjugated Fab fragment donkey anti-mouse IgG (H+L). Store at 4 °C.

11. **Primary antibodies:** mouse anti-mouse ISL1/2, goat anti-mouse VEGFR2 and rabbit anti-mouse ERG. Store at -20 °C.

12. **Secondary antibodies:** Alexa Fluor® 488 FAB donkey anti-mouse, Alexa Fluor® 647 FAB donkey anti-goat, Alexa Fluor® 555 FAB donkey anti-rabbit. Store at 4 °C.

3. Methods

All animal work described below should be carried out according to institutional ethical and legal guidelines, such as those from the Animal Welfare Ethical Review Body (AWERB) and Home Office in the United Kingdom.

3.1 Mouse embryo generation

To isolate E10.5 embryos, perform timed matings. The morning after the mating, check for the presence of a vaginal (copulation) plug which signifies a potential pregnancy. Consider the day of finding the vaginal plug as E0.5 and harvest embryos 10 days later. To account for the rapid changes during early development, stage match the embryos by carefully counting the number of somite pairs that have formed ***(12)***. Embryos on day 10.5 of gestation usually have a range of 34 to 38 somite pairs.

3.2 Embryo dissection and fixation

1. To dissect E10.5 mouse embryos, you will need a stereoscope, Watchmaker's forceps (size 50 or 55), 60 mm disposable dishes, Pasteur pipettes (cut tip), a 24-well plate, 2 mL safe-lock tubes, a bucket full of ice, cold 1 x PBS and 4 % formaldehyde.

2. Euthanise the pregnant female by cervical dislocation and confirm death with a secondary method.

3. Spray the abdominal area with 70 % ethanol to prevent fur from entering the abdominal cavity. Use forceps to lift the skin of the lower abdomen and make a 'v' shape incision using scissors to expose the abdominal cavity (*see* **Note 3**). Excise the gravid uterine horns and transfer them to a fresh dish filled with cold 1 x PBS. Place the dish with the uterine horns on ice (Fig. 1A).

4. Use fine forceps to make perpendicular cuts between each individual embryo still surrounded by the uterus and transfer them one by one into a fresh dish with cold 1 x PBS for further dissection under the stereoscope (Fig. 1A').

5. Use a pair of forceps to remove the uterine tissue that surrounds the decidua. Locate the placenta attached to the embryo and remove it. Remove Reichert's membrane from the yolk sac. Finally, remove the yolk sac and amniotic sac and excise the allantois and umbilical vein, so that only the embryo proper remains (Fig. 1B). **Tip:** the yolk sac can be used for genotyping.

6. Use a Pasteur pipette with a cut tip end (to fit the embryo without damaging it) to collect each embryo and transfer it to a 24-well plate filled with 1 x PBS where it is essential to identify individual embryos, for example, where a litter has different genotypes (Fig. 1B'). Repeat the process for the rest of the embryos in the litter.

7. Replace 1 x PBS with freshly thawed, cold 4 % formaldehyde and incubate for 2 hours at 4 °C with gentle agitation e.g., in an ice box on a rocking platform (*see* **Note 4**).

8. Following fixation, wash embryos twice with 1 x PBS for 5 minutes at room temperature to remove any residual formaldehyde. Embryos can be stored in 1 x PBS at 4 °C (≤ 2 weeks). For long-term storage, embryos can be dehydrated down a methanol gradient comprised of 25 %, 50 %, 75 % (v/v) MeOH in 1 x PBS and then stored in 100% MeOH in -20 °C (*see* **Note 5**). (Fig. 1C)

3.3 Embryo staining

This protocol is optimised for the following antibodies: mouse anti-mouse ISL1/2, goat anti-mouse VEGFR2 and rabbit anti-mouse ERG. You may need to alter conditions when using other antibodies. Use 0.02 % NaN_3 when incubating to avoid microbial growth that will damage the antibody and/or tissue.

Prepare embryos for the staining protocol:

- Embryos stored in MeOH should be rehydrated using a MeOH gradient comprised of 100 %, 75 %, 50 % and 25 % (v/v) MeOH and finally 1 x PBS.

- Use a Pasteur pipette (cut tip) to transfer the embryo from the 24-well plate to a 30 mm dish filled with 1 x PBS.

- Count the number of somites and record it in your lab book.

- Use forceps to trim off the top of the head, without damaging the PA area; remove the limbs; remove the trunk after the hindlimbs (*see* **Note 6**). This step aids antibody penetration (Fig. 1D).

- Use a Pasteur pipette (cut tip) to transfer the embryo to a labelled 2 mL tube.

Attention: Perform all steps carefully and avoid touching the embryo while pipetting solutions in and out of the tube. Always leave a small amount of liquid surrounding the embryo – do not let the embryo dry at any point.

1. To permeabilise the embryo, use a pipette to remove 1 x PBS and add 1 mL of PBST containing 0.02 % NaN_3. Leave the tube shaking gently at 4 °C overnight.

2. To prevent non-specific binding of antibodies, prepare blocking solution with 0.02 % NaN_3 and include unconjugated Fab Fragment Donkey Anti-Mouse IgG (H+L) 1:200

to prevent background staining caused by the secondary antibody binding to endogenous mouse IgG.

3. Centrifuge blocking solution at > 10,000 g before use to remove particulates. Remove PBST from the tube and add 300 µL of the prepared blocking solution. Block with gentle agitation at 4 °C overnight.

4. Prepare primary antibody solution by adding the antibodies in the blocking solution with 0.02 % NaN_3 and include unconjugated Fab Fragment Donkey Anti-Mouse IgG (H+L) 1:200. To stain and quantify endothelial cells, use antibodies against VEGFR2 (early endothelial marker) and ERG (endothelial cell nuclear marker). To stain for and identify ectoderm/endoderm use ISL1/2. Dilute anti-VEGFR2 and ERG antibodies 1:200 and anti-ISL1/2 1:50. Centrifuge primary antibodies at > 10,000 g in a microfuge before adding them to the blocking solution to remove particulates. Vortex to mix the solution well.

5. Remove blocking solution and replace with 300 µL of blocking solution with the primary antibodies. Incubate with gentle agitation at 4 °C for 72 hours.

6. Remove primary antibody solution and wash the embryo 5 times for 1 hour with 1 mL of fresh PBST at room temperature with gentle agitation.

7. Prepare secondary antibody solution comprised of 1:200 anti-mouse Alexa Fluor 488, anti-goat Alexa Fluor 647 and anti-rabbit Alexa Fluor 555 in blocking buffer. Centrifuge secondary antibodies at > 10,000 g before use to remove particulates. Remove PBST and replace with 300 µL of blocking solution with the secondary antibodies. Incubate embryo with gentle agitation at 4 °C for 48 hours. From this point onwards, protect the samples from light (either keep the 2 mL tube with the embryo in a container that does not allow light to reach the embryo or wrap the tubes in aluminium foil).

8. Remove secondary antibody solution and wash the embryo every hour with 1 mL of fresh PBST with gentle agitation at room temperature five times.

Embedding embryos in agarose

This step is particularly important. It allows handling of the embryo without damaging it after it becomes transparent following the BABB clearing.

1. Place 1 % low-melting agarose solution in the microwave for 45 seconds or until it is melted. It will become a clear bubbling solution when it is hot. Cool it down until is still warm enough to be liquid but not hot, approximately 55 °C (*see* **Note 7**).

2. Use a Pasteur pipette (cut tip) to carefully transfer the embryo to a plastic paraffin mould. Place the embryo in a sagittal position (Fig. 1E). Carefully remove PBST from around the embryo using a pipette. Quickly, add enough amount of warm agarose to the mould to cover the embryo and fill the mould. Ensure that there are no air bubbles around the embryo (*see* **Note 8**).

3. Carefully place the mould on ice until the agarose solidifies. Cover the ice box to protect the fluorescently stained embryo from light exposure.

Dehydration and tissue clearing

Methanol dehydration

1. Use a clean scalpel to cut the agarose from around the embryo. Cut close to the embryo but leave enough agarose to be able to pick the sample up without touching the embryo. Use blunt forceps to gently grab the agarose containing the embedded embryo and place it into a 2 mL tube with 1 mL of 25 % MeOH.

2. Incubate embryo with gentle agitation for 10 minutes in the dark at RT.

3. Remove 25 % MeOH and add 1 mL of 50 % MeOH. Incubate embryo with gentle agitation for 10 minutes in the dark at RT.

4. Remove 50 % MeOH and add 1 mL of 75 % MeOH. Incubate embryo with gentle agitation for 10 minutes in the dark at RT.

5. Remove 75 % MeOH and add 1 mL of 100 % MeOH. Incubate embryo with gentle agitation for 10 minutes in the dark at RT.

6. Repeat 100 % MeOH wash twice (*see* **Note 9**).

Clearing with BABB

Reminder: BABB is toxic and corrosive. Take extra care when using it (*see* **Note 10**).

1. In a fume hood, remove 100 % MeOH from the tube and add 1 mL of 100 % BABB. Incubate embryo with gentle agitation for 10 minutes.

2. In a fume hood safety, replace with fresh 100 % BABB and incubate for 2 hours in the dark at room temperature or at 4 °C overnight (Fig. 1F).

Note: Optional stopping point. Embryos can remain in 100 % BABB in tubes for up to a week.

Mounting embryos for imaging

Mount the embryo between two coverslips in order to allow imaging the embryo on both sides (*see* **Note 11**). It is important to use a divider between the coverslips to prevent the embryo from being squashed. Make sure the divider does not melt when in contact with the corrosive reagent BABB, e.g., use FastWells™ reagent barriers (Fig. 1G).

1. Peel off the adhesive tape from a FastWell™ reagent barrier and attach it to a 24 mm x 60 mm number 1.5 glass cover slip. Tap with your fingers around the barrier and apply gentle pressure to make sure it is attached properly (*see* **Note 12**). Label the

coverslip with important information about the staining such as embryo age (number of somites) and antibodies used.

2. Remove the 100 % BABB from the 2 mL tube containing the embryo and discard it according to your institutional guidelines for safe handling. Locate the embryo and use forceps to pick it up and place it in the middle of the barrier on the coverslip. Add a small drop of 100 % BABB on top of the embryo to cover the agarose area only.

3. Remove the adhesive tape from the other side of the barrier and attach carefully the second coverslip on top. Press gently with your finger around the barrier area only and be careful not to break the glass. Coverslips with the sample can be stored flat in the dark at RT for up to a year if the seal is tight.

3.4 Data acquisition

To image the stained and cleared embryo, use a confocal microscope equipped with a 10x water immersion objective and appropriate software (e.g., NIS-Elements AR or ZEN).

Image the left and right side of the embryo separately by flipping the coverslip sandwich.

1. Place a clear glass slide on the platform and add the coverslip sandwich with the embryo on top of the glass slide. Use a Pasteur pipette to add a drop of distilled water onto the cover slip above the area where the embryo is located. Select a 10x water immersion objective and set the stage position.

2. Select wide-field fluorescence and bring the embryo into the field of view to locate the PA of interest, e.g., the 4th PA. Centre the field of view to the PAA of interest, e.g., the fourth PAA.

3. Set up the acquisition parameters (ND Acquisition tab):

    a) Pinhole size - 1.0 Airy Unit

    b) Frame – X 1024 * Y 1024

    c) Speed – 7

    d) Adjust zoom and specimen orientation

    e) Select the channels – 488, 647, 555

4. Set up Z-stack (Setup Manager):

    a) Select 488 channel only and click ‘live’.

    b) Turn the focus knob on the microscope to adjust the focus. Start from the top of the specimen and move towards the middle. When you see the pharyngeal arch forming, click ‘set first’ to determine the top position. Carry on turning the focus

knob until you reach the middle of the embryo (end of the pharyngeal arch) and press ‘set last’ to determine the bottom position.

c) Adjust the gain master throughout the Z-stack. Set the laser intensity and gain at the middle of the Z-stack for each channel (488, 647 and 555).

d) Run the Z Correction option.

e) Click ‘start experiment’.

f) Save file as <filename>.lsm, including information of parameters and channels used, date, embryo identification, embryo orientation etc.

g) Repeat this procedure for the other side of the embryo.

3.5 Analysis using Imaris software

Imaris is a comprehensive imaging software that allows the visualisation, analysis and interpretation of 3D microscopy images (alternative imaging software may be available that is suitable for this step, but such suitability would need to be established by the reader).

For the analysis of the PAA, e.g., the fourth PAA, three main functions of Imaris are needed:

Surface: to create surfaces of the regions of interest

Mask: to visually separate the regions of interest

Spot: to quantify the number of endothelial cells (EC) in each region of interest

Here, we describe the procedure for fourth PAA analysis.

1. Use Imaris Converted (download for free) to convert <filename>.lsm file to <filename>.ims file.

2. Open Imaris 9.7.0 software and import the <filename>.ims file.

3. The image will open by default with the 3D view and the camera to perspective mode (45 ^{o}). Change the camera to the orthogonal view (Fig.2A) (*see* **Note 13**).

4. Click edit and select ‘Show Display Adjustment’. A new window will pop up with all the three channels: Green (488) – ISL1/2, Red (555) – ERG and Blue (647) – VEGFR2. You can use the display adjustment window to select which channels you want to be visible and adjust them (*see* **Note 14**).

5. Tick off the ISL1/2 channel and use the pointer selection mode to rotate the image until you locate the fourth PAA parallel to the x-axis in the view of plane (dorsal to ventral).

6. Use the orthoslicer function to define the area around the fourth PAA and exclude the outside volume (Fig. 2B):

    a) Go to 'Properties' and click 'Add new Ortho Slicer'  (*see* **Note 15**).

    b) A new line will appear inside the 'Properties' box named "Ortho Slicer 1". Below, a new box named 'Settings' for Ortho Slicer 1 will appear with three plane options (YZ, XZ, XY), as well as a bar which allows you to choose the slice position.

    c) Select 'YZ Plane' and move the slice position to the dorsal side until the 4th PAA meets the dorsal aorta. Information beyond this point is not needed to create the fourth PA and PAA area. Click 'Add new Ortho Slicer' to create Ortho Slicer 2 and choose 'YZ plane'. This time move the slice position towards the ventral side and stop at the point where the fourth PAA meets aortic sac.

    d) Go to 'Display Adjustment' and select the ISL1/2 channel. Click 'Add new Ortho Slicer' to create Ortho Slicer 3 and choose 'XZ plane'. Move the slice position towards the cranial position and stop in the middle of the third PAA. Click 'Add new Ortho Slicer' to create Ortho Slicer 4 and choose 'XZ plane'. Move the slice position towards the caudal position and stop in the middle of the sixth PAA (if not yet formed, below the fourth PA).

Surfacing the fourth Pharyngeal Arch

1. Go to 'Properties' and select 'Add New Surface'  (Fig. 3A).

2. A new line will appear inside the 'Properties' box named "Surfaces 1". Double click on it and rename it "PA". Bellow, a new box will appear named 'Create'. Click 'Skip automatic creation, edit manually' which takes you automatically to the 'Draw'  mode → 'Contour'→ 'Board'. Click 'YZ' under 'Orientation' and use the 'Slice Position' bar to place the board close to Ortho Slicer 1 to mark the beginning of the manual drawing towards the ventral side. At this point tick off Ortho Slicer 1 and 'Volume'  .

3. Use 'Pointer Selection Mode' to rotate the image and bring the YZ plane into view.

4. Go to 'Draw'→ 'Contour'→ 'Mode' and choose 'Distance Drawing Mode'. Set the vertex spacing to 10 µm.

5. Go back to 'Draw' mode → 'Contour'→ 'Board' to begin drawing the surface. Select 'Draw' under the 'Slice Position' and check that a red dot appears under the brush icon which will indicate that the draw mode is on. Use the 'Slice Position' bar to move through the slices. Trace the perimeter of the fourth PA with the mouse cursor and repeat it every 3 slices (9 µm) until the arch is fully traced.

6. Click ‘Create Surface’ to generate the surface of the traced region (Fig. 3B) (*see* **Note 16**). **Tip:** to change the colour of the created surface, go to ‘Properties’→ ‘PA’→ ‘Colour’  .

7. Remove all four Ortho Slicers – not needed any more.

8. Go to ‘Properties’→ ‘PA’→ ‘Statistics’  to get information about the size of the arch.

Attention: remember to ‘Export’ and ‘Save’ the image (*see* **Note 17**).

Create the fourth PA channels

Use the ‘Mask’ function to create the PA channels (*see* **Note 18**):

1. Go to ‘Properties’→ “PA”→ ‘Edit’  and select ‘Mask All…’. A new window will appear.

2. Tick ‘Duplicate channel before applying mask’ and select Green channel. Go to ‘Mask Settings’→ ‘Constant inside/outside’→ ‘Set voxels outside surface to zero’→’OK’.

3. A new channel will appear in the ‘Display Adjustment’ panel named “Masked ISL1/2 channel”. Double click to rename “PA ISL1/2 channel”. Repeat the same process for the ERG and VEGR2 channel. The new channels include only information of the region of interest.

4. ‘Export’ and ‘Save’.

5. Tick off ISL1/2, ERG and VEGR2 channels and keep only the PA ISL1/2, PA ERG and PA VEGFR2 channels. Tick off ‘PA’ surface and turn ‘Volume’ back on.

6. Go to ‘Edit’→ ‘Crop 3D’ and crop it around the PA volume only (*see* **Note 19**).

7. Export and save the new file as “CROPPED”.

Surfacing the fourth Pharyngeal Arch Artery

1. Go to ‘Properties’ and click ‘Add New Surface’ (Fig. 3C).

2. A new line will appear inside the ‘Properties’ box named “Surfaces 1”. Double click on it and rename it “PAA”. Bellow, a new box will appear named ‘Create’. Click ‘Skip automatic creation, edit manually’ which takes you automatically to the ‘Draw’ mode → ‘Contour’→ ‘Board’. Click ‘YZ’ under ‘Orientation’ and use the ‘Slice Position’ bar to place the board at the beginning of the artery and turn off the ‘Volume’. Go to ‘Display Adjustment’ and tick off the ISL1/2 channel (information not useful anymore).

3. Use ‘Pointer Selection Mode’ to rotate the image and bring the YZ plane into view.

4. Go to ‘Draw’→ ‘Contour’→ ‘Mode’ and choose ‘Distance Drawing Mode’. Set the vertex spacing to 10 µm.

5. Go back to ‘Draw’ mode → ‘Contour’→ ‘Board’ to begin drawing the surface. Click ‘Draw’ under the ‘Slice Position’ and check that a red dot appears under the brush icon, which will indicate that the draw mode is on. Use the ‘Slice Position’ bar to move through the slices. Trace the perimeter of the fourth PAA with the mouse cursor and repeat it every slice until the artery is fully traced.

6. Click ‘Create Surface’ to generate the surface of the traced region (Fig. 3D).

7. Go to ‘Properties’→ ‘PAA’→ ‘Statistics’ to get information about the size of the artery.

Attention: ‘Export’ and ‘Save’ image.

Create the fourth PAA channels

Use the ‘Mask’ function to create the PAA channels (*see* **Note 20**):

1. Go to ‘Properties’→ ‘PAA’→ ‘Edit’ and select ‘Mask All…’. A new window will appear.

2. Tick ‘Duplicate channel before applying mask’ and select ‘PA ISL1/2 channel’. Go to ‘Mask Settings’→ ‘Constant inside/outside’→ ‘Set voxels outside surface to zero’→’OK’.

3. A new channel will appear in the ‘Display Adjustment’ panel named ‘Masked PA ISL1/2 channel’. Double click to rename ‘PAA ISL1/2 channel’. Repeat the same process for the PA ERG and PA VEGR2 channel.

4. ‘Export’ and ‘Save’.

Create a channel for the Plexus

Use the ‘Mask’ function to create the Plexus channel (*see* **Note 21**):

1. Go to ‘Properties’→ ‘PAA’→ ‘Edit’ and select ‘Mask All…’. A new window will appear.

2. Tick ‘Duplicate channel before applying mask’ and select “PA VEGR2 channel”. Go to ‘Mask Settings’→ ‘Constant inside/outside’→ ‘Set voxels inside surface to zero’→’OK’.

3. A new channel will appear in the ‘Display Adjustment’ panel named ‘Masked PA VEGFR2 channel’. Double click to rename “Plexus”.

4. ‘Export’ and ‘Save’.

Surfacing the Plexus (automatic creation)

1. Go to ‘Properties’ and click ‘Add New Surface’.

2. A new line will appear inside the ‘Properties’ box named “Surfaces 1”. Double click on it and rename it “Plexus”. Bellow, a new box will appear named ‘Create’. A series of four boxes will appear: first – blue arrowhead (back), second – blue arrowhead (next), third – two green chevrons (finish) and fourth – orange ‘x’ (cancel) which signifies the automatic creation.

3. Click next and go to ‘Source Channel’ and choose “Plexus”.

4. Click next and go to ‘Threshold’→ ‘Threshold (Absolute Intensity)’ and set it manually.

5. Click next and set the number of Voxels.

6. Click finish to complete the automatic creation.

7. ‘Export’ and ‘Save’.

Attention: automatic creation should not be used for quantification, as it not sufficiently accurate.

Quantification of endothelial cell numbers

For the number of fourth pharyngeal arch EC

1. Go to ‘Properties’ and click ‘Add New Spots’  (Fig. 4A).

2. A new line will appear inside the ‘Properties’ box named “Spots 1”. Double click on it and rename it “PA spots”. Bellow, a new box will appear named ‘Create’. A series of four boxes will appear: first – blue arrowhead (back), second – blue arrowhead (next), third – two green chevrons (finish) and fourth – orange ‘x’ (cancel) which signifies the automatic creation.

3. Click next and go to ‘Source Channel’ and choose “PA ERG channel”. Go to ‘Spot Detection’→ ‘Estimated XY Diameter’ and set it to 3 µm (diameter of an endothelial cell).

4. Click next and go to ‘Filter Type’→ ‘Quality’ and select manually the endothelial cells.

5. Click finish to complete the automatic creation.

6. Go to ‘Properties’→ “PA Spots”→ ‘Statistics’ to obtain the number of endothelial cells.

7. ‘Export’ and ‘Save’.

Attention: make sure that each spot is positive for both EC markers (ERG and VEFGR2). In case the automatic creation has selected a spot that it is not positive for both EC markers, manually delete the spot. To do this, go to 'Properties'→ 'PA Spots'→ 'Edit'. Hold down the 'command' key and select the spot that needs to be deleted. The dot will become yellow. Go to 'Edit'→ 'Selection' and click delete. **N.B.** yellow colour on Imaris indicates selection.

For the number of fourth pharyngeal arch artery endothelial cells

1. Create a channel for the fourth PAA endothelial cells:

    a) go to 'Properties'→ 'PA Spots'→ 'Edit' and select 'Mask All…'. A new window will appear.

    b) tick 'Duplicate channel before applying mask' and select "PAA ERG channel". Go to 'Mask Settings'→ 'Constant inside/outside'→ 'Set voxels outside surface to zero'→'OK'.

    c) a new channel will appear in the 'Display Adjustment' panel named "Masked PAA ERG channel". Double click to rename "PAA spots".

1. Go to 'Properties' and click 'Add New Spots' (Fig. 4B).

2. A new line will appear in the 'Properties' box named "Spots 1". Double click on it and rename it "PAA spots". Bellow, a new box will appear named 'Create'. A series of four boxes will appear: first – blue arrowhead (back), second – blue arrowhead (next), third – two green chevrons (finish) and fourth – orange 'x' (cancel) which signifies the automatic creation.

3. Click 'next' and go to 'Source Channel' and choose 'PAA Spots channel'. Go to 'Spot Detection'→ 'Estimated XY Diameter' and set it to 3 µm (approximate diameter of an embryonic endothelial cell in vivo).

4. Click next and go to 'Filter Type'→ 'Quality' and select manually the endothelial cells.

5. Click finish to complete the automatic creation.

6. Go to 'Properties'→ 'PAA Spots'→ 'Statistics' to get the number of endothelial cells.

7. 'Export' and 'Save'.

Notes

1. Transfer the scales and hotplate with magnetic stirrer to the fume hood to avoid exposure to paraformaldehyde. Wear gloves at all times and avoid inhaling it. Use freshly made or freshly thawed formaldehyde. Old formaldehyde solution causes stronger endogenous fluorescence. N.B. formaldehyde is hazardous and must be disposed of in accordance with institutional regulations.

2. Place the lid loosely on top of the glass bottle to keep the bottle from exploding. Pause the microwave half-way through to check that solution hasn't overspilled and to stir the bottle. Solution is ready when big bubbles are formed, and solution is clear.

3. It is important to make upward movements with the scissors to avoid damaging the embryos.

4. E10.5 mouse embryo fixation for this protocol and antibody combination was optimised to 2 hours fixation with 4 % formaldehyde at 4 °C. Fixation time should be checked and adjusted to the antibodies being used.

5. Store fixed embryos in methanol for maximum of 3 months. Longer storage may result in degradation of the proteins used for immunostaining.
6. Trimming the head and trunk of the embryo facilitates better antibody penetration. Cutting the limbs off the embryo allows the sample to be embedded flat, which is important for imaging.

7. Prior to adding the 1 % low melting agarose, you should be able to touch the bottle with your hand (wearing gloves) without burning; alternatively, place bottle into a water bath or oven set to 55 °C for 30 minutes. This is particularly important because very hot agarose can damage the embryo.

8. It is important to add just enough agarose to cover the embryo, but not too much, otherwise imaging will be difficult and results not clear for analysis.

9. Make sure the embryos embedded in agarose are fully dehydrated before adding BABB solution to the 2 mL tube. If not properly dehydrated the embryos will become milky instead of transparent. If this does happen, repeat the final step of the dehydration process and try clearing with BABB again.

10. Use glass or polypropylene plastic that will not dissolve when in contact with BABB. BABB is toxic and corrosive, take extra care when using it.

11. Due to the thickness of the embryo, imagining the two sides individually produces clearer results and more accurate analysis. To achieve that, you should mount the embryo between two coverslips instead of using a slide. This way you can simply flip the coverslip sandwich and image either side.

12. Take extra care when trying to seal the coverslip on top of the barrier. Coverslips are very thin and extremely easy to break. Apply gentle pressure on the area of the coverslip that is occupied by the barrier only. If you touch elsewhere, it is very likely that the glass will break.

13. N.B. the camera view is a matter of personal preference. The analysis can be carried out either way.

14. To change the name or channel display colour, double-click on the current channel's name. A new window will appear with the option to rename the channel and change the colour.

15. N.B. orthoslicers do not add information to the image, they just define the area of interest.

16. If you are not happy with the surface created or want to change an area, go to 'Properties'→ 'Surface'→ 'Creation'  → 'Rebuild'→ 'Skip automatic creation, edit manually'→ 'Contour'→ 'Board'→ 'Visibility'→ 'All'. When you locate the area that needs modification, select 'Delete…' and then repeat drawing procedure. Once modifications are completed click 'Create'. Then modified surface will appear.

17. Creating surfaces manually is time consuming, therefore it is advisable that you save your work immediately after you create them in case the software or computer crashes.

18. To remove unwanted information, you should create a new channel for the pharyngeal arch that will only display the information inside the arch. Use the 'Mask' function to select and set everything outside the arch to zero, which deletes it.

19. This step helps the program to run with a smaller volume of information and therefore minimize the risk of the software crashing. N. B. recommended computer specifications to operate Imaris are 16-32 GB RAM, 3.3 GHz CPU (Intel or AMD) 4 to 6 Cores, graphics: NVIDA Quadro P2000 5 GB, Multiple Fast Hard Disks or (SATA) SSDs, monitor: single 1280 x 1024 pixels up to dual 1920 x 1200.

20. To create a new channel that shows information about the pharyngeal arch artery only, use the 'Mask' function and set everything outside the artery to zero, which deletes it. N. B. you do not need to create a new pharyngeal arch artery channel for ISL1/2 (green) because there is no green staining inside the artery.

21. To see the volume of the plexus for the automatic creation, use the 'Mask' function to create a new channel and set everything inside the artery to zero, which deletes it. You should now only have information about the plexus without the artery.

Acknowledgments

We thank Sophie Astrof and her lab members as well as Ariadna Navarro-Aragall for teaching us this method, Laura Denti and the staff of the Biological Resources Unit for animal husbandry and the Confocal Microscopy Unit for maintaining the imaging equipment. EI is funded by a PhD studentship from the British Heart Foundation, ref. FS/19/29/34367. CR is supported by a Wellcome Investigator Award, ref. 205099/Z/16/Z.

References

1. Hiruma, T., Nakajima, Y., & Nakamura, H. (2002) Development of pharyngeal arch arteries in early mouse embryo. Journal of Anatomy, 201(1), 15–29. https://doi.org/10.1046/j.1469-7580.2002.00071.x
2. Hutson, M. R., & Kirby, M. L. (2007) Model systems for the study of heart development and disease. Cardiac neural crest and conotruncal malformations. Seminars in Cell & Developmental biology, 18(1), 101–110. https://doi.org/10.1016/j.semcdb.2006.12.004
3. Wang, X., Chen, D., Chen, K., Jubran, A., Ramirez, A., & Astrof, S. (2017) Endothelium in the pharyngeal arches 3, 4 and 6 is derived from the second heart field. Developmental Biology, 421(2), 108–117. https://doi.org/10.1016/j.ydbio.2016.12.010
4. Jerome, L. A., & Papaioannou, V. E. (2001) DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. Nature Genetics, 27(3), 286–291. https://doi.org/10.1038/85845
5. Lindsay, E. A., Vitelli, F., Su, H., Morishima, M., Huynh, T., Pramparo, T., Jurecic, V., Ogunrinu, G., Sutherland, H. F., Scambler, P. J., Bradley, A., & Baldini, A. (2001) Tbx1 haploinsufficieny in the DiGeorge syndrome region causes aortic arch defects in mice. Nature, 410(6824), 97–101. https://doi.org/10.1038/35065105
6. Weninger, W. J., Maurer-Gesek, B., Reissig, L. F., Prin, F., Wilson, R., Galli, A., Adams, D. J., White, J. K., Mohun, T. J., & Geyer, S. H. (2018) Visualising the Cardiovascular System of Embryos of Biomedical Model Organisms with High Resolution Episcopic Microscopy (HREM). Journal of Cardiovascular Development and Disease, 5(4), 58. https://doi.org/10.3390/jcdd5040058
7. Phillips, H. M., Stothard, C. A., Shaikh Qureshi, W. M., Kousa, A. I., Briones-Leon, J. A., Khasawneh, R. R., O'Loughlin, C., Sanders, R., Mazzotta, S., Dodds, R., Seidel, K., Bates, T., Nakatomi, M., Cockell, S. J., Schneider, J. E., Mohun, T. J., Maehr, R., Kist, R., Peters, H., & Bamforth, S. D. (2019) Pax9 is required for cardiovascular

development and interacts with Tbx1 in the pharyngeal endoderm to control 4th pharyngeal arch artery morphogenesis. Development, 146(18), dev177618. https://doi.org/10.1242/dev.177618

8. Azaripour, A., Lagerweij, T., Scharfbillig, C., Jadczak, A. E., Willershausen, B., & Van Noorden, C. J. (2016) A survey of clearing techniques for 3D imaging of tissues with special reference to connective tissue. Progress in histochemistry and cytochemistry, 51(2), 9–23. https://doi.org/10.1016/j.proghi.2016.04.001
9. Richardson, D. S., & Lichtman, J. W. (2015) Clarifying Tissue Clearing. Cell, 162(2), 246–257. https://doi.org/10.1016/j.cell.2015.06.067
10. Ertürk, A., Becker, K., Jährling, N., Mauch, C. P., Hojer, C. D., Egen, J. G., Hellal, F., Bradke, F., Sheng, M., & Dodt, H. U. (2012) Three-dimensional imaging of solvent-cleared organs using 3DISCO. Nature Protocols, 7(11), 1983–1995. https://doi.org/10.1038/nprot.2012.119
11. Kuwajima, T., Sitko, A. A., Bhansali, P., Jurgens, C., Guido, W., & Mason, C. (2013) ClearT: a detergent- and solvent-free clearing method for neuronal and non-neuronal tissue. Development, 140(6), 1364–1368. https://doi.org/10.1242/dev.091844
12. Ramirez A, Astrof S (2020) Visualization and Analysis of Pharyngeal Arch Arteries using Whole-mount Immunohistochemistry and 3D Reconstruction. Journal of Visualized Experiments: JoVE (157):10.3791/60797. https://doi.org/10.3791/60797
13. Chan, A., Dong, M., Wang, L., & Chan, W. (2004). Somite as a Morphological Reference for Staging and Axial Levels of Developing Structures in Mouse Embryos. Neuroembryology and Aging*, 3(2)*, 102–110. https://doi.org/10.1159/000089005

Figure legends

Figure 1: Embryo immunofluorescent staining.

(A) Place the culled mouse on its back and use forceps to lift the skin of the lower abdomen, then make a 'v' shape incision (dotted lines). Excise the gravid uterine horns and transfer them to a dish with cold PBS (A'), placed on ice. Use forceps to separate the embryos still in their uterus (A').

(B) Dissect the embryos in a fresh dish with cold PBS under the stereoscope to remove the indicated tissue layers surrounding the embryo. Use a Pasteur pipette with a cut tip to transfer the embryos to a 24-well plate with cold PBS (B'); when all embryos are dissected, replace the PBS with cold 4 % formaldehyde and incubate for 2 h at 4 °C with gentle agitation.

(C) Embryos can be dehydrated through an increasing methanol gradient and stored in methanol in -20°C but should be rehydrated before use in a decreasing methanol gradient and then transferred into PBS. Count the number of somites, then use forceps to trim the embryo along the dotted lines. Perform immunostaining (not shown).

(E) Use a Pasteur pipette (cut tip) to transfer the immunostained embryo into a plastic mould (sagittal position). Remove residual liquid and add warm agarose to fill the mould. Once set, trim excess agarose away and use blunt forceps to transfer the agarose block into a 2 mL tube containing 1 mL of 25% methanol in PBS and dehydrate through an increasing methanol gradient.

(F) In a fume hood, replace methanol with 1 mL of 100% BABB.

(G) Mount the embryo between two coverslips using the FastWells™ reagent barrier with a small drop of 100% BABB on top to cover the agarose area only.

Figure 2: Analysis using Imaris software.

(A) Imaris desktop 3D view after the confocal image is imported. The properties box (outlined in orange) allows choosing functions such as volume, slicers, spots, creating surfaces etc. The navigation box (outlined in red) allows choosing between different camera views. The display adjustment box (outlined in green) allows adjusting the visible channels.

(B) Demonstration of how to use the ortho slicer function to define the area of interest. The settings box (outlined in blue) allows choosing the plane of view, slice position etc.

Figure 3: Surfacing the 4th pharyngeal arch and artery.

(A,B) Manual drawing of the 4th pharyngeal arch. (A) From the properties box (outlined in orange), select the function 'Add New Surface' (circled in red) and subsequently the function 'draw' (circled in white). The blue line (inside the yellow outlined box) illustrates how to draw around the arch for manual creation every 3 µm. (B) Final product of the 4th pharyngeal arch surface after tracing the relevant region in (A) through the sections of the confocal z- stack; the arch surface is shown after 90 $^{\circ}$C rotation relative to (A).

(C, D) Manual drawing of the 4th pharyngeal arch artery. (C) Use the method shown in (A,B); the blue line illustrates how to draw around the artery every 1 µm. (D) Final product of the 4th pharyngeal arch artery surface after tracing the relevant region of the confocal z-stack in (C); the artery surface is shown after 90 °C rotation relative to (C).

Figure 4: Quantifying endothelial cell numbers using the spotting function of Imaris.

(A) From the properties box (outlined in orange), select the function 'Spots' (circled in white) and then the source channel (white arrow). The diameter is set to 3 µm (blue arrow). The spots are then created automatically.

(B) Spots in the pharyngeal arch artery area are shown in white, spots in the pharyngeal arch area are shown in red. A record of the total number of the spots of interest can be obtained by selecting the 'statistics' icon (circled in green).

Figure 1

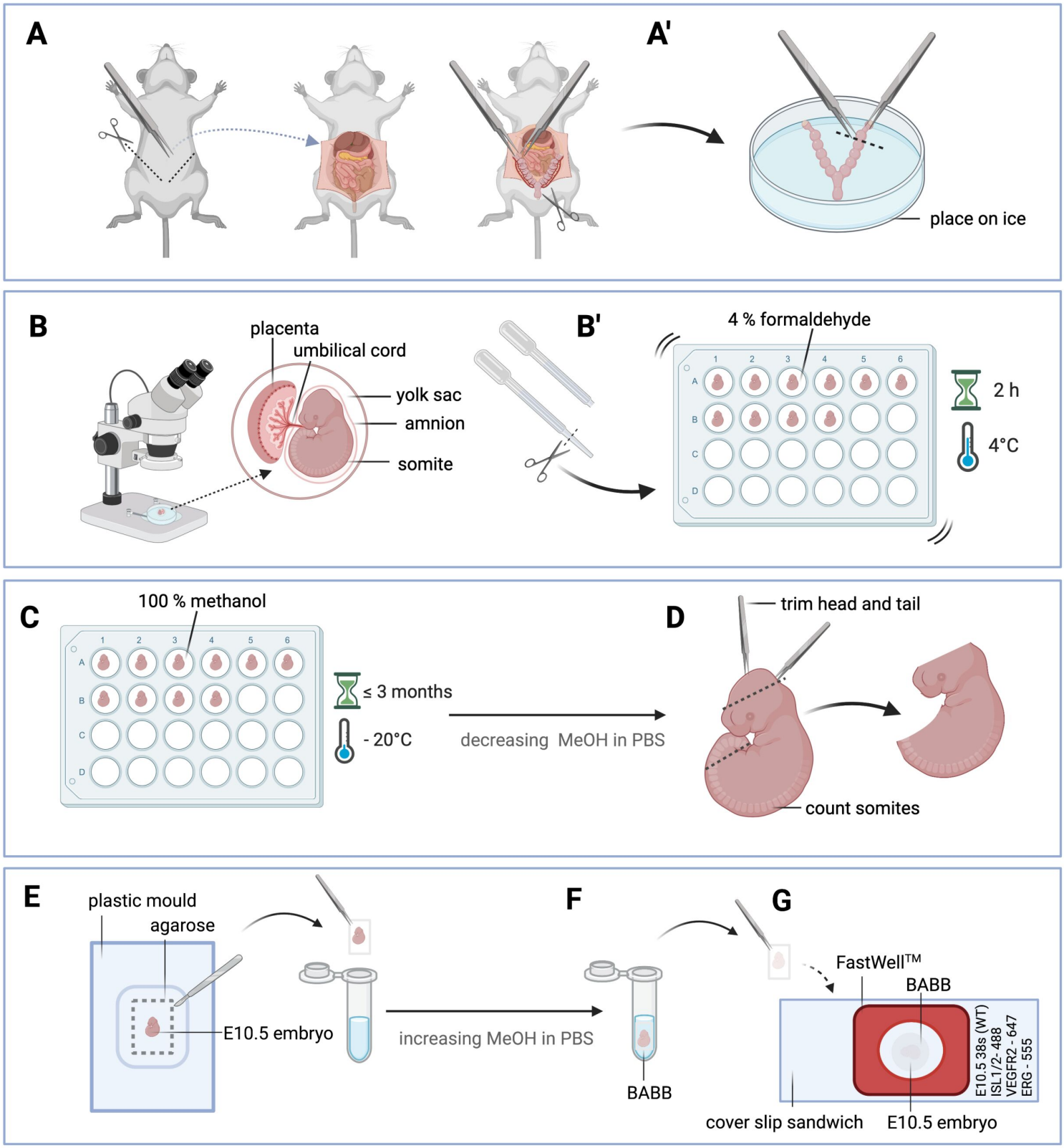
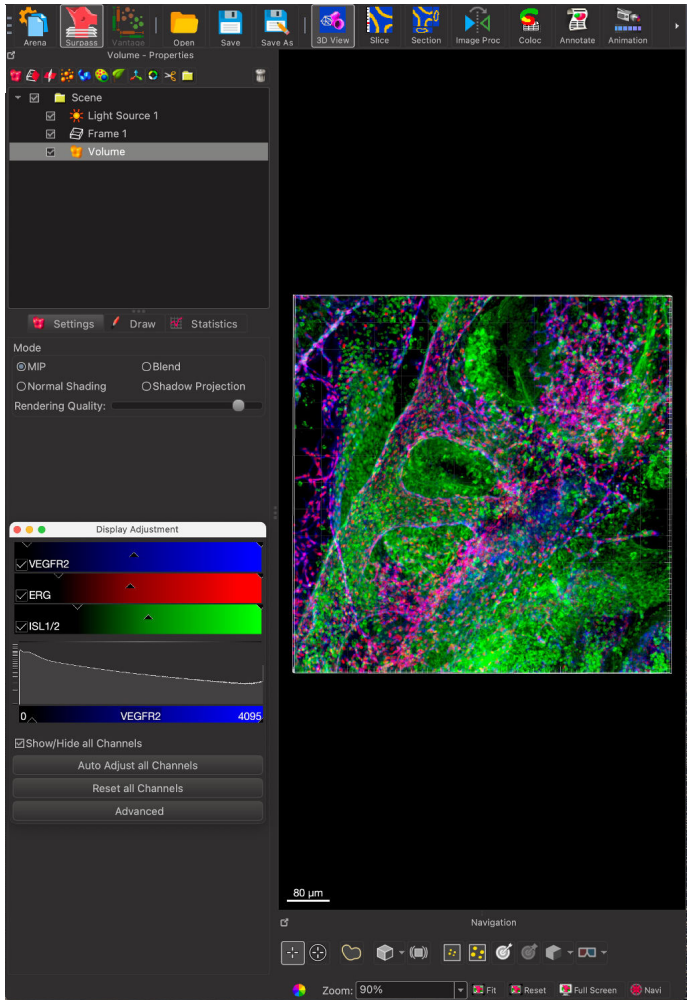

A
A'
place on ice
B
placenta
umbilical cord
yolk sac
amnion
somite
B'
4 % formaldehyde
2 h
4°C
C
100 % methanol
≤ 3 months
- 20°C
decreasing MeOH in PBS
D
trim head and tail
count somites
E
plastic mould
agarose
E10.5 embryo
increasing MeOH in PBS
F
BABB
G
FastWell™
BABB
E10.5 38s (WT)
ISL1/2- 488
VEGFR2 - 647
ERG - 555
cover slip sandwich
E10.5 embryo

Figure 2

A


Arena
Surpass
Vantage
Open
Save
Save As
3D View
Slice
Section
Image Proc
Coloc
Annotate
Animation
Volume - Properties
Scene
Light Source 1
Frame 1
Volume
Settings
Draw
Statistics
Mode
MIP
Blend
Normal Shading
Shadow Projection
Rendering Quality:
Display Adjustment
VEGFR2
ERG
ISL1/2
0
VEGFR2
4095
Show/Hide all Channels
Auto Adjust all Channels
Reset all Channels
Advanced
80 µm
Navigation
Zoom:
90%
Fit
Reset
Full Screen
Navi

B

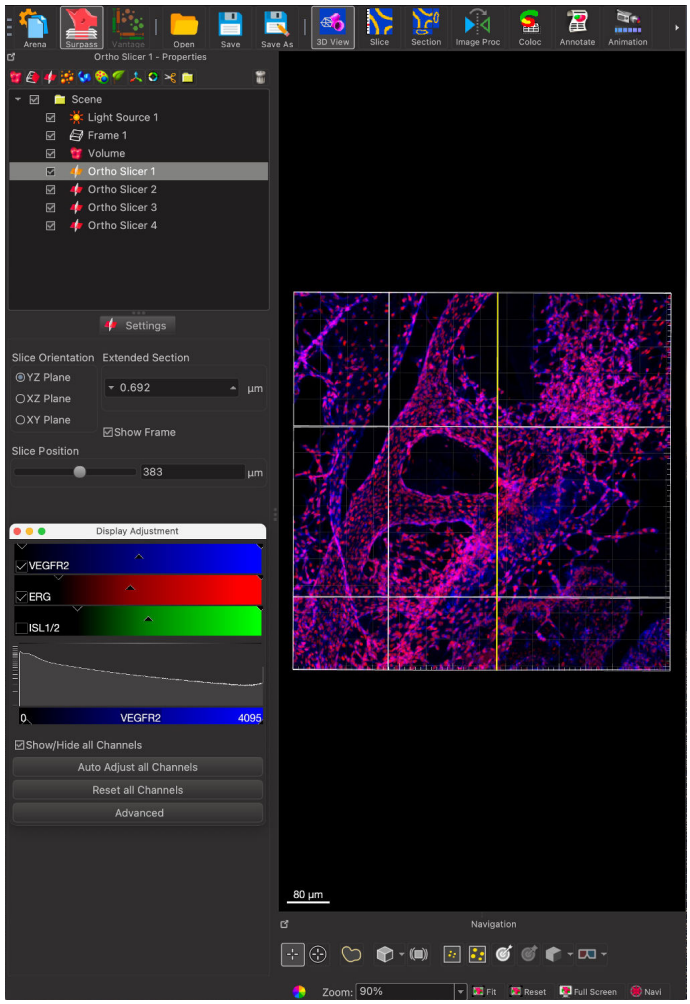

Arena
Surpass
Vantage
Open
Save
Save As
3D View
Slice
Section
Image Proc
Coloc
Annotate
Animation
Ortho Slicer 1 - Properties
Scene
Light Source 1
Frame 1
Volume
Ortho Slicer 1
Ortho Slicer 2
Ortho Slicer 3
Ortho Slicer 4
Settings
Slice Orientation
Extended Section
YZ Plane
XZ Plane
XY Plane
0.692
µm
Show Frame
Slice Position
383
µm
Display Adjustment
VEGFR2
ERG
ISL1/2
0
VEGFR2
4095
Show/Hide all Channels
Auto Adjust all Channels
Reset all Channels
Advanced
80 µm
Navigation
Zoom:
90%
Fit
Reset
Full Screen
Navi

Figure 3

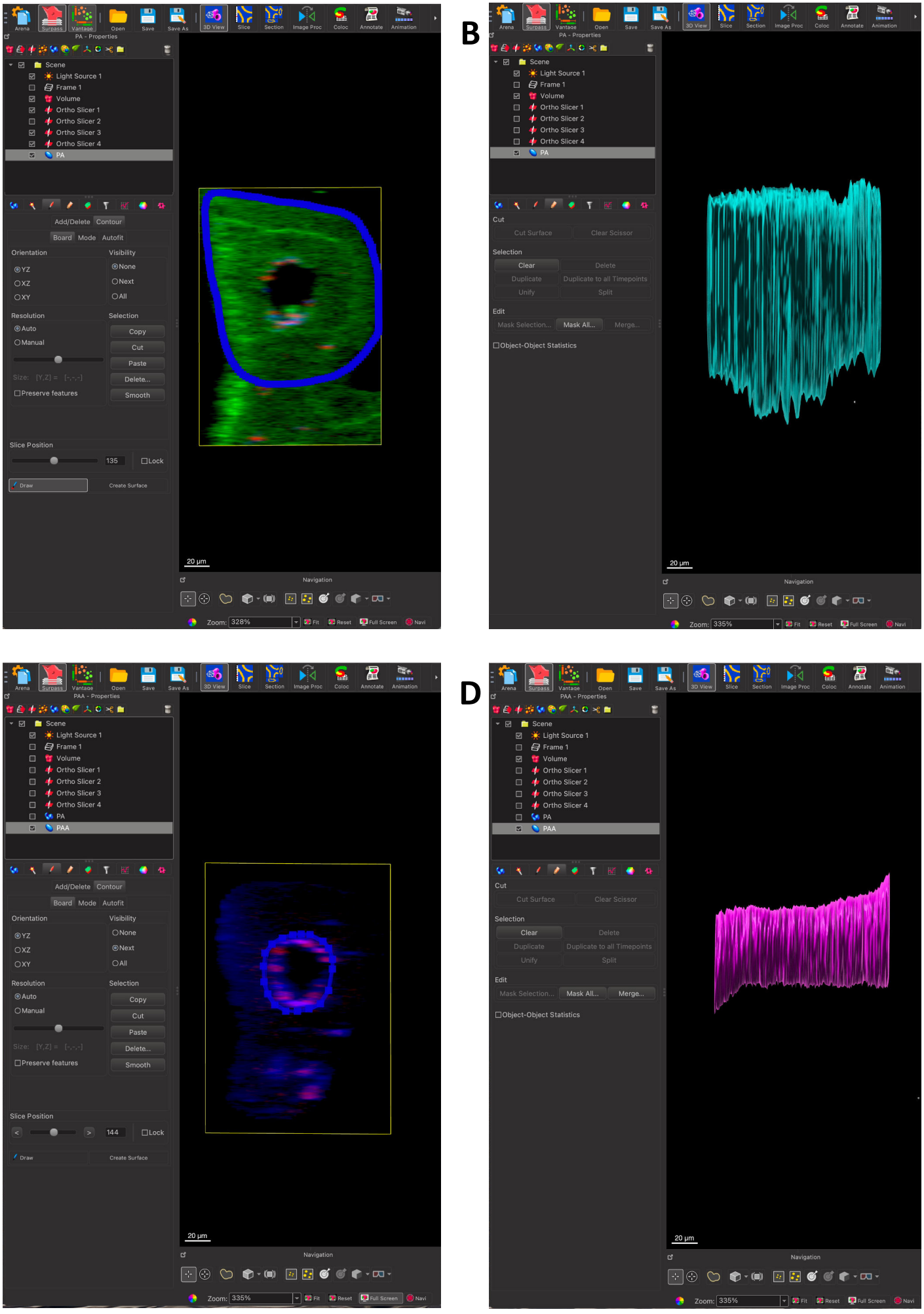
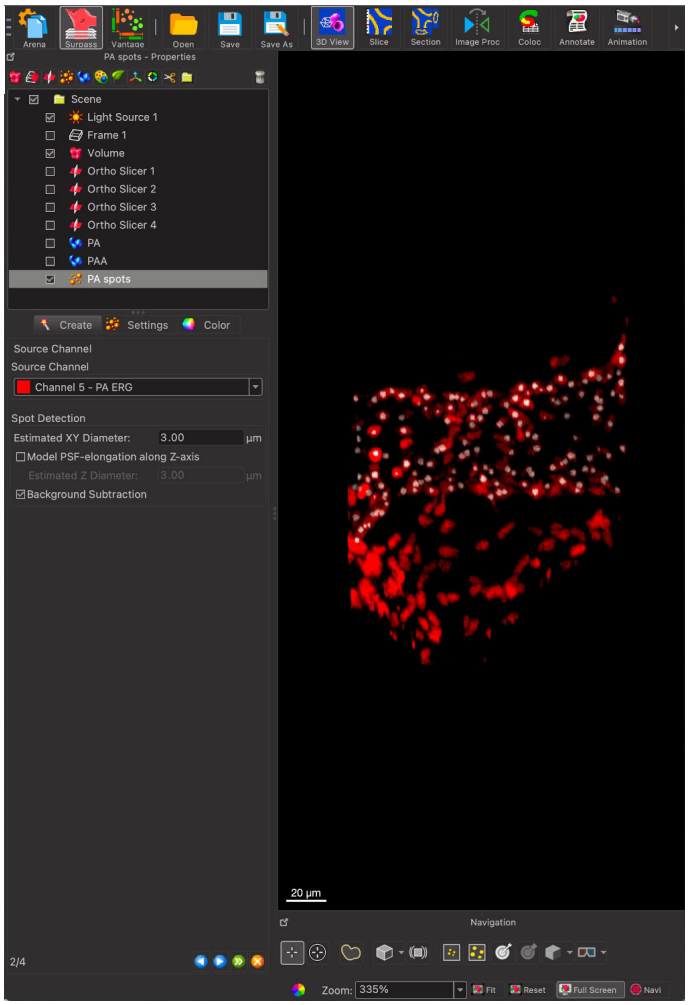
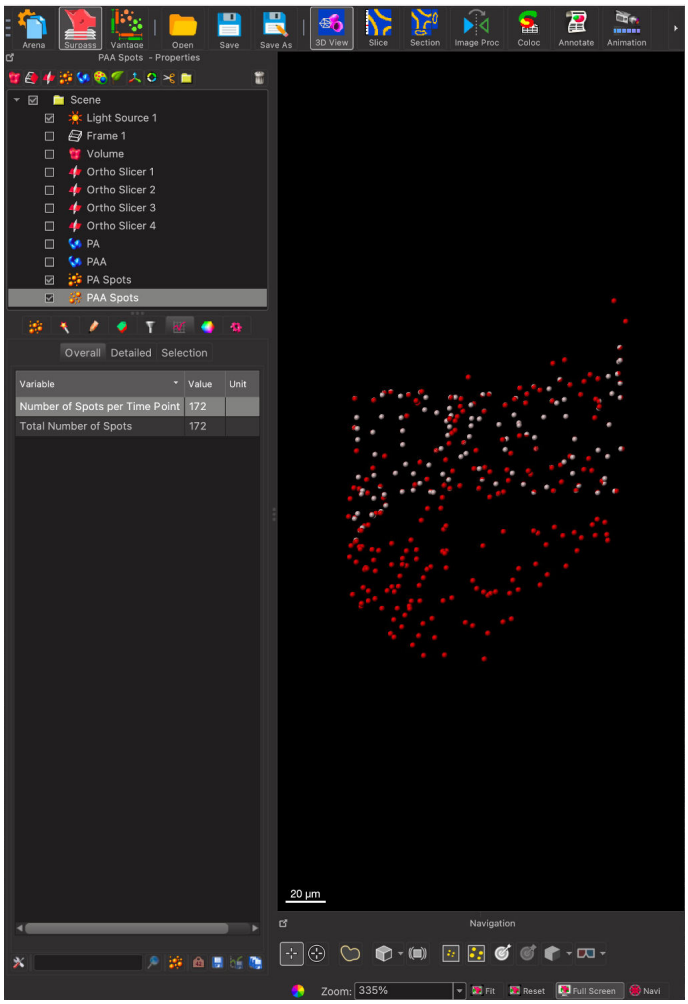

A
B
C
D
Arena
Surpass
Vantage
Open
Save
Save As
3D View
Slice
Section
Image Proc
Coloc
Annotate
Animation
PA - Properties
PAA - Properties
Scene
Light Source 1
Frame 1
Volume
Ortho Slicer 1
Ortho Slicer 2
Ortho Slicer 3
Ortho Slicer 4
PA
PAA
Add/Delete
Contour
Board
Mode
Autofit
Orientation
Visibility
YZ
XZ
XY
None
Next
All
Resolution
Auto
Manual
Selection
Copy
Cut
Paste
Delete...
Smooth
Preserve features
Slice Position
135
144
Lock
Draw
Create Surface
Cut
Cut Surface
Clear Scissor
Clear
Delete
Duplicate
Duplicate to all Timepoints
Unify
Split
Edit
Mask Selection...
Mask All...
Merge...
Object-Object Statistics
20 µm
Navigation
Zoom: 328%
Zoom: 335%
Fit
Reset
Full Screen
Navi

Figure 4

A


Arena
Surpass
Vantage
Open
Save
Save As
3D View
Slice
Section
Image Proc
Coloc
Annotate
Animation
PA spots - Properties
Scene
Light Source 1
Frame 1
Volume
Ortho Slicer 1
Ortho Slicer 2
Ortho Slicer 3
Ortho Slicer 4
PA
PAA
PA spots
Create
Settings
Color
Source Channel
Source Channel
Channel 5 - PA ERG
Spot Detection
Estimated XY Diameter:
3.00
µm
Model PSF-elongation along Z-axis
Estimated Z Diameter:
3.00
µm
Background Subtraction
2/4
20 µm
Navigation
Zoom:
335%
Fit
Reset
Full Screen
Navi

B


Arena
Surpass
Vantage
Open
Save
Save As
3D View
Slice
Section
Image Proc
Coloc
Annotate
Animation
PAA Spots - Properties
Scene
Light Source 1
Frame 1
Volume
Ortho Slicer 1
Ortho Slicer 2
Ortho Slicer 3
Ortho Slicer 4
PA
PAA
PA Spots
PAA Spots
Overall
Detailed
Selection
Variable
Value
Unit
Number of Spots per Time Point
172
Total Number of Spots
172
20 µm
Navigation
Zoom:
335%
Fit
Reset
Full Screen
Navi