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Dual labeling of neural crest cells and blood vessels within chicken embryos using chickGFP neural tube grafting and carbocyanine dye Dil injection

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Abstract:	All developing organs need to be connected to both the nervous system (for sensory and motor control) as well as the vascular system (for gas exchange, fluid and nutrient supply). Consequently both the nervous and vascular systems develop alongside each other and share striking similarities in their branching architecture. Here we report embryonic manipulations that allow us to study the simultaneous development of neural crest-derived nervous tissue (in this case the enteric nervous system), and the vascular system. This is achieved by generating chicken chimeras via transplantation of discrete segments of the neural tube, and associated neural crest, combined with vascular Dil injection in the same embryo. Our method uses transgenic chickGFP embryos for intraspecies grafting, making the transplant technique more powerful than the classical quail-chick interspecies grafting protocol used with great effect since the 1970s. ChickGFP-chick intraspecies grafting facilitates imaging of transplanted cells and their projections in intact tissues, and eliminates any potential bias in cell development linked to species differences. This method takes full advantage of the ease of access of the avian embryo (compared with other vertebrate embryos) to study the co-development of the enteric nervous system and the vascular system.
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TITLE:

Dual labeling of neural crest cells and blood vessels within chicken embryos using chick^{GFP} neural tube grafting and carbocyanine dye Dil injection

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SHORT ABSTRACT:

Here we report dual labeling of neural crest cells and blood vessels using chick^{GFP} neural tube intraspecies grafting combined with intra-vascular Dil injection. This experimental technique allows us to simultaneously visualize and study development of the NCC-derived (enteric) nervous system and the vascular system, during organogenesis.

LONG ABSTRACT:

All developing organs need to be connected to both the nervous system (for sensory and motor control) as well as the vascular system (for gas exchange, fluid and nutrient

supply). Consequently both the nervous and vascular systems develop alongside each other and share striking similarities in their branching architecture. Here we report embryonic manipulations that allow us to study the simultaneous development of neural crest-derived nervous tissue (in this case the enteric nervous system), and the vascular system. This is achieved by generating chicken chimeras *via* transplantation of discrete segments of the neural tube, and associated neural crest, combined with vascular Dil injection in the same embryo. Our method uses transgenic chick^{GFP} embryos for intraspecies grafting, making the transplant technique more powerful than the classical quail-chick interspecies grafting protocol used with great effect since the 1970s.

Chick^{GFP}-chick intraspecies grafting facilitates imaging of transplanted cells and their projections in intact tissues, and eliminates any potential *bias* in cell development linked to species differences. This method takes full advantage of the ease of access of the avian embryo (compared with other vertebrate embryos) to study the co-development of the enteric nervous system and the vascular system.

INTRODUCTION:

The chicken embryo is an invaluable model organism in vertebrate developmental biology, not least because its development *in ovo* permits experimental manipulations that are otherwise impossible to perform in vertebrates that develop *in utero*. This accessibility and ease of manipulations has led to the chick embryo playing key roles in many seminal discoveries in the field of developmental biology. Amongst the most powerful techniques has been the use of quail-chick chimeric embryos to study cell fate, a method pioneered by Professor Nicole Le Douarin in the 1970's¹⁻³. In particular, quail-chick chimeras have been especially useful to genetically mark and follow highly migratory neural crest cell (NCC) populations during early development. NCC are a multipotent population of migratory cells, arising in the dorsal ectoderm at the margins of the neural tube, that give rise to a wide range of cell types throughout the vertebrate embryo. These include craniofacial structures (cartilage, bone, muscles), neurons and glia (in the sensory and autonomic nervous systems), melanocytes, and a subpopulation of cells of the endocrine system^{2,4,5}. One of the most important factors influencing NCC fate is their initial location along the anterior-posterior axis of the neural tube. For example, enteric NCC, which give rise to the neurons and glia of the enteric nervous system (ENS), arise from two discrete sub-populations: the first located in the vagal (caudal hindbrain) region, and the second in the sacral region of the neural tube⁶⁻¹³. Inter or intra-species grafting of the corresponding regions of the neural tube have been the techniques of choice to permanently label these cells and subsequently allow tracking, from their birth on the margins of the neural tube, to their final destinations within the digestive tract^{6,7,10}.

Another embryonic manipulation easier to perform in chick, compared to other animal models, is the vital labeling of the vascular system. Indeed, as the chick embryo develops, it lays on top of an extra-embryonic vascular network that circulates oxygen and nutrients from the yolk. This accessible vascular network, located on the surface of the yolk, can be used as a gateway to label the embryo's developing vascular system during organogenesis^{12,14-17}. Intravascular injection of various dyes, such as the lipophilic dye Dil, makes it possible to delineate/stain all the luminised vessels of the

nascent vascular network.

Because developing organs need to be connected to both the nervous system (for sensory and motor control) as well as the vascular system (for gas exchange, fluid and nutrient supply), the two networks develop alongside each other and share striking similarities in their branching architecture¹⁸⁻²⁰. Here we report embryonic manipulations that allow us to study the simultaneous development of the NCC-derived ENS, along with the vascular system, during organogenesis. This is achieved by generating chicken chimeras via transplantation of discrete segments of the neural tube, including the neural crest, combined with vascular Dil injection. As an advance from quail-chicken chimeras, our method uses transgenic GFP chick embryos for intraspecies grafting, making the transplant technique more powerful, in terms of imaging cells and their projections, and eliminating any potential bias linked to species differences.

PROTOCOL:

1. Preparation of micro-scalpel for neural tube ablations

1.1. Shape a micro-scalpel from a commercially available steel sewing needle.

1.1.1. First flatten the needle on both sides using a grinding wheel mounted on a powered bench grinder.

1.1.2. Start shaping the scalpel, first on a coarse grade Arkansas stone using a controlled circular motion, in alternate directions, on both sides of the needle.

1.1.3. Carry on the same sharpening movements on an extra fine grade Arkansas stone to shape an ultra fine micro-scalpel, with a well defined cutting edge (Figure 1A,B).

Note: Alternatives to the micro-scalpel could be electrolytically sharpened needles, commercially available tungsten needles, or pulled glass needles.

2. Incubate wild type and GFP eggs to the desired stage

2.1. Store fertilized chicken eggs and transgenic GFP chicken eggs in a cooled incubator at 14-15 °C prior to incubation as development is halted at this temperature. Store eggs for a few days, up to one week.

2.2. To commence development, place wild type and GFP eggs on a tray horizontally and incubate simultaneously in a humidified (58-60%) incubator at 37.5 °C, so that embryos are at matching stages for neural tube grafting.

2.3. To obtain embryos at the 10-12 somite stage of development for carrying out vagal neural tube grafting, incubate eggs for 1.5 days (33-38 hours) and stage embryos according to the development tables of Hamburger and Hamilton²¹.

3. Prepare eggs for windowing and grafting

3.1. Move one egg at a time to a custom-made egg holder for windowing. Make a small hole in the egg shell by repeatedly tapping, with straight scissors, on the upper surface of the pointed end of the egg.

3.2. Remove 2-3 ml of albumin from the egg with an 18½ G hypodermic needle and 5 ml syringe. Removing the albumin lowers the yolk within the egg and facilitates subsequent windowing without causing any damage to the embryo.

3.2.1. Discard the albumin. Seal the hole with a small strip of clear tape cut to size with fine scissors.

3.3. Using curved scissors, tap another hole in the upper surface of the egg shell. Insert the tip of the scissors into the hole and, keeping the scissors parallel to the bench, work in a circular motion to cut a ~2 cm diameter window on top of the shell.

3.3.1. Keep the scissors in a stationary position and rotate the egg. Discard the removed disk of egg shell. At E1.5, the embryo is recognizable as a darker yellow disk on top of the yolk.

3.3.2. Remove any shell debris that has fallen within the egg using tweezers. Discard any unfertilized eggs (identified by a small white spot on top on the otherwise light yellow yolk).

4. Prepare the host embryo to receive grafted tissue

4.1. Adjust the stereo-microscope to eye level and optimize the orientation of gooseneck light source to adequately illuminate the embryo without causing reflections.

4.2. To visualize the embryo *proper*, inject a small amount of Indian ink under the center of the darker yellow disk, using a mouth tube and a pulled glass micro-pipette (Figure 1C, Oii).

4.2.1. Prepare the ink 50:50 with PBS containing Penicillin/Streptomycin at 100 µg/ml final concentration. Insert the micro-pipette through the yolk membrane outside the perimeter of the blastoderm then carefully angle its tip directly beneath the embryo.

4.2.2. Deliver ink underneath the embryo by blowing on the mouth tube. If mouth pipetting is not permitted, use a 1 ml syringe instead. Be careful not to introduce any air bubbles underneath the embryo, which can lead to contamination, then carefully remove the glass micro-pipette. This is a delicate step that can lead to the death of the embryo if not done with precision.

4.3. Stage the embryo by reference to Hamburger and Hamilton²¹ and record the

stage in a lab book.

4.4. Using a custom made micro-scalpel (or a fine tungsten needle) mounted on a needle holder, make a very small gash in the vitelline membrane, next to the area where the micro-surgery will be performed.

4.4.1 Carefully apply 2-3 drops of PBS over the membrane tear (using a glass micro-pipette and the mouth tube) to create space between the embryo and the membrane. Cut a larger window in the membrane to expose the entire region where the micro-surgery will take place.

4.5. Remove the neural tube region of interest using the micro-scalpel, starting with rostral and caudal transverse incisions across the entire dorsal neural tube (at the level of somite 1 to 7 in the video).

4.5.1. Cut bilaterally between the neural tube and the somites to separate the neural tube from surrounding tissues, without damaging the somites.

4.5.2. Very gently separate the neural tube from the underlying notochord, which should remain intact. Note that successful neural tube excision will leave all surrounding tissues perfectly intact (Figure 2).

4.6. Remove the excised neural tube by aspirating it into a glass micro-pipette, then discard.

4.7. Record the level of neural tube ablation in a lab book. The host embryo is now ready to receive the donor neural tube.

5. Prepare the donor graft tissue

5.1. Select a windowed, stage-matched GFP embryo by viewing under a fluorescent stereo-microscope with FITC filter. The GFP fluorescence makes it very easy to visualize the somites and stage the embryo.

5.2. Once a stage-matched embryo has been identified, remove the embryo from the egg by making 4 incisions, with Pascheff-Wolff Spring Scissors (Figure 1C, I) in a rectangle shape around the embryo then gently pick it up with an embryo spoon.

5.3. Place the embryo in a square watch glass with a sylgard polymer base. Gently shake the embryo with Dumont #5 tweezers to remove any attached yolk. Remove the vitelline membrane and pin the embryo onto the polymer base using stainless minuten pins (Figure 1C).

5.4. Using the Spring Scissors, make 4 incisions in a rectangular shape around the neural tube and surrounding somites, in the same region that has been removed from the host embryo.

5.5. Using a plastic transfer pipette, transfer the neural tube and somite tissues from the donor GFP embryo into a watch glass containing 0.2% pancreatin in Pen/Strep PBS.

5.6. Allow enzymatic digestion to proceed for 10 minutes at room temperature to help separate the tissues. After incubation in enzyme, use stainless minuten pins mounted on a handle to manually separate the neural tube from all adjacent tissues.

5.7. Using a glass micro-pipette, transfer the dissociated neural tube to another watch glass containing DMEM+10% serum (e.g. goat, horse or fetal calf) on ice, to rinse the excess pancreatin and stop enzymatic digestion. After 5 minutes, the dissected neural tube is ready to be grafted orthotopically into the chick host (Figure 2, Figure S1).

6. Graft the tissue

6.1. Using a glass micro-pipette, carefully transfer the dissected neural tube from the watch glass to the host embryo. Position the neural tube in the correct anterior-posterior orientation and gently push the explant adjacent to the excised region of the chick host using the micro-scalpel. Leave a small fragment of ectoderm attached to, or by cutting a small nick in, the dorsal surface to identify the orientation of the neural tube.

6.1.1. If necessary, use the micro-scalpel to trim the explant to the exact size of the excised region.

6.2. Gently guide the neural tube into the ablated region and position it such that the dorsal side is correctly orientated. Use a glass micro-pipette, mounted to a mouth tube, to remove PBS and/or fluid surrounding the graft. This helps the donor and host tissues to adhere and the graft to become established.

6.3. Seal the entire window with 24 mm wide clear tape to prevent dehydration and contamination.

6.4. Label the chimeric embryo by marking with a pencil on the eggshell and record its number in the lab book. Return the egg to the incubator for further development.

7. Inject Dil into blood vessels of host embryo

7.1. At the desired experimental time point (here, 3-10 days later), retrieve the chimeric embryo from the incubator and remove the clear tape using straight scissors to gain access to the embryo within the egg.

7.2. If necessary, enlarge the window in the shell using the scissors. Be careful not to damage the chorioallantoic membrane if it is attached to the shell, which would result in hemorrhage and jeopardize the blood vessel labeling.

7.3. Choose an accessible vein on the yolk making sure the blood flow is directed towards the embryo. Choose a branching point of one of the vitelline veins (Figure 3B,C).

Note: At E6.5-E7.5, the chorioallantoic membrane may need to be gently moved aside with tweezers to access the yolk veins. After E8.5, the only option is to inject into one of the chorioallantoic membrane veins since, by this stage, the chorioallantoic membrane fully covers the embryo.

7.4. Remove the vitelline membrane above the chosen injection point using two Dumont #5 tweezers by tearing in opposite directions.

7.5. Break a pulled glass needle using a Dumont #5 and adjust its diameter to the approximate size of the vein prior to loading with CellTracker CM-Dil. Make the Dil stock solution at 40 $\mu\text{g}/\mu\text{l}$ in DMSO and store at -20 °C. Prepare the working solution in 0.3 M sucrose/PBS at a concentration of 4 $\mu\text{g}/\mu\text{l}$.

7.5.1. Aspirate between 5-10 μl of Dil in 0.3 M sucrose/PBS into the needle using suction with a mouth tube. Older embryos might require up to 25 μl or more. From E8.5, embryos have larger, more muscular veins, which may need to be held in position with a Dumont #5 before stabbing with the Dil-loaded glass needle.

7.6. Swiftly insert the needle into the vein and blow steadily with the mouth tube to allow the Dil to join the blood flow slowly without forming a clot. Alternatively, use a pressure injector for Dil delivery.

8. Harvest embryos for sectioning or wholemount examination

8.1. To retain as much Dil within the embryo as possible, harvest the embryo immediately after the injection by scooping it onto a perforated spoon and cutting the blood vessels and connective tissues with a pair of straight scissors, to free the embryo from the yolk.

8.2. Remove any loose membranes and dissect out the organs of interest (i.e., the lungs and digestive tract in this tutorial), taking great care not to compress the tissue, which creates diffusion of the Dil. Immediately fix the tissues by immersion in 4% PFA for 1-2 hours at room temperature.

8.3. Rinse the tissue for 5 min in PBS, then 15 min in PBS containing 5 $\mu\text{g}/\text{ml}$ DAPI. Mount the samples on a bridged microscope slide for whole mount examination or embed them for cryo-sectioning.

REPRESENTATIVE RESULTS:

Figure 1 shows typical instruments required to carry out microsurgical isolation and transplantation of the neural tube. Figure 2 shows the transplant procedure. Following transplantation embryos are screened for transplant success. This involves examining

the embryo under a stereo fluorescence microscope, typically the morning after microsurgery, for the presence of graft-derived (GFP+) NCC. If transplantation has been a success, then GFP+ NCC can be observed in the vicinity of the neural tube and in early migration pathways leading toward the foregut. If the procedure has not been successful, GFP+ NCC will not be observed outside the neural tube, or if they are present in the host they may be in smaller numbers. These unsuccessful embryos are discarded. Typically, 5-8 neural tube transplants are performed in one day, and of these 80% are successful. Reasons for unsuccessful neural tube transplantation include death of the embryo due to tissue damage incurred during microsurgery, or failure of the neural tube to integrate into the host embryo. The latter can result from poor placement of the neural tube within the host or from a poor quality neural tube due to poor dissection technique or from excessive exposure to dissociation enzyme. The initial screening step, as well as similar later examinations for GFP+ cells, is useful as it means that time and resources are not wasted by performing experiments on embryos that do not have GFP-labelled NCC within the gut.

Figure 3 shows the procedure for Dil injection of the blood vessels. The success of the Dil injection technique depends on optimizing the diameter of the injection needle to the size of the targeted vein; careful insertion of the needle into the vein (so as not to pierce the other side); avoiding the needle getting plugged during the injection (by blowing through the mouth tube at a constant rate). If any of these parameters is done incorrectly, the embryo will bleed excessively causing death, or it will require several hours to recover before a second attempt at labeling can be made. Following Dil injection, embryos should be viewed immediately under a stereo fluorescence microscope and successfully labeled embryos should be rapidly harvested. With successful labeling, Dil labeled blood vessels are present throughout the embryo (Figure 3C,D) including the capillary beds (Figure 3D).

Upon harvesting of embryos and examination of tissue sections or wholemount gastrointestinal tracts, typical results reveal GFP+ NCC within the primitive ENS and the fine structure of the Dil-labeled gut blood vessel networks (Figure 4.) Wholemount preparations can be examined using confocal microscopy whereby image stacks produce three dimensional (3D) reconstructions showing the interrelationships between the fine projections of GFP+ ENS cells and the Dil stained vascular system (Figure 4 A-C; G-I; Videos 1 and 2).

Figure 1. Recommended microsurgery instruments. A micro-scalpel shaped from a sewing needle. B fine Arkansas stone for shaping a micro-scalpel. C a) straight scissors, b) curved scissors, c) 5ml syringe with 181/2 G hypodermic needle, d) plastic pipette, e) custom made egg holder, f) black ink, g) square watch glass, h) square watch glass with black sylgard base, i) micro-scalpel on needle holder, j) minutien pins, k) minutien or tungsten needle on needle holder, l) Pascheff-Wolff spring scissors, m) Dumont #5 tweezers, n) perforated spoon, oi) short fire-pulled transfer needle, oii) long fire-pulled inking needle, p) mouth tube.

Figure 2. Intraspecies neural tube transplant. Chick embryo/GFP neural tube images

have been modified from Delalande *et al.*¹². Vascularization is not necessary for gut colonization by enteric neural crest cells.

Figure 3. Intravenous Dil injection. **A** Recommended instruments: a) CellTracker CM-Dil drop on parafilm, b) pulled glass injection needle, c) mouth tube. **B** Schematic diagram of intravenous Dil injection into E4 chimeric chick embryo. **C** *in ovo* Dil intravenous injection showing fine glass needle containing Dil inserted into vein (arrow). **D** E4 embryo post Dil injection (red). **E** Dil stained fine blood vessel network in a live embryo, 24 hours post-injection. Br: brain; H: heart; LB: limb bud; A: allantois. Images in C and D have been modified from Delalande *et al.*¹² Vascularization is not necessary for gut colonization by enteric neural crest cells.

Figure 4: Representative results in the stomach and caecum of an E5.5 chick embryo. **A-C** 3 dimensional (3D) reconstruction of a confocal image stack in the region of the stomach showing (D) the GFP+ enteric neural crest cells (ENCC) (E) the Dil stained vascular system and (F) a merged image of both networks **D-F** Histological sections at the level of the stomach showing (G) the GFP+ ENCC (H) the Dil stained vascular system and (I) a merged image of both networks. Nuclei are stained with DAPI (cyan). **G-H** 3D reconstruction of a confocal image stack in the caecum region showing (A) the GFP+ ENCC migration front in green, (B) the Dil stained vascular system in red, and (C) a merged image of both networks. Images A-F have been modified from Delalande *et al.*¹² Vascularization is not necessary for gut colonization by enteric neural crest cells.

Figure S1: Isolation of a donor GFP+ neural tube from the surrounding tissues by enzymatic digestion and micro-dissection. **A** GFP+ neural tube and adjacent somites dissected from the donor embryo. **B** Isolated neural tube after pancreatin digestion and micro-dissection using stainless minuten pins. So: somites; NT: neural tube; Nc: Notochord.

Video 1: 3-dimensional 360° rotation of the image in Figure 4C, showing the vascular system and the ENCC in the stomach at E5.5 (HH27-28).

Video 2: 3-dimensional 360° rotation of the image in Figure 4I, showing the vascular system and the ENCC migration front in the region of the caecum at E5.5 (HH27-28).

DISCUSSION:

The method of intraspecies neural tube grafting, combined with blood vessel labeling described here, takes full advantage of the ease of access of the avian embryo within the egg (compared with other vertebrate embryos) to study the co-development of an element of the autonomic nervous system (the ENS) and the vascular system.

For labeling NCC derivatives, the chick^{GFP}-chick intraspecies grafting method we describe has a number of advantages over the classical quail-chick chimera method that was established over 40 years ago¹⁻³. First, under FITC light, GFP fluorescence is extremely bright, to the extent that GFP+ cells are easily discernable in live chimeric

embryos. This allows the success of the graft to be checked *in ovo*, whereas quail-chick grafting requires the embryo to be killed, processed and immunostained using QCPN, before the success of the graft can be ascertained². Second, GFP expression in the transgenic chick^{GFP} is cytoplasmic, therefore it not only labels cell bodies, but also allows the projections of the transplanted cells to be visualized²². This permits intricate neuronal networks to be observed at high resolution (note that fine projections are best visualized when the sample is immunostained with anti-GFP antibody). As QCPN labeling is restricted to the quail cell nucleus, such networks are not revealed using quail-chick chimeras. Third, intraspecies grafting eliminates any potential species differences between cells within the chimeric embryo. Since quail embryos have a shorter incubation period than chick (19 days *versus* 21 days) it has been suggested that quail cells have a higher proliferation rate than chick cells, which could potentially affect the development of the chimeric tissues²³. Interestingly also, it has been shown in plants that interspecies grafting could produce extensive alterations in DNA methylation patterns in the host²⁴. Fourth, chick^{GFP} facilitates back-transplant experiments to address topics such as NCC fate and cell commitment²⁵. Fifth, the transgenic chick^{GFP} is also useful for many other techniques including FACS sorting of GFP+ cell subpopulations, organotypic culture of organs containing GFP+ cells, genetic manipulation of GFP+ grafted tissue *via* electroporation of expression plasmids²⁶, and other imaging technologies such as optical projection tomography²⁷.

The neural tube transplantation approach can be modified by microsurgically replacing shorter amounts of neural tube. By using smaller segments of neural tube the microsurgery is potentially less damaging to the embryo and survival can be improved. However, the downside of transplanting less neural tube is that the numbers of GFP+ NCC in the host will be reduced. Users could try and achieve a balance between the amount of neural tube transplanted to give optimum survival of embryos, and the numbers of GFP+ NCC within host intestine sufficient to give informative results.

For vessel painting, Dil has the advantage that its fluorescence is very bright and robust. Also, it has the capacity to diffuse during fixation assuring staining of the finest opened capillaries. Since it is a vital dye, embryos can survive the injection procedure and carry on developing with a stained vascular system (up to 24 hours in our hands, although the staining becomes more punctate over time, see Figure 3E). The combination of chick^{GFP} grafting with Dil vascular painting is therefore compatible with live imaging. Besides all these advantages, it is important to note that vascular injection only labels luminised vessels and therefore does not identify unopened capillaries, endothelial tip cells or isolated endothelial cells. However, further progress in avian transgenesis could provide new ways to circumvent such issues, as exemplified by experiments using Tg(tie1:H2B-eYFP) quail embryos to study vascular morphogenesis²⁸. Another limitation of this technique is that, for effective vessel labeling in embryos at E7.5 and beyond, larger amounts of dye need to be injected, which can make experiments expensive. However, a modification of the technique could include low cost blood vessel labeling using highlighter ink¹⁴, although this approach has not been tried in our hands.

Critical steps of the procedures include the process of visualizing the embryo by injecting ink underneath the blastodisc. If the membrane covering the yolk is torn by the ink-filled needle at this stage then embryo survival is severely compromised. Also, it is important, when preparing a donor neural tube, that the tissue is not left for an excessively long time in pancreatin (consider approximately 10 minutes as a maximum). Prolonged exposure to pancreatin damages the tissue and the neural tube is then difficult to handle and it will not incorporate well into the host. Critical parameters of Dil injection are Dil volume and needle diameter. These should be assessed and optimized on wild type stage matched controls before attempting injection of chimeric embryos.

In conclusion, our dual labeling method of neural tube transplantation and Dil vessel painting in live chick embryos can be used to investigate the inter-relationships between NCC and blood vessel networks during organogenesis. Considering the mechanisms responsible for establishing correct target innervation and vascularization during organ development are still largely unknown, this methodology holds potential for future discoveries in this field.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure

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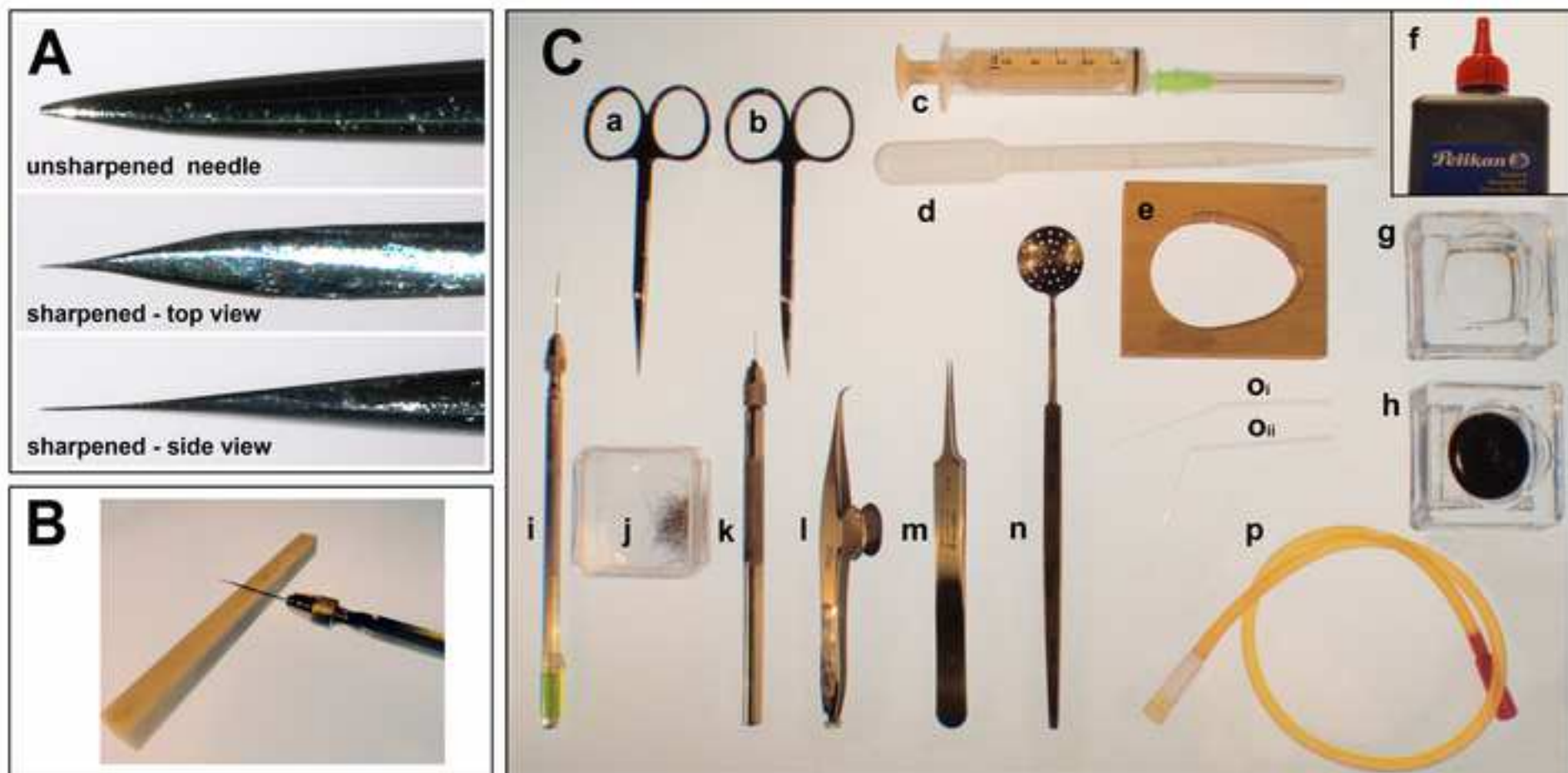
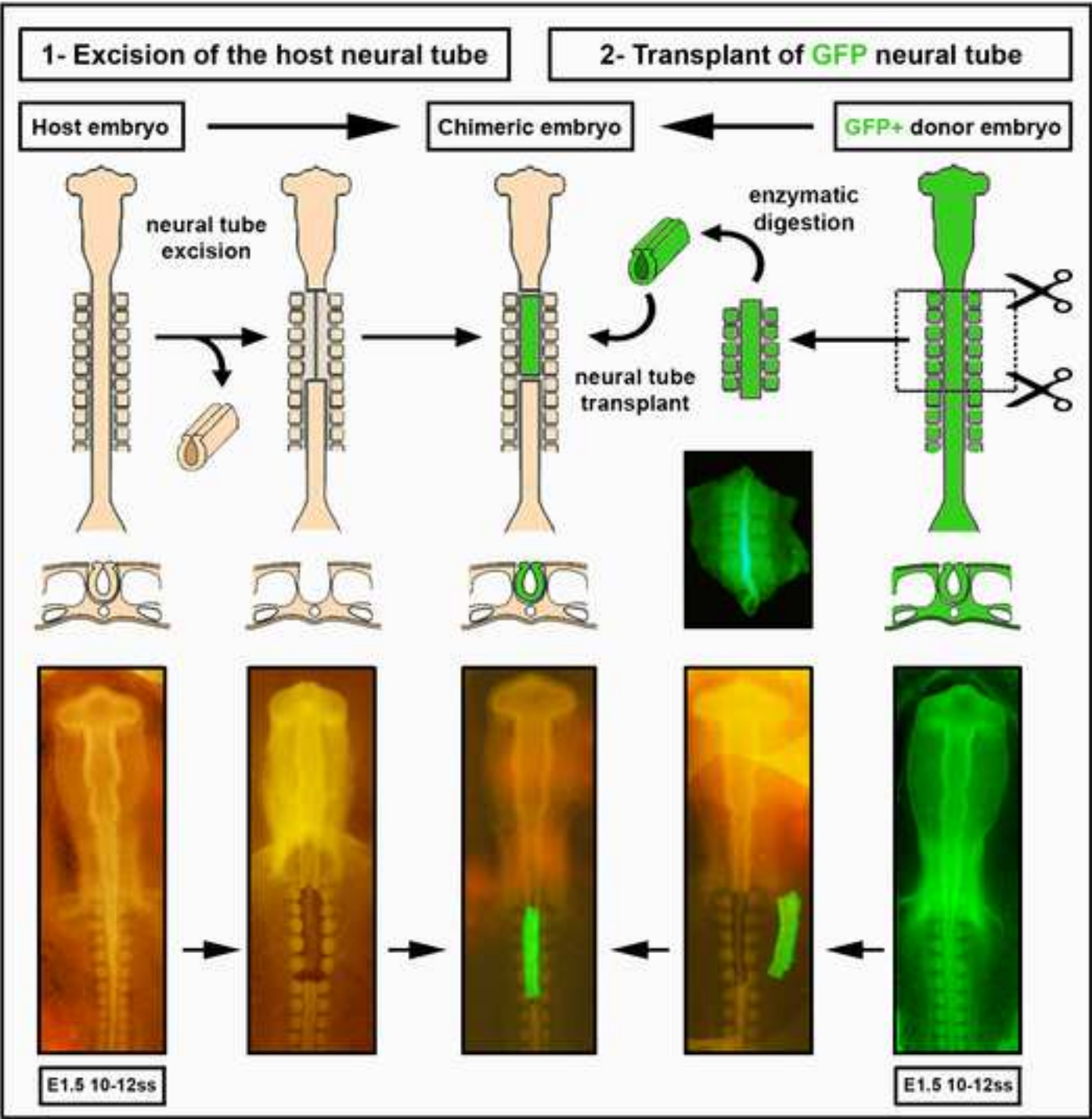


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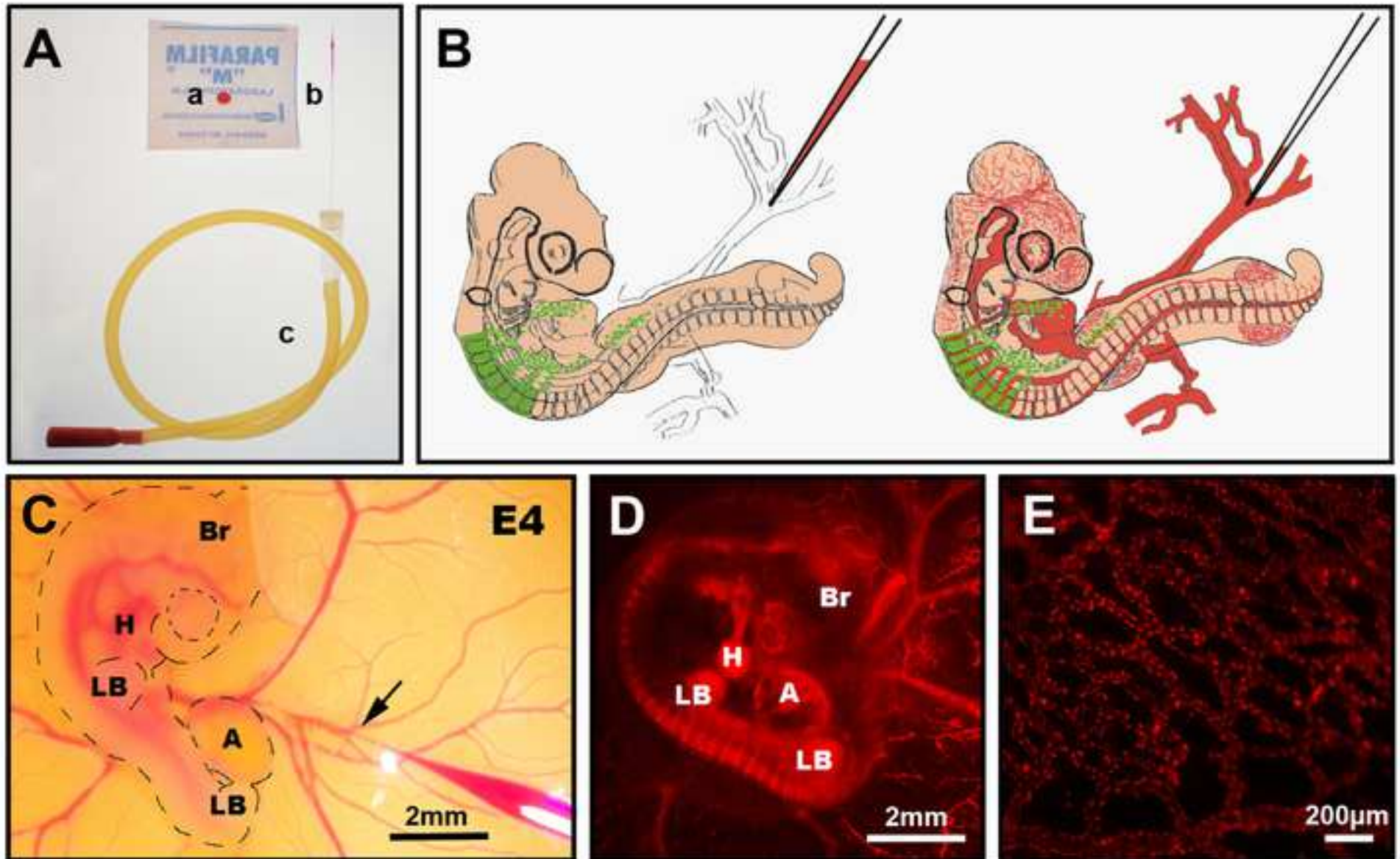
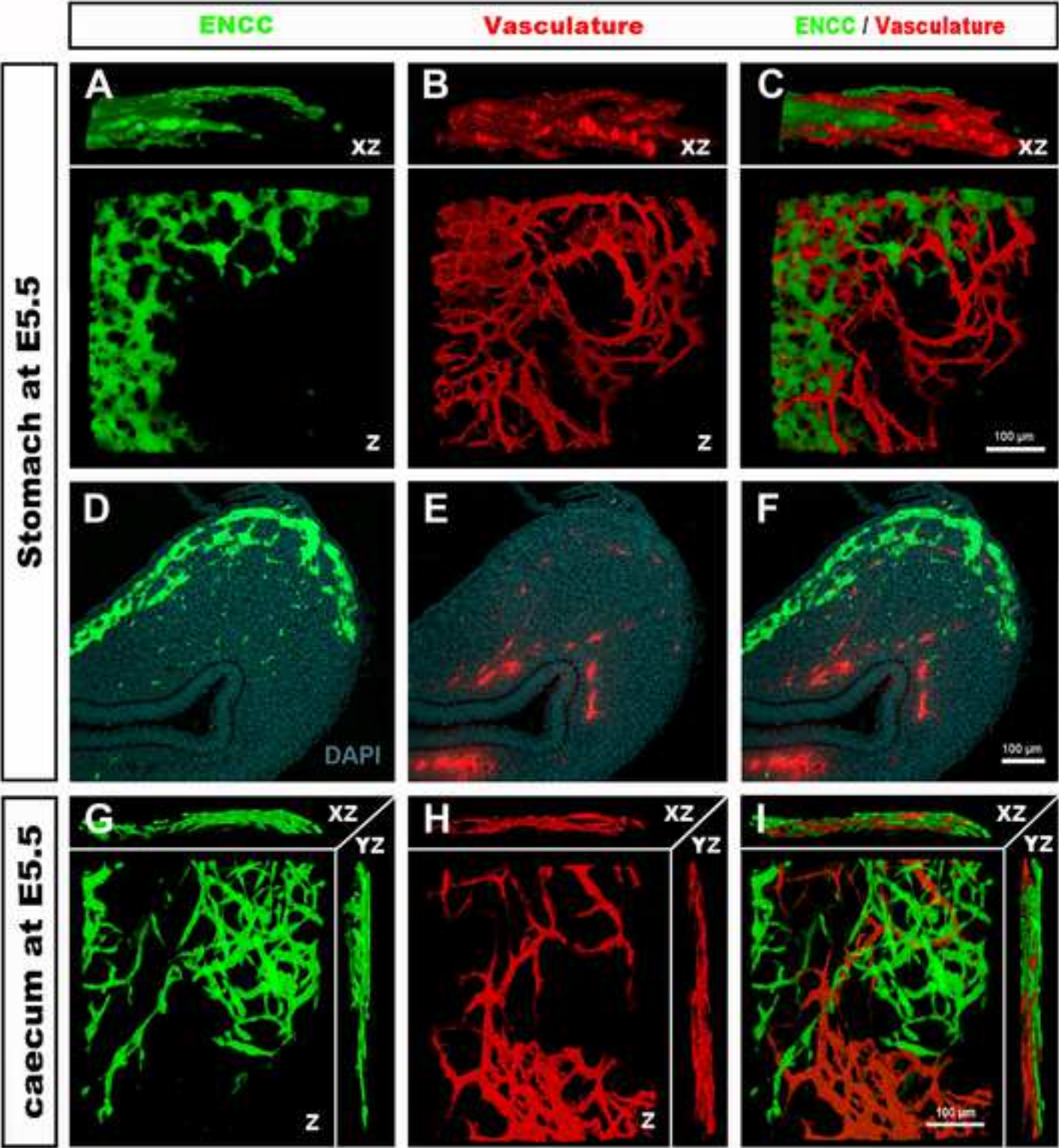
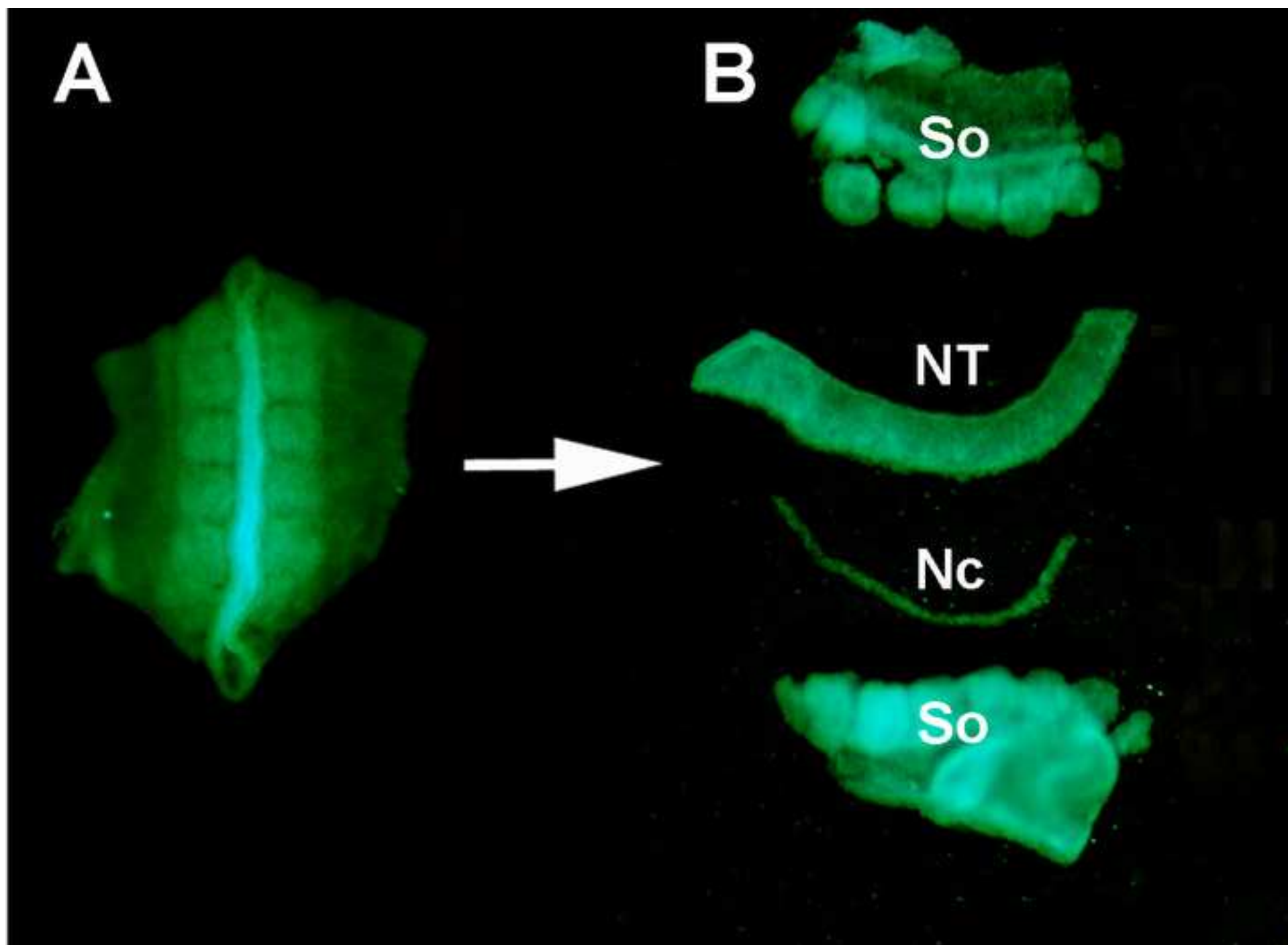


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Name of the Material/Equipment
Fertilised chick eggs
Fertilised GFP chick eggs
Egg incubator (Profi-H Hatcher)
14C Incubator
Stereo-microscope
Digital Camera
Image acquisition software
Goose neck halogen cold light source
18½ G hypodermic needle
Pancreatin
DMEM
Goat serum
5ml syringe
Mouth tube
Sigma Pasteur pipettes non-plugged, L 5 3/4 in.
Transfer pipettes, polyethylene
Borosilicate glass capillaries, thin wall without filament
Iris Scissors - ToughCut
Curved Iris Scissors - ToughCut
Needle holders (Nickel-plated pin holder)
Pascheff-Wolff Spring Scissors
Dumont #5 forceps
Minutien pins
Dumont AA forceps, Inox Epoxy- coated
Perforated spoon
Tungsten needles (0.125mm diameter)
Sellotape (clear, 24mm width)
Pen/Strep (Penicillin, Streptomycin) Solution
Sylgard 184 silicone elastomer kit
Pelikan black ink
CellTracker CM-Dil
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)
Settings for glass needle puller
Heat 950; Pull 150; Velocity 100; Time 200; Pressure 500

Company	Catalog Number
Henry Stewart and Co, Louth, UK	
The Transgenic Chicken Facility, The Roslin Institute, The University of Edinburgh	
Lyon Technologies, CA, USA	910-033
Precision Cooled Incubator, Leec Ltd., Nottingham, UK	Model LT2
LEICA	Model MZ 12.5
LEICA	DC500
LEICA	IM50
Advanced Imaging Concepts, Inc	KL 1500 LCD
SIGMA - ALDRICH	HSWNH181
SIGMA - ALDRICH	P3292
SIGMA - ALDRICH	D5030
SIGMA - ALDRICH	G6767
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SIGMA - ALDRICH	A5177
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Fine Science Tools	26018-17
Fine Science Tools	15371-92
Fine Science Tools	11251-30
Fine Science Tools	26002-15
Fine Science Tools	11210-10
Fine Science Tools	10370-18
Fine Science Tools	10130-05
Any Supplier	
VWR international	101447-068
Dow Corning	S09 512 516
Pelikan	211-169
Molecular Probes	C-7001
Molecular Probes	D1306
Sutter Instruments	Flaming/Brown micropipette puller model P-86

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Author(s):

Jean-Marie DELALANDE, Nikhil THAPAR & Alan J. BURNS

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This outstanding group of investigators provides an excellent and detailed description of the technique for vagal neural tube grafting and vascular injections in the avian embryo. The text is well-written and the figures are outstanding. With appropriate videos to highlight the method, this will be a very useful addition to the literature.

Major Concerns:

- 1) There is reference to a Figure S1, but I did not find this figure in the pdf. **This figure was uploaded along with all the others. I do not know why it was not**

built into the pdf. I will upload it again.

Minor Concerns:

1) Lines 128-129: change the sentence to "Store fertilized chicken eggs, obtained from commercial breeders and from transgenic GFP chicken eggs...." I changed the wording slightly and removed the "obtained from commercial breeders" as the sources of WT eggs and GFP eggs is given in the Table.

2) It would be helpful if the authors provide information on where the GFP chickens can be obtained. See point 1 above.

3) Line 206: the word "micro-pipette" is repeated twice. Deleted.

Reviewer #2:

Manuscript Summary:

The authors present an interesting combination of marking both migrating neural crest cells and the vascular network in developing chick embryos using transgenic GFP chick- WT chick neural transplants and injection of Dil stain in the vasculature.

Experiments and expected results are clearly described and nicely illustrated. Comments of the results are useful to emphasize critical points of the methods and the discussion is interesting.

This article should be of great value to developmental biologists working on the avian model as in ovo grafting and injection procedures quite benefit from visualized and detailed protocols.

Major Concerns:

None

Minor Concerns:

Description of experiments

- cf 5.7 : can goat serum be replaced by FCS or HS? If probably this is the case, please add a note to text. Yes it can be. This has been added to 5.7.

- cf 6.1: indicated to "keep the orientation of the transplanted GFP+ neural tube". But, how can one keep the anterior-posterior orientation? (by means of some label? or are they anatomical features to identify anterior and posterior

parts of the excised neural tube?) I have added the following to 6.1 “The orientation of the neural tube can be identified by leaving a small fragment of ectoderm attached to, or by cutting a small nick in, the dorsal surface”.

- cf 6.3: about the tape: please indicate its width; are any type of tape convenient? as I would guess some could be toxic to the embryo due to glue solvent ? In our experience any type of clear adhesive tape is fine. I have never been aware of any toxicity problems with tape. We use 24mm wide Sellotape so that one strip covers the entire window in the egg. I have added this width to 6.3.

- cf 7.6 and Fig3C: Adaptation of micropipette and injected vein diameter sizes seems critical to Dil labelling of the vascular network. And the choice of the diameter of the vein should be critical too, in order to avoid fatal bleeding after injection into a large vein. It would be thus useful that the authors indicate the range of appropriate diameter sizes of vessels/pipettes. I think Figure 3 reflects the appropriate diameter of vessels and their location, as well as the pipette size needed. It would be very difficult not only to measure the tip diameter, but also to break off a pipette tip to an exact, predetermined size. This aspect is somewhat trial and error, but it soon becomes apparent what is most appropriate and practical.

Figures : All are fine and useful (except that FigS1 was unavailable to this reviewer). This figure was uploaded along with all the others. I do not know why it was not built into the pdf. I will upload it again.

Materials for imaging (eg, camera and software for in ovo imaging) are not mentioned and should be indicated in the Table. These are now given in the Table.

There are some inconsistencies in the abbreviations: NCC appears in the text from line 71 (but would be needed line 38) and thereafter is not always used; similarly ENS is missing line 350. We have now included NCC abbreviations throughout after first use.

In Fig4, ENCC is not mentioned in the legend. Changed to mention ENCC.
Line 440, typing error "embryos" Corrected.

Reviewer #3:

Manuscript Summary:

The authors describe a technique for dual labeling of neural crest cells and

blood vessels within chicken embryos using transgenic GFP chick neural tube grafting and Dil injection of the vasculature.

Major Concerns:

none

Minor Concerns:

One alternative to India Ink injection that the authors may want to mention is the use of neutral red agar pads to lightly stain embryos for visualization. In our personal experience, this method is preferred because the tissue itself is stained and can be seen during surgery under brightfield, and there is less foreign material introduced into the egg. Agar pads containing neutral red are cut slightly smaller than the embryo, placed on top of the vitelline membrane and embryo for a few minutes until the desired level of staining intensity is reached, and then removed from the egg. Both the donor and host can be stained. **This sounds like a very useful method. However, I have never used this approach for embryo visualization, or seen it being used. I would be happy to include a reference for this if the reviewer is willing to provide one.**

Line 101: "via" does not need to be italicized **Corrected.**

Line 105: "bias" does not need to be italicized **Corrected.**

129: chicken eggs --> chickens **I don't know why this change is suggested. It seems correct as is. We store the chicken eggs in an incubator. We don't have any chickens. I therefore didn't make any change.**

172: point out which micropipette in Fig 1C **Done.**

219: refer to figure **Done.**

222: please describe somewhere how to make or where to buy sylgard base watch glasses **The commercial source of sylgard base is now included in the Table.**

224: refer to figure **Done.**

338: "so _as_ not" **Done.**

467: critical steps of Dil --> critical parameters for Dil **Done.**

Reviewer #4:

The protocol presented by Dleanlande et al focuses on dual labeling of neural crest and blood vessels in the chick embryo. This is important because the nervous and vascular systems develop together in many contexts and it is

important to be able to visualize both in the same embryo. In general, this is a well written protocol and the images add to the descriptions. However, there are places where more details are required. Below are some suggestions:

1. Please note that the tungsten needle would also need to be electrolytically sharpened or purchased from a source that should be listed (Fine Science tools, pin section). Also can use pulled glass needles, pulled closed. Custom egg holders or watch glasses can be used. **The following has been added to 1.2: "Alternatives to the micro-scalpel could be electrolytically sharpened needles, commercially available tungsten needles, or pulled glass needles"**
2. Many places do not allow mouth pipetting any longer. One can also use a bent 1cc syringe filled with ink. **The following has been added to 4.2.2: "If mouth pipetting is not permitted in your Institution a 1ml syringe can be used to instead"**.
3. Add source of pancreatin **Done.**
4. Again, please state an alternative to mouth injection of Dil. Pressure injection would be one. For these glass needles, please add settings for the use of pulled glass needles. **The following has been added to 7.6: "A possible alternative to the mouth tube for Dil delivery could be a pressure injector". The settings for making pulled glass needles is given in the Table.**
5. An important detail that has been left out is how the authors tell dorsal from ventral neural tubes so as not to transplant neural tubes upside down. **I have added the following to 6.1 "The orientation of the neural tube can be identified by leaving a small fragment of ectoderm attached, or by cutting a small nick in the dorsal surface"**.

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