Tridimensional Visualization and Analysis of Early Human Development

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Summary

Generating a precise cellular and molecular cartography of the human embryo is essential to understanding of the mechanisms of organogenesis in normal and pathological conditions. Here, we combined whole-mount immunostaining, 3DISCO clearing and light-sheet imaging to start building a three-dimensional cellular map of the human development during the first trimester of gestation. We provide high-resolution 3D images of the developing peripheral nervous, muscular, vascular, cardiopulmonary and urogenital systems. We found that the adult-like pattern of skin innervation is established before the end of the first trimester, showing important intra- and inter- individual variations in nerve branches. We also present evidence for a differential vascularization of the male and female genital tracts concomitant with sex determination. This work paves the way for a cellular and molecular reference atlas of human cells, which will be paramount for understanding human development in health and disease.

Introduction

For centuries, the intricacies of human development have remained an enigma and its study unexplored due to sociocultural, religious and moral reasons (Morgan, 2009). In the second half of the twentieth century, a growing interest for experimental embryology and technological developments (King and King, 1986) led to the first scientific descriptions of human embryo development (His, 1881; Keibel and Elze, 1908). In the mid of the 20th century, Franklin Mall and colleagues at the Carnegie Institute collected, sectioned and stained thousands of human embryos and fetuses (Morgan, 2009). Wax reconstructions and 3D embryo models were generated (Norman, 1923) to define stages of human embryogenesis, still in use today (Keibel and Mall, 1910; O'Rahilly and Müller, 1987). Similar collections of human embryos were initiated in other countries (Blechschmidt, 1977; Fujimoto, 2001). These data still appear in all modern textbooks and atlases of embryogenesis and developmental biology. More recently, histological sections were digitized and reconstructed in 3D (de Bakker et al., 2016). Immunohistochemical studies have also been performed but lack the 3D information which is essential to understand organogenesis. *Ex vivo* 3D images of paraformaldehyde (PFA)-fixed human embryos were recently obtained using magnetic resonance imaging or phase-contrast-X-ray radiographic computed tomography, however, the resolution is still far from being at the cellular level (Kanahashi et al., 2016; Yamada et al., 2010).

Whole mouse brains and embryos rendered transparent with various procedures, can be imaged with excellent cell resolution (Richardson and Lichtman, 2015). Solvent-based clearing methods such as 3DISCO (Three-dimensional imaging of solvent cleared organs) (Ertürk et al., 2012) and its derivatives iDISCO (Renier et al., 2014), iDiSCO+ (Renier et al., 2016) and uDISCO (Pan et al., 2016) are fully

compatible with whole-mount immunofluorescence (Belle et al., 2014; Renier et al., 2014). The rise of light sheet fluorescence microscopy (LSFM) has led to the development of imaging setups compatible with large field objectives and thick (>1 cm) specimens (Dodt et al., 2007). We set out to develop a protocol which combines antibody labeling and tissue clearing for better analyses in human embryology.

Here we have performed whole-mount immunostaining on 36 human embryos and fetuses ranging from 6 to 14 weeks of gestation with over 70 antibodies most of which had never been used in humans (see STAR methods and Table S1). We generated 3D images of human embryos at an unprecedented cellular resolution. This work offers a unique opportunity to start building a molecular and cellular atlas for the study of human development. The possible applications of this new method in the field of embryology are countless. They open new avenues for the study of molecular mechanisms regulating the development of human embryos in physiological and pathological conditions.

Results and Discussion

Development of the human peripheral nervous system

We used a protocol for whole-mount immunostaining and 3DISCO clearing to analyze the development of human embryos (first 8 weeks of gestation) and fetuses (from 8.5 to 14 weeks, see methods). We first studied the development of the peripheral nervous system (PNS) using antibodies against the neuron-specific intermediate filament protein peripherin (Prph), which in rodents is expressed by sensory and autonomic axons (Parysek et al., 1988). In human fetuses, Prph labels auditory and vomeronasal nerves (Casoni et al., 2016; Locher et al., 2013).

We first used embryos at gestation week 6 (GW6 or Carnegie stage CS17, n=1; see STAR Methods and experimental procedures for stage determination), GW7 (CS19, n=2), GW7.5 (CS21, n=1) and GW8 (CS23, n=3). As previously reported for mice (Belle et al., 2014; Renier et al., 2014), tissue-shrinkage was observed (20-40% of its original size) and remained homogeneous throughout the embryo. This shrinkage can be avoided using iDISCO+ (Renier et al., 2016), however, the cleared embryos produced by this method are more fragile and their fluorescence less stable than with 3DISCO. In addition, the methanol treatment needed for iDISCO+ is not compatible with some antibodies. Last, the size reduction after 3DISCO allowed whole embryos to be imaged using LSFM and Prph+ peripheral nerves to be visualized from the brainstem and spinal cord to their distal extremities (Figures 1A-1C and S1A, S1B and Movie S1).

We next tested specific markers of sensory and motor axons, focusing primarily on the innervation of the developing feet and hands from GW6-GW12. Motor and sensory axons, were respectively labeled with antibodies against choline acetyl transferase (ChAT; n=14 cases) and transient adhesion glycoprotein-1 (Tag-1/Contactin 2; n=22 cases) (Karagogeos et al., 1997). Double staining for Prph and Tag-1 (n=14) confirmed that both markers overlapped in peripheral nerves, although from GW9 the thinnest axonal branches were better seen with Tag-1 (Figure 1D). In contrast, there was no overlap between ChAT+ and Prph+ (Figure 1E; n=6) or ChAT+ and Tag-1+ (Figure 1F and S1C; n=7) immunoreactive axons. Motor nerve roots were not immunoreactive for Prph (data not shown), suggesting that in humans, Prph is differentially expressed between spinal cord and cranial motor axons.

3D virtual dissection of organ innervation with Imaris segmentation plugins was performed to trace, artificially color and reconstruct individual nerve fascicles (STAR

Methods). This method was applied to GW7 (CS19; n=2) and GW8 (CS23; n=1; Figure 1G) cranial nerves (Figure 1G and S1A and Movie S1). Fetuses older than GW8, too large for our LSFM microscope, were dissected prior to processing to image limbs and other large organs.

Segmentation-based tracing was used to reconstruct the sensory innervation of the hand from GW6 to GW11.5. The human hand contains the ulnar (or cubital), median and radial nerves, which innervate a different hand areas in a pattern highly conserved between individuals (Dogan et al., 2010).

Sensory nerves and their branches in the hands of 14 embryos and fetuses were reconstructed in 3D using Prph (GW7-GW11, n=11) or Tag-1 (GW6-9.5, n=3) immunostaining (Figures 2A, 2B and S1D), and used to create a developmental time-lapse of sensory innervation in the human hand (Figure 2B and Movie S2). Furthermore, we reconstructed the motor (ChAT+) and sensory (Tag-1+) innervation of two GW8.5 and GW9 fetuses (Figures 2C, 2D and S1C and Movie S2) to determine where and how the two types of nerves diverge.

We next compared the nerve branching patterns in 11 hands and found, in all individuals analyzed, striking heterogeneity between the left and right hands. At all ages, the median nerve was the most similar (Figure S2A and not shown), whereas noticeable differences in the number and length of branches existed between the left and right ulnar or radial nerves (Figures S2A, S2B and Movie S2). The low number of cases analyzed did not allow detecting any correlation with hand laterality. Interestingly, unlike in adult, the musculocutaneaous nerve extended into the hand, at least until GW11 (Figure 2B).

The adult cutaneous innervation is topographically organized with each of the three nerves innervating non-overlapping skin territories. To determine how this map is

established, we pseudo-colored the domains overlying Tag-1+ axons in hands at GW6, GW8.5 and GW11.5 (Figure S2C and Movie S2). At GW8.5, most of the hand surface was already covered with nerves whose topography appeared mature by GW11.5. The middle and index fingers are particularly interesting as their dorsal surfaces are shared by several nerves. The median nerve which normally only arborizes ventrally, projects to the dorsal side of these fingers. At GW6 the median nerve had not yet reached the tip of the index on the palmar (ventral) side but started to branch dorsally, ahead of the radial nerve, between future phalanges (Figure S2D). By GW8 the median nerve reached the tip of the index finger ventrally and started to cover the dorsal and distal part, whereas the radial nerve only innervated the caudal part of the dorsal side of the index finger. This pattern was unchanged at later ages (Figure S2D).

Together, our novel protocol allows 3D visualization of the development of the peripheral innervation of early human fetal stages (first trimester of life) in an unprecedented level of precision and detail and revealed several unknown features of the development of limb innervation. First, an adult-like pattern of the domains of the skin covered by each nerve is already established during the late embryonic-early fetal period. However, within each domain the pattern of nerve branching can be highly divergent between individuals but also within the same individual. This is particularly striking at the level of the hands, where the arborization of the ulnar and radial nerves significantly differ between the right and left hands. This suggests that the development of the PNS innervation is, at least to some extent, stochastic and not profoundly influenced by specific guidance cues distributed in the developing limbs and extremities (Hassan and Hiesinger, 2015). Moreover, in digits that are shared by multiple nerves there is no significant overlap of the branches originating

from the different nerves. Our data infer that median nerves grow faster and start invading the dorsal part of some digits prior to the other nerves and that this may prevent them from entering these territories. Finally, significant remodeling of the sensory innervation occurs. The length of human embryogenesis, compared to rodents, will facilitate the analysis of tissue remodeling during development.

Next, we further validated our method by testing a large palette of antibodies against proteins expressed by many cell types in developing mouse and human embryos (STAR Methods).

3D analysis of human muscle development using LSFM

The current knowledge of skeletal and head muscle development in humans is based on histological studies conducted during the first half of the twentieth century (Gasser, 1967; Gilbert, 1957), and immunostaining with a few muscle cell markers (Abe et al., 2010; De La Cuadra-Blanco et al., 2013). The distribution of muscle cell progenitors in the embryo, however, has never been reported. The transcription factor (TF) Pax7, is a muscle stem cell marker and key initiator of myogenesis in jawed vertebrates (Bryson-Richardson and Currie, 2008). We observed Pax7 immunoreactive nuclei homogenously distributed within each developing muscle of GW9.5 and GW10.5 arms and legs (Figures 3A, 3B and Movie S3; n=1). As expected, all developing Pax7+ muscles were contacted by ChAT+ motor branches (Figure 3B). GW9 leg and arm (n=1) were also triple-immunostained for ChAT, Tag-1 and myogenin (Myog), a myogenic TF expressed by differentiating myoblasts (Bryson-Richardson and Currie, 2008) (Figure 3C and Movie S3). This immunostaining shows that clusters of Myog expressing cells are found at the extremities of each motor nerve branch.

Doublecortin (Dcx) is an X-linked gene which encodes a microtubule-associated (Gleeson et al., 1998; Des Portes et al., 1998). Mutations in *DCX* impede neuronal migration and cause lissencephaly. Dcx may also influence the development of the neuromuscular junction in humans (Bourgeois et al., 2015). Dcx immunostaining on limbs at GW8 (n=1) GW9.5 (n=1), GW10 (n=1), GW10.5 (n=1) and GW13.5 (n=1) showed that developing muscles express Dcx (Figure 3D and data not shown). Sensory nerves were also immunoreactive for Dcx (Figure 3D). Myosin heavy chain (MHC) was previously detected in skeletal, heart and craniofacial muscles from human fetuses (Abe et al., 2010). Here we studied the distribution of MHC+ muscles throughout the body at GW8-GW14 (Figure 3E and Movie S3; n=9 cases). All muscles in the upper half of a GW8 embryo (n=1) were labeled and identified with an unprecedented resolution (Figure 3E and Movie S3). Whole-mount MHC muscle staining was also performed on GW11 and GW14 arms and legs (Figures S3B, 3C). Developing muscles and motor innervation could be simultaneously visualized following MHC/ChAT double immunostaining (Figure 3F and not shown), and importantly, the muscles within each hand and foot of a GW9.5 fetus could be segmented (Figures 3F and S3A, S3D and Movie S3). Moreover, the shape of the developing bones, which appear as black areas on optical sections, could also be extracted and visualized in 3D (Figure S3D).

3D analysis of the vasculature in human embryos

Existing data based on the filling of large arteries and veins (Mall, 1905), 3D reconstructions of histological sections (de Bakker et al., 2016) and vascular corrosion casting (Zawiliński et al., 2001) provide an incomplete view of the early development of human vasculature. In chick and mouse, blood vessels arise from

endothelial cell progenitors (angioblasts) which aggregate to form a dense vascular meshwork covering the embryo, a process known as vasculogenesis (Chung and Ferrara, 2011). Functional continuity occurs later through the remodeling of this initial endothelial network. As the embryo grows, new vessels sprout from existing ones, in a process called angiogenesis. Here, we first performed whole-mount labeling with antibodies against plasmalemma vesicle-associated protein (Plvap; also known as PV-1 or PAL-E), a transmembrane glycoprotein expressed by fenestrated microvascular endothelial cells (Elima et al., 2005). Whole embryos at GW8 (n=2), or various organs from GW9.5-GW14 fetuses (n=8) were labeled and imaged with LSFM. At GW8, an extremely dense Plvap+ vascular network of endothelial cells extended throughout the body (Figure 4A and Movie S4), except in developing bones and corneas (Figure 4B and Movie S4). This supports the existence of a phase of vasculogenesis preceding angiogenesis in human embryos. Among other endothelial cell markers tested, Collagen IV also labels capillaries, but less efficiently than Plvap (Figure 4C). The 3D organization of developing arteries on GW9-GW14 organs (n=6 fetuses) were be assessed with immunostaining for smooth muscle-specific α-actin (SMA; Figure 4D and Movie S4).

Mammalian lymphatic vasculature development is documented for several species (Yang and Oliver, 2014) but not much is known about human lymphangiogenesis (Sabin, 1909). The lymphatic system is crucial for immune response and understanding its development might be relevant to gain some insights into some human diseases. To start investigating lymphangiogenesis in human fetuses, we next labeled the gastrointestinal tract of a GW14 fetus with antibodies against the glycoprotein podoplanin, a lymphatic cell marker (Breiteneder-Geleff et al., 1999; Schuster et al., 2015) and found many isolated podoplanin+ cells covering the gut

and stomach (Figure 4E). Lymphatic cells could also be seen using Prox-1 staining (data not shown). The lack of podoplanin+ capillaries at this age suggests that, as in other mammals, the human lymphatic system might mature later than the vascular system.

Lung development in human embryos

In human embryos, the lung primordia and the trachea emerge from the foregut endoderm around CS11/GW4 (O'Rahilly and Müller, 1987) and airway branching proceeds from GW6 to GW16 during the pseudoglandular stage (Galambos and Demello, 2007). New bronchial branches, which bud from existing ones, further divide and lead to an expansion of the tree. Only a few immunohistological studies of human embryo lungs have been conducted (Sparrow et al., 1999). Here we performed whole-mount immunostaining with 11 antibodies on lungs from 8 embryos and fetuses at GW8-GW14.

In the mouse, the sex determining region Y (SRY)-box 9 (Sox9) TF is expressed in alveolar epithelial cell progenitors at the distal part of each lung branch (Rockich et al., 2013), whereas Sox2 is restricted to the proximal part (Maeda et al., 2007). We observed a similar result in human lungs where all terminal bronchial buds were Sox9+ (Figures 5A-5D, 5H, and S4A and Movie S5), whereas Sox2 was expressed in a complementary pattern (Figures S4A and S4D). Strikingly, double staining for Sox9 and Dcx, which was not yet reported to be present in the lung epithelium, showed that Dcx was also expressed in the proximal epithelial portion of each airway (Figures 5A and 5B). We also visualized in 3D the vasculature of the lung with Plvap (Figures 5A-5C and S4B). The small capillary plexuses in the lung mesenchyme wrapping around terminal buds at GW8 are not isolated but form a continuous network with the

larger vessels (Figures 5A-5C and S4B), thereby supporting an angiogenesis mode of lung vascularization as observed in mice (Parera et al., 2005).

The complete reconstruction of the mouse airway lineage revealed that the branching pattern is highly stereotyped with new branches budding according to 3 modes: domain branching (daughter branches form in a row), planar and orthogonal bifurcation budding (Metzger et al., 2008). We performed such a lineage analysis in human whole-lungs using the segmentation of Sox9/Dcx (or Sox9/Sox2) bronchioles (Figures 5D and 5E and Movie S5). Similarly to the mouse, we observed three branching modes in humans (Figure 5E), but with some evidence of asymmetric bifurcations (Figure 5E). We next performed immunolabeling for smooth muscle markers SMA and myosin smooth muscle (MyoSM). In both cases, smooth muscles surrounding the bronchi and the proximal part of airway epithelial tubules were labeled (Figures 5F and 5G and S4A). The absence of smooth muscles on distal buds was confirmed using Sox9 immunostaining (Figure 5H). Incidentally, smooth muscle staining also allowed to visualization of lung arteries and arterioles (Figures 5F and 5G and Movie S5).

The lung is primarily innervated by sympathetic and parasympathetic axons traveling through the vagus nerve. Parasympathetic ganglia also contribute to the innervation of the trachea and main bronchi (Aven and Ai, 2013). Due to the complexity of the airway tree, understanding its innervation can only be achieved in 3D. We visualized lung innervation using immunostaining against Prph, Tag-1 and ßIII-Tubulin. Immunoreactive vagus axons extended to the most distal portion of the airway branches (Figures S4C and S4D). Preliminary results indicate that the development of the vasculature of the human heart and its innervation (using immunostaining for

Tyrosine hydroxylase) can also be studied with this 3D method (Figures 5I and 5K and S4E and Movie S5).

3D Analysis of urogenital system development in human embryos

In adult mammals, the urogenital system is comprised of the kidneys, urinary tracts gonads and reproductive ducts. Before GW7-GW8, the human gonadal ridges are undifferentiated. Genital ducts appear morphologically similar in both sexes with two paired structures: the Wolffian ducts (WD) which are derived from the mesonephros and the Müllerian ducts (MD) whose differentiation is induced and guided by the WD. A major reorganization of the genital tracts accompanies sex determination (Jacob et al., 2012; Orvis and Behringer, 2007). In males, MD regress under the influence of an anti-Müllerian hormone produced by the testis. In females, the WD degenerate and the MD transform into the female reproductive ducts (Georgas et al., 2015; O'Rahilly, 1983).

Knowledge of urogenital system development is primarily based on histological and electron microscopy analysis (Fritsch et al., 2012; Hashimoto, 2003), and has not yet been studied at a cellular and molecular level in humans unlike in mice (Georgas et al., 2015; Little et al., 2007). We studied the development of the human urogenital system using antibodies against Pax2 TF. Pax2 mRNA was previously detected in the mesonephros and WD at GW6-7 (Tellier et al., 2000). We used embryos and fetuses of either sex, including 11 males (GW8-GW14) and 9 females (GW7-GW14). All major components of the urogenital system were visualized in 3D (Figures 6 and 7, and S5 and Movie S6), and individually segmented. In the youngest male embryo (GW8), the caudal tip of the MD was in close contact with the WD but had not yet fully elongated (Figures 6A and 6B) and mesonephric tubules stemming from the WD

covered the testis primordium. Kidneys still occupied a ventral position adjacent to the genital ridges (Figure 6A). At GW9.5 (n=1), the MD further elongated along the WD but did not yet join (Figures 6C and 6D). In GW10 male fetuses (n=2), the two MD were fused and extended medially between the two WD up to the urogenital sinus. However, in both cases, the MD started to fragment dorsally (Figure 6E, F and Figure S5A), and this remnant of the fused MD might give rise to the prostatic utricle. At older ages, such as GW14 (n=1), the mesonephric nephrons of the WD have regressed while the epididymis ducts and *vas deferens* have emerged (Figure 6G). As previously reported (Jacob et al., 2012), an apical MD remnant is still present next to the *rete testis* by GW14 (Figures 6G and 6J and Movie S6). Sox9 is essential to testes differentiation in males and its mRNA was first detected around CS19-CS21 (GW7-7.5) (Hanley et al., 2000). Using whole-mount immunostaining for Sox9, on GW10, GW11, GW13.5 and GW14 fetuses (n=1 for each) we visualized the 3D organization of the testis cords, containing Sox9+ Sertoli cells (Figure 6H and 6J and Movie S6 and not shown). We also stained GW10.5-GW13 female fetuses with Pax2 (n=6) and followed in 3D the reorganization of the urogenital tract (Figures 7 and S5). In our youngest female fetus (GW10.5) the MD have already fused to form the uterovaginal canal (Figure 7A). The WD were still continuous but mesonephric tubules began to regress. At GW11.5, the regression of the mesonephros and WD was more pronounced and the length of the two MD increased (Figure 7B). At GW13, the distal part of the WD had dissolved whereas its cranial part transformed into the Fallopian tubes (Figures 7C and S5B and Movie S6). The vascularization of the developing gonads is thought to play a role in their maturation (Brennan et al., 2002; Coveney et al., 2008). We performed double staining for Pax2 and Plvap to study the interaction between the vasculature and the genital tracts. Interestingly, at GW8 and

GW10, the developing testes and WD were embedded in a dense capillary meshwork but the MD was avascular (Figures 7D and 7E). In contrast, in a GW10.5 female fetus, both the MD and the WD were vascularized (Figures 7E and 7F) and a dense vascular network ensheathed the MD by GW13 (Figures 7F and 7G and Movie S6). While we could not study younger female embryos, these results suggest that MD angiogenesis may be sex dependent. This differential vascularization of the developing MD in males and females at GW10 suggest that their regression in males, which starts around this stage could be linked to or facilitated by the lack of vascularization. By contrast, the WD are vascularized in both sexes. Interestingly, the distal fused part of the Müllerian ducts does not seem to regress and likely gives rise to the prostatic utricle as previously proposed (Jacob et al., 2012).

Information on the early development of human nephrons is scarce (Ludwig and Landmann, 2005). Staining of GW10-GW13.5 kidneys (n=5) showed that Pax2 and Sox9 are both expressed in the epithelium of the ureteric tree (Figure S6). Pax2, together with another TF, Six2, was also found in nephron precursors of the cap mesenchyme surrounding the ureteric tips in the nephrogenic zone of the developing kidney (O'Brien et al., 2016). Staining of the endothelial cells with Plvap revealed the presence of developing glomeruli and their morphology (Figures S6D and S6E).

Transcription factors: new tools to study human embryology

During development, cell differentiation toward specific lineages is controlled by a cascade of transcription factors. We used antibodies against 19 transcription factors to follow the development of multiple organs (see also above).

In mouse and human embryos, Sox10 is expressed by oligodendrocytes, neural crest cells and some of their derivatives (Betters et al., 2010; Bondurand et al., 1998).

SOX10 mutations cause various diseases such as neurocristopathies, melanoma and peripheral demyelinating neuropathies (Bondurand and Sham, 2013). Here we performed double immunostaining for Sox10 and Prph on limbs from five fetuses, at GW9-GW12. Sox10+ cells were widely distributed throughout the PNS, at the level of the dorsal root ganglia and all along Prph-labeled nerves and branches (Figure S7A and Movie S7). These Sox10+ cells most likely correspond to Schwann cell precursors, which in the mouse migrate along PNS axons (Espinosa-Medina et al., 2014).

In the developing tongue, taste bud primordia and papillae have been identified at GW9-GW10 using electron microscopy (Hersch and Ganchrow, 1980). No markers of developing human taste buds are known. In order to visualize tongue papillae, we performed immuno-labeling with antibodies against Prox1 and Sox2, both of which control mouse taste bud differentiation (Nakayama et al., 2015; Okubo et al., 2006). Both Prox1 and Sox2 were detected in lingual papillae (including circumvallate papillae) covering the surface of the tongue (Figure S7B and S7C and Movie S7) in a similar pattern, suggesting that they are co-expressed in taste bud precursors, as observed in mice.

Whole-mount immunostaining for Sox2, which is known to be expressed by many types of cell progenitors (Sarkar and Hochedlinger, 2013), was also performed on other tissues from GW9.5, GW10 and GW12 fetuses. Strikingly, Sox2 expression was detected at the tip of each digit, in a narrow ring of cells lining the edge of the developing nails (Figure S7D and Movie S7). This indicates that Sox2 is a marker of fingernail stem cells in human fetuses, suggesting a role for this TF in nail development. Sox2 was also highly expressed in the ventricular zone of the developing brain (Figure S7E). Finally, we focused on the digestive system, which

was collected in embryos and fetuses from GW7 to GW14 (n=13). Staining with Sox9 clearly revealed the 3D organization of the developing villi of the gut epithelium where stem cells express this TF (Figure S7F and Movie S7)(Bastide et al., 2007). The enteric nervous system (ENS) has recently been the focus of many studies in part because of its frequent involvement in several genetic diseases (Heanue and Pachnis, 2007). ENS neurons are derived from neural crest cells which colonize the gut around GW4. Double labeling for Phox2b (paired-like homeobox 2b) and Prph revealed the dense network of ENS neurons, which formed interconnected clusters covering the surface of the gut (Figure S7G and Movie S7). Prph staining also revealed the parasympathetic innervation of the digestive system (Figure S7G).

Conclusion

Here, we have combined immunolabeling with whole-body and whole-organ 3D imaging to provide a new comprehensive and unbiased method to study the early development of multiple human biological systems.

Non-invasive medical imaging of embryos and fetuses *in utero* has made remarkable progress. This includes 3D/4D obstetrical ultrasonography which can generate holographic images of the embryo (Baken et al., 2015; Pooh et al., 2011) but mostly provides information about surface features and cavities, and 3D power Doppler which visualizes the embryo vasculature (Weisstanner et al., 2015). In addition, *in utero* magnetic resonance imaging (MRI) provides a good appreciation of the developing CNS in the fetus (Weisstanner et al., 2015) and diffusion tensor imaging (DTI) tractography is now used as a prenatal diagnostic of callosal dysgenesis as early as GW20 (Jakab et al., 2015). Whilst these techniques represent valuable tools to visualize the gross anatomy of the fetus, they lack in resolution and are insufficient

to resolve the molecular signature of individual cells or to know their organization within organs. Recently, whole-organ histology and 3D-reconstruction analysis based on the digital alignment of embryo sections has been performed (de Bakker et al., 2016), but it is extremely time consuming. Our method, which preserves the 3D organization of the organs while achieving a great cellular resolution, is rapid, highly reproducible and should provide clinicians with a reliable spatial framework for the correlation of *in utero* and *postmortem* 3D images of embryos and fetuses.

The simplicity of this method, its robustness (for example, we could label endothelial cells in a fresh frozen GW5 embryo kept for 11 years at -80°C, unpublished data) and sensitivity will facilitate its transfer to human embryology laboratories. Its use for the analysis of embryos and fetuses with genetic diseases and malformations will improve our understanding of their etiology.

Currently, the main limitations of our method are the availability of human embryos, the number of antibody combinations (a maximum of four at this time), the compatibility of the antibodies with our protocol (Table S1) and the storage of large size light-sheet image datasets. Nevertheless, the spectrum of future investigations and applications of this method in the field of embryology and fetology are countless. For instance, defining how many cells give rise to an individual organ and understanding how cell numbers are regulated during development is essential to understanding the process of organogenesis. Here we show that whole-mount immunostaining with transcription factors and proliferation markers (KI67, H3P) expressed in specific cell types (such as stem cells and muscle precursors) can be efficiently carried out in cleared embryos and fetal organs. This opens the possibility to obtain the number of cells expressing these factors, as well as mapping their localization in the body and estimating the rate of cell proliferation during the first

trimester of human gestation -- a critical period for various diseases in which tissue growth is perturbed. The recent surge of Zika virus infection and reports of its deleterious effects in the brain (Oliveira Melo et al., 2016) has demonstrated how incomplete our understanding of human embryo development is, and how little we know about the mechanisms through which pathogens, toxins and mutations impact embryogenesis. Our work shows that it should be possible in the near future to build a reference 3D atlas of the developing human. As a first step in this direction, all our 3D datasets are made available on a dedicated website (https://transparent-human-embryo.com/) which will also serve as a repository for additional embryology 3D data generated from our laboratory and others. This reference 3D atlas of the developing human and specific organs and systems not only represents a powerful educational online tool for researchers, educators and students worldwide, but will allow 3D printing of anatomical models for didactic purposes in health sciences education programs.

Author contributions

S.A.M., P.G. and F.C. collected, staged and fixed the embryos and fetuses. M.B. and S.A.M. did the clearing and immunostaining. M.B. and D.G. performed the LSFM acquisitions and Imaris data processing. A.C. and M.B. did the figures. A.C., M.B., D.G. and G.C. analyzed the data. A.C. and P.G. wrote the manuscript. All authors corrected the manuscript.

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Table S1. Related to Figure 1.

Primary antibodies tested but not working with our protocol

Movie S1.

3D analysis of human cranial nerve development.

Related to Figures 1 and S1.

Movie S2

3D development of hand and foot innervation.

Related to Figures 2 and S1 and S2.

Movie S3

3D imaging of the developing muscles.

Related to Figures 3 and S3.

Movie S4

3D imaging of the developing vasculature.

Related to Figure 4.

Movie S5

3D imaging of the developing cardiopulmonary system.

Related to Figures 5 and S4.

Movie S6

3D imaging of the developing urogenital system.

Related to Figures 6 and 7 and S5.

Movie S7

3D analysis of transcription factor expression in human embryos.

Related to Figure S7.

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Figure legends

Figure 1

3D analysis of peripheral nervous system development in human embryos.

All panels are LSFM images of solvent-cleared embryos.

(A) Surface shading image (left) and Prph labeling of peripheral nerves (right) at GW7. (B) Overlay of the surface shading image (grey) and Prph labeling (green) at GW8. (C) High magnification images of Prph+ innervation at GW8. The middle and right panels are overlays of the surface contrast image (grey) and Prph labeling (green). (D) GW9.5 thumb labeled for Prph and Tag-1. The two markers co-localize but the thinnest branches (arrowheads) are better labeled with Tag-1. (E) Dorsal view of a GW9.5 hand double-labeled for ChAT (motor axons) and Prph (sensory axons), showing the lack of co-localization. (F) Dorsal view of a GW9 foot labeled for ChAT and Tag-1. The two markers are not co-expressed. (G) Right view of the head and cranial nerves at GW7 (Prph staining). On the right panel, cranial nerves are segmented and highlighted with specific pseudo-colors. Abbreviations: N, nostrils; M,

mouth; V2 (maxillary) and V3 (mandibular), second and third branches of the trigeminal nerve.

Scale bars,1000 μm in A and G, 2000 μm in B, 500 μm in C, 300 μm in D, 200 μm in E and 150 μm in F.

See also Figure S1.

Figure 2

3D analysis of the innervation of the developing human hand and foot.

All panels are LSFM images of solvent-cleared embryos.

(A) Images (Dorsal view) illustrating the segmentation process of individual sensory nerves in a GW8 hand labeled for Prph. Raw LSFM images (left), individual segmentation and 3D rendering of the radial (blue), median (magenta) and ulnar (green) nerves (right panels). (B) Time series illustrating the developing innervation of the right hand from GW7-GW11 at a similar scale. The musculocutaneous nerve (arrows) transiently extends into the hand. (C and D) Segmentation of the sensory (Tag-1, green) and motor (ChAT, red) nerves in a GW9 foot (C) and GW8.5 hand (D). (C) Dorsal views (upper panels) and right side views (lower panels). (D) Trajectory (dorsal view) of individual motor and sensory axons (lower panel).

Scale bars, 700 μm in A, 400 μm in B, 500 μm in C, 200 μm in D.

See also Figure S2.

Figure 3

3D analysis of muscle development in human embryos.

All panels are LSFM images of solvent-cleared embryos.

(A) Lateral view of the right foot (left panel) and dorsal view of the right hand (right panel) of a GW9.5 fetus labeled for Pax7. (B) Plantar view of a GW10.5 foot labeled for Pax7 (blue) and ChAT (Red). (C) Dorsal view of a GW9 foot labeled for Myog, ChAT and Tag-1. Myog+ nuclei cluster at the tip of ChAT+ motor branches (arrowheads). (D) Dorsal view of a GW9.5 foot double-labeled for Doublecortin (Dcx) and ChAT. Dcx is found in muscles (asterisks) and sensory nerves (arrows) but not in ChAT+ motor axons (arrowheads). (E) Images from a GW8 embryo immunolabeled for MHC and Tag-1. Front view (left). High-magnification view of the oculomotor muscles (upper middle panel). The dotted lines mark the borders of the muscles where the light sheet is weakened by the pigmented epithelium. Lateral view (lower middle panel) of muscles and sensory nerves. The right panel shows muscles and sensory nerves in the left arm. (F) GW9.5 hand labeled for MHC and ChAT showing muscles and motor innervation. Muscles were individually segmented and pseudo colored (see Figure S3A for muscle names), and developing bone contours are visualized (see methods).

Abbreviations: SR, superior rectus; LR, lateral rectus; IR, inferior rectus; MR, medial rectus; IO, inferior obliquus; SO, superior obliquus; Om, oculomotor muscles; Dia, diaphragm; Bi, biceps; Tri, triceps.

Scale bars, 300 μm in A and D, 500 μm in B and E, 200 μm in C, 400 μm in F.

See also Figure S3.

Figure 4

3D analysis of the developing vascular system in human embryo.

All panels are LSFM images of solvent-cleared embryos. (A, B) GW8 embryo immunolabeled for Plvap, a marker of endothelial cells. Plvap+ cells form a very

dense network throughout the embryo. The right panels in A illustrate the right arm and hand. (B) The left panel is a z projection of 1400 µm through the left leg. The vascular network penetrates all tissues except the developing bones (asterisks). The right panel is at the level of the face. Note the absence of vessels on the developing cornea (arrowhead). (C) Vessels labeled with Collagen IV at the surface of the ribs in a GW11 fetus. (D) Arteries in the right leg/knee and foot of a GW11.5 fetus immunolabeled for MyoSM (left) or SMA (right). The right panel shows the result of the segmentation and individual colorization of the main arteries of the foot (dorsal view). (E) Image at the level of the digestive tract of a GW14 fetus labeled for podoplanin, a marker of lymphatic cells. Podoplanin cells are found above the stomach (Sto), and Gut. The right panel shows that podoplanin+ cells have not yet developed into vessels. Abbreviations: No, nostrils; Mo, mouth; Fem, Femorale arteria; Sup Lat Gen, arteria superiori lateralis genus; Pop, arteria popliteal; Pan, pancreas.

Scale bars, 1500 µm in A (left panel), 500 µm in A (right and middle panels) B, D and E (left panel), 200 µm in C, 50 µm in E (right panel).

Figure 5

3D analysis of the developing cardiopulmonary system in human embryos.

All panels are LSFM images of solvent-cleared embryos.

(A-E) Left lung from a GW9.5 fetus immunostained for Sox9, Dcx and Plvap. Sox9 is expressed in the distal bud of the epithelial tubules whereas Dcx is found in their proximal part. The entire lung vasculature can be labeled with Plvap. (B) Optical section (250 µm z projection) showing one lung epithelial tubule. (C) Illustrates the capillary network (red) at the level of Sox9+ terminal buds (blue). (D, E) 3D Analysis

of airway branching. One lung lobe (green and blue) is isolated with Imaris (right panel), and in (D) a single bronchus is isolated (appearing in red). (E) Higher magnification images of the isolated bronchus viewed under two different angles. The rightmost panels illustrate three types of branching patterns. (F) GW9.5 lungs stained for smooth muscle myosin (MyoSM). The two main bronchi (arrowheads) and their ramifications are seen. (G) SMA staining revealing the pattern of airway smooth muscle branching in a GW11.5 left lung. Muscles around arteries (arrowheads) and proximal airways (arrows) are seen. (H) GW10 lung branches. The terminal buds are labeled with Sox9. SMA+ smooth muscles (yellow) are only found around the proximal portion (Sox9-) of the tubules. (I-K) LSFM images of the developing heart. (I) GW10 heart vasculature stained with anti-SMA antibodies. (J-K) Evolution of the dopaminergic innervation (TH, tyrosine hydroxylase) of heart between GW10.5 (J) and GW14 (K). The right panel in K illustrates the sympathetic innervation (green) on large vessels of the heart (red, see also Figure S4E).

Abbreviations: RC, right coronary artery; LC, left coronary artery, IV, interventricular artery.

Scale bars, 500 µm in A, D (left panel), G (left panel), I and J, 150 µm in B, C, G (right panel) and K (right panel), 200 µm in d (right panel), 50 µm in E, 300 µm in F, 100 µm in H, 1000 µm in K (left panel).

See also Figure S4 and Figure S7.

Figure 6

3D analysis of the urogenital system development in male embryos.

All panels are LSFM images of solvent-cleared embryos.

(A, B) GW8 embryo stained for Pax2. Raw LSFM image (left) and 3D rendering

image (right). The middle panel shows the segmentation and pseudocolorization of the kidney and ureters (yellow), Müllerian ducts (magenta, MD), and Wolffian ducts (blue, WD; the mesonephric tubules are indicated by an arrowhead). (B) High magnification of the MD/WD junction (arrow). (C, D) Segmented (left) and 3D (right) images of the GW9.5 urogenital system labeled with Pax2. (D) The apical tip has enlarged (arrow). The distal tips of the MD (arrowheads in C and D) have extended along the WD (compare with A) but have not yet fused. (E) Segmented (left) and 3D (right) images of the GW10 urogenital system labeled with Pax2. The MD have fused distally (arrow) and began to fragment (arrowheads). (F) More advanced stage of MD regression and fragmentation (arrowhead) in a second GW10 fetus. (G) 3D image of the GW14 urogenital system labeled with Pax2. A short fragment of the Müllerian duct persists (arrowhead) and the *vas deferens* (VD) appears more developed (arrow). (H) GW10 testis (Te) labeled with Pax2 (red) and Sox9 (green). Sox9+ Sertoli cells are seen in the developing testis cords (arrowheads). (I) Single optical section (2 µm z projection) through a GW10 fetus testis labeled for Pax2, Sox9 and Plvap (endothelial cells). The arrow indicates the *rete testis*. (J) GW14 testis labeled for Sox9 and Pax2 segmented. The Müllerian (magenta) and Wolffian (cyan) ducts (Pax2+) have been segmented and pseudocolored.

Abbreviations: Kid, kidney; MD, Müllerian duct; PU, prostatic utricle; WD, Wolffian duct; ♂, male.

Scale bars, 400 µm in A, B, 500 µm in C and E, 100 µm in D and H (right panel), 300 µm in F, G, H (left and middle panels) and I, 350 µm in J.

See also Figure S5 and Figure S6.

Figure 7

Comparative 3D analysis of the urogenital system development in female and male embryos.

All panels are LSFM images of solvent-cleared embryos.

(A) Original LSFM image (left), segmented/colorized (middle) and 3D (right) images of the urogenital system in a GW10.5 female fetus labeled with Pax2. The Wolffian ducts (cyan) are continuous and the Müllerian (magenta) ducts have fused. (B) Genital system of a GW11.5 female fetus stained with Pax2 after segmentation and 3D rendering. The arrow indicates the developing uterus and upper vagina. The Wolffian ducts start to regress (arrowheads). (C) Genital system of a GW13 female fetus stained with Pax2 after segmentation and 3D rendering. The size of the future uterus has increased and the Wolffian ducts have significantly regressed (arrowheads). The right panel is a high magnification at the level of the apical part of the Müllerian duct showing the developing fimbriae of the oviduct (arrow). (D-E) Vasculature of the developing gonads. (D) GW8 testis labeled for Pax2 and Plvap. A dense Plvap+ capillary network covers all the testis (arrow on the left) and the Wolffian duct (WD). By contrast, the Müllerian duct (MD) is not vascularized (arrowheads). The right panel is an optical section (1.2 µm) through the right testis and MD. (E) At GW10, the MD is still devoid of capillaries in male. (F, G) GW10.5 and GW13 ovaries labeled for Pax2 and Plvap. A dense vascular network covers the ovary (Ov). Unlike in males, both the Müllerian and Wolffian ducts are densely vascularized. Abbreviations, ♀, female ; ♂, male; MD, Müllerian Ducts; WD, Wolffian Ducts; OV, Ovary.

Scale bars, 500 µm in A, B and C (left panel), 270 µm in C (right panel), 100 µm in D (middle and right panels), 160 µm in D (left panel), 200 µm in E-G

See also Figure S5 and Figure S6

Supplementary figure legends

Figure S1. Related to Figure 1

Peripheral innervation of the tongue and hand of 3DISCO-cleared embryos.

All panels are images acquired by light sheet fluorescence microscopy (LSFM) of 3DISCO-cleared embryos.

(A) GW7 embryo stained for Prph. The left panel is a segmentation of the four nerves innervating the tongue (right side). The trigeminal nerve (V3, mandibular subdivision) and the chorda tympani of the facial nerve (VIIb or Wrisberg nerve) contribute to the lingual nerve. The two other nerves are the glossopharyngian (IX) and hypoglossus (XII). The right panel is a ventral view of the tongue. (B) Dorsal views of the tongue in a GW9.5 fetus labeled for Prph (left) and ChAT (right). (C) Dorsal view of the left hand at GW8.5 labeled for Tag-1 (sensory nerves) and ChAT (motor nerves). All nerves (ulnar, median, and radial) are shown on the upper panels. The lower panels represent the ulnar, the median and the radial nerves of the hand individually. The ulnar and the median nerves are comprised of both sensory and motor axons. (D) LSFM images of a GW11 right hand labeled for Prph after segmentation and pseudocolorization. The median (magenta), radial (blue) and ulnar (green) nerves are shown together. From left to right, images correspond to a counterclockwise rotation of an angle indicated on the panels from the dorsal view position on the left.

Scale bars, 500 μm in A (left panel), 300 μm in A (right panel) and B, 200 μm in C.

Figure S2. Related to Figure 2.

Development of sensory nerve branches in the human hand

(A, B) LSFM images after segmentation and 3D rendering of the innervation of GW6-GW9.5 embryos (dorsal views). The upper panels are mirror images of left hand nerves and the lower panels show right hands from the same individuals. Red arrowheads indicate branches found uniquely in one of the hands. (A) At GW6, the median nerve branches (magenta) are very similar in both the right and the left hand whereas the radial (blue) and ulnar (green) nerves have distinct branching patterns between the left/right hands. (B) Radial nerves at GW7, GW8 and GW9.5. Some branches are only found in either the left or right hands. (C) Dorsal and palmar 3D views of GW6, GW8.5 and GW11.5 right hands in which the surface of the skin overlaying the radial (blue), ulnar (green) and median (magenta) nerves have been pseudo-colored. (D) Side views illustrating the development of the innervation of the medial (magenta) and radial (blue) nerves in the right index finger at GW6, GW8, GW9 and GW11. Short arrows indicate the dorsal branches of the median nerve that extend ahead of the radial nerve and arrowheads the border between, the radial and medial nerves.

Scale bars, 200 μm in C (left), 300 μm in C (middle) and 1000 μm in C (right).

Figure S3. Related to Figure 3

Segmentation of the musculature in human embryos.

All panels are LSFM images of 3DISCO-cleared embryos.

(A) Palmar view of the left hand in a GW9.5 fetus labeled for MHC. Each panel shows the segmentation of individual muscles or group of muscles. (B) Dorsal view of the right foot of a GW11 fetus labeled with antibodies against MHC and Tag-1. Muscles (magenta) and sensory nerves (Green) can be seen. (C) Dorsal view of the muscles of the right hand of a GW14 embryo labeled for MHC. (D) Dorsal and plantar

views of the right foot of a GW9.5 fetus labeled for MHC. All muscles have been individually segmented, pseudo-colored and bones contours were extracted from LSFM images. Abbreviations: I-V, toes. Scale bars, 400 µm in A,B, 1000 µm in C, 500 µm in D.

Figure S4. Related to Figure 5

Development of the cardiopulmonary system in human embryos.

All panels are LSFM images of 3DISCO-cleared embryos and fetuses.

(A) Left lung from a GW14 fetus immunostained for Sox2, Sox9 and SMA. Sox2 is expressed along the airway tubules except in the distal buds which only express Sox9. SMA+ smooth muscles ensheathe the Sox2+ epithelium (arrowheads) but not the Sox9+ buds. The right panel is an optical section (3 µm z projection). (B) Vasculature of the lungs from a GW8 embryo labeled for Plvap. (C) Innervation of the lungs from a GW9.5 fetus labeled with anti-Prph. The vagus nerves (arrows) send branches along the lung airways. The lungs and their innervation have been segmented and pseudo-colored in green. (D) Innervation of the airway branches in a GW14 embryo, visualized with immuno-histochemistry with anti-ßIII tubulin and anti-Sox2. ßIII-tubulin+ axons extend along the Sox2+ epithelial tubules to the level of the distal epithelial buds (not labeled with Sox2). (E) Dopaminergic (TH) innervation of the heart of a GW14 fetus. The large vessels are labeled with anti-SMA (see also Fig. 6K).

Scale bars, 1000 µm in A and C (left panels), 200 µm in A (middle panels), 100 µm in A(right panel), 400 µm in B, 300 µm in C (right panel) and D, 1200 µm in E.

Figure S5. Related to Figure 6 and Figure 7

Development of the Müllerian and Wolffian ducts in male and female human embryos.

All panels are LSFM images of the genital tracts of 3DISCO-cleared embryos and fetuses labeled with Pax2 antibody, segmented and pseudocolored. All images are at the same magnification. (A) In males the Müllerian duct (MD) extends ventrally along the Wolffian duct (WD) and fuses with the opposite MD around GW9.5. The MDs then degenerate except the fused domain that will become the prostatic utricle. (B) In females, the MDs fuse to form the uterus and vagina and the WDs regress.

Scale bars, 400 μm in A and B.

Figure S6. Related to Figure 7

LSFM of the developing human kidney.

All panels are LSFM images of 3DISCO-cleared fetuses.

(A, B) GW11.5 kidney labeled for Sox9, Pax2 and MyoSM. Sox9 and Pax2 are expressed in the developing kidney (Kid) but are absent from the suprarenal gland (SG). MyoSM labels arteries vascularizing the kidney. (B) Is a single optical section (1 μm) through the cortex. Sox9 and Pax2 are expressed in the distal part of the ureteric buds (arrowheads). Pax2 is also expressed in the cap mesenchyme and developing nephrons (arrow). (C) A GW11 kidney labeled for Pax2 and Six2. Both TFs are expressed in developing cap mesenchyme surrounding ureteric tips and renal vesicles (arrowheads). (D) Single optical section (2 μm) through the kidney of a GW13.5 fetus labeled for Plvap, Six2 and Pax2. Six2 and Pax2 are co-expressed in the cap mesenchyme (arrowheads) but Pax2 is also expressed in tubules. Plvap staining shows the dense renal capillary network and glomeruli primordia (arrows).

Scale bars, 500 μm in A, 50 μm in B, 180 μm in C and 70 μm in D.

Figure S7. Related to Figure 5

Transcription factor expression in human embryos.

All panels are LSFM images of solvent-cleared embryos.

(A) GW9 and GW12 fetuses labeled for Sox10 and Prph. The left and middle panels illustrate Sox10+ cells at the level of the spinal cord (Sc) and dorsal root ganglia (Drg). The right panel shows Sox10 cells (including Schwann cell precursors) migrating along sensory nerves in the foot. (B) Tongue of a GW9.5 fetus labeled for Prox1 and Tag-1. Prox1 is expressed by taste bud cell precursors and Tag-1 by sensory axons. (C) Tongue of a GW10 fetus labeled with Sox2 antibodies. The pattern of developing Sox2+ taste buds is seen. (D) Sox2 and Prph immunostaining of the index of a GW12 embryo. Sox2 is found in a narrow ring of cells (arrowheads) at the base of the presumptive finger nails (Prph stains sensory nerves). The right image is a higher magnification of the Sox2 cells. (E) Sox2 staining of a GW9 hindbrain. Sox2 is highly expressed in progenitors of the ventricular zone lining the IVth ventricle (IV) and cerebellum (Cer). (F) Sox9 immunostaining of the intestine of a GW11.5 embryo labeled for Sox9. Sox9 is highly expressed in the epithelium of the developing villi. The upper right panel is a high mag of intestinal villi. The bottom right panel is a 3.5 µm z projection. (G) Whole-mount staining for Phox2b and Prph on a GW13 gut. The dense network of Phox2b immunoreactive enteric neurons is shown and their Prph+ connections. The vagus nerve innervating the gut is also seen (arrow).

Scale bars, 150 µm in A, 80 µm in B, 500 µm in C, E, F (left panel), 300 µm in D (left panels), 50 µm in D (right panel), 100 µm in F (right panels), 800 µm in G (left panel, 200 µm in G (middle panel), 70 µm in G (right panels).

STAR Methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr Alain Chédotal (alain.chedotal@inserm.fr).

All data has been deposited in https://transparent-human-embryo.com/ and access to the high-resolution movies and image stacks is regulated via an MTA agreement available online.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human embryos (<GW8.5) and fetuses (≥GW8.5) were obtained with the parent's written informed consent (Gynaecology Hospital Jeanne de Flandres, Lille, France) with approval of the local ethic committee (protocol N° PFS16-002). Tissues were made available in accordance with the French bylaw (Good practice concerning the conservation, transformation and transportation of human tissue to be used therapeutically, published on December 29, 1998). Permission to utilize human tissues was obtained from the French agency for biomedical research (Agence de la Biomédecine, Saint-Denis La Plaine, France). For this study, we used 7 embryos at Carnegie Stage 17 (CS17) corresponding to 41 days of development (E46) or gestational week 6 (GW6) (n=1)(O'Rahilly and Müller, 1987), CS19/E46/GW7 (n=2), CS21/E51/GW7.5 (n=1), CS23/E56/GW8 (n=3), and 29 fetuses at GW8.5 (n=2), GW9 (n=3), GW9.5 (n=5), GW10 (n=6), GW10.5 (n=2), GW11 (n=3), GW11.5 (n=1), GW12 (n=1), GW13 (n=1), GW13. (n=1) and GW14 (n=4). This included 9 females and 11 males (sex was undetermined for the others).

METHOD DETAILS

Tissue collection and processing

For embryos younger than 8 gestation weeks (GW), the developmental age was assessed using the Carnegie stages (CS)(O'Rahilly and Müller, 1987). Gestation weeks of each embryo or fetus studied were estimated based on the information about: 1) menstrual weeks, 2) morphology, 3) length (crown-rump), 4) craniofacial structures, and 5) position of the tongue. Embryos and fetuses, were fixed by immersion in 4% PFA at 4°C for 1 to 5 days depending on size. For fetuses between GW9-14, tissues were dissected after fixation and post-fixed in 4% PFA at 4°C overnight. Embryos and fetuses, did not present any malformations.

Sex determination

Sex determination was obtained by isolating DNA from extracted tissues using lysis buffer containing 0.1mg/ml proteinase K, 5M Sodium Chloride, 20% Sodium dodecyl sulfate, 1M Tris, pH 8.0 solution in water and stored at 54°C over night. DNA was precipitated with isopropanol (1:1) and re-suspended in RNase/DNase-free water for 3 hr at 65°C. A PCR was performed in a thermocycler (Biorad) using the following steps: 94°C for 3 min and 35 cycles of 94°C for 1 min; 56°C for 30 s; 72°C for 30 s and 72°C for 5 min. For genotyping the following primers were used: SRY sense 5'-AGCGATGATTACAGTCCAGC-3' and antisense 5'-CCTACAGCTTTGTCCAGTGG-3'; FGF16 sense 5'- CGGGAGGGATACAGGACTAAAC-3' and antisense 5'-CTGTAGGTAGCATCTGTGGC-3'. Only 20 embryos could be assessed, as prior tissue processing prevented DNA extraction for other cases.

Bleaching

To remove pigmentation and reduce signal-to-noise ratio related to hematomas, the

tissue bleaching was carried out (Renier et al., 2014). The samples were dehydrated for 1hr at RT in ascending concentrations of methanol in 1XPBS (50%, 80%, 100%). The samples were then treated overnight at 4°C with a 6% hydrogen peroxide solution in 100% methanol. The following day, samples were re-hydrated for 1hr at RT in descending concentrations of methanol (100% twice, 80%, 50%) and washed in 1XPBS during 1hr. Samples were kept at 4°C for further processing.

Whole-mount immunostaining

Samples were permeabilized and blocked by rotation at 70 rpm in 1XPBS containing 0.2% gelatin (Prolabo), and 0.5% Triton X-100 (Sigma-Aldrich) (PBSGT) at RT (Belle et al., 2014). For immunostaining, samples were transferred to a solution containing 0.1% saponin (10mg/mL) in PBSGT together with the primary antibodies (listed in the Key Resource Table and in Table S1) and placed at 37°C (Benchmark, Incu-Shaker Mini), with rotation at 70 rpm, for 7 to 14 days depending on tissue size and density. This was followed by six washes of 30 min in PBSGT at RT. Next, secondary antibodies (listed in the Key Resource Table) were diluted in a solution containing 0.1% saponin (10mg/ml) in PBSGT and passed through a 0.22 µm filter. Samples were incubated at 37°C (Benchmark, Incu-Shaker Mini) in the secondary antibody solution overnight or for 2 days depending on sample size and density. After six washes of 30 min in PBSGT at RT, samples were stored in the dark at 4°C in until tissue clearing. The protocol was similar for single and multiple labeling.

Agarose embedding

As previously described (Belle et al., 2014; Renier et al., 2014) small samples were embedded in agarose prior to clearing and processing with the LSFM. To embed samples, 1.5% agarose (Roth) was prepared in TAE 1X (Invitrogen).

Tissue clearing

3DISCO

For all tissue clearing, a modified 3DISCO clearing protocol was used. All incubation steps were performed in dark conditions at RT in a fume hood, on a tube rotator (SB3, Stuart) at 14 rpm, using a 15 ml centrifuge tube (TPP, Dutscher). Samples were first dehydrated in ascending concentrations (50%, 80%, and 100%) of tetrahydrofuran (THF; anhydrous, containing 250 ppm butylated hydroxytoluene inhibitor, Sigma-Aldrich) diluted in H_2O. The initial 50% THF bath was done overnight while the 80% and 100% THF incubations were left for 1.5 hr each. Samples next underwent a delipidation step of 30 min in dichloromethane (DCM; Sigma-Aldrich) followed by an overnight clearing step in dibenzyl ether (DBE; Sigma-Aldrich). The next day, samples were stored in individual light-absorbing glass vials (Rotilabo, Roth) at RT. In these conditions, samples could be stored and imaged for up to 9 months without any significant fluorescence loss.

Methanol clearing

For large tissues, methanol clearing was used to achieve higher transparency using a modification from the iDISCO+ protocol (Renier et al., 2016). Whole embryos (≤GW8) and tissues (>GW8) were dehydrated in methanol/1XPBS series (n=7) : 20%, 40%, 60%, 80%, 100% x2 for 1 hr each at RT on a tube rotator (SB3, Stuart) at 14 rpm, using a 15 ml centrifuge tube (TPP, Dutscher) covered with aluminum foil to avoid contact with light. Then samples were incubate overnight in 2/3 DCM/ 1/3 Methanol. After 30 min in 100% DCM, samples were transferred to DBE.

3D imaging and image processing

3D imaging was performed as previously described. Acquisitions were performed by using an ultramicroscope I (LaVision BioTec) with the ImspectorPro software

(LaVision BioTec). The light sheet was generated by a laser (wavelength 488, 561 or 640nm, Coherent Sapphire Laser, LaVision BioTec) and focused using two cylindrical lenses. Two adjustable protective lenses were applied for small and large working distances. A binocular stereomicroscope (MXV10, Olympus) with a 2x objective (MVPLAPO, Olympus) was used at different magnifications (0.63x, 1x, 1.25x, 1.6×, 2x, 2.5x, 3.2x, 4x, 5x, and 6.3x). The corresponding zoom factors and numerical apertures are available at http://lavisionbiotec.com/ultramicroscope-ii-specifications.html.

Samples were placed in an imaging reservoir made of 100% quartz (LaVision BioTec) filled with DBE and illuminated from the side by the laser light. A PCO Edge SCMOS CCD camera (2,560 × 2,160 pixel size, LaVision BioTec) was used to acquire images. The step size between each image was fixed at 1 and 2 µm. All tiff images are generated in 16-bit. For large samples, a platform was created using PDMS (Sylgard) fixed to a sample holder from LaVision.

Image processing

Images, 3D volume, and movies were generated using Imaris x64 software (version 8.0.1, Bitplane). Stack images were first converted to imaris file (.ims) using ImarisFileConverter and 3D reconstruction was performed using the "volume rendering" function. To facilitate image processing, images were converted to an 8-bit format. To obtain opaque visualizations, the normal shading view was applied. Optical slices were obtained using the "orthoslicer" tool. To isolate a specific region of the tissue, the surface tool was used and the mask option was selected. For isolation of smaller structures, i.e. nerve segmentation, the surface tool was manually applied and each nerve was subsequently pseudo-colored. For the hand, the foot and the

urogenital system, nerves and organs were initially segmented manually and visualized in 3D using the 3D rendering tool. In order to visualize bone structures in 3D, skin is artificially removed by segmentation and the contrast is modified using normal shading. 3D pictures and movies were generated using the “snapshot” and “animation” tools. Movie reconstruction with .tiff series are done with ImageJ (1.50e, Java 1.8.0_60, 64-bit), titles and transitions addition have been done with iMovie (version 10.1.1).

QUANTIFICATION AND STATISTICAL ANALYSIS

N/A

DATA AND SOFTWARE AVAILABILITY

All movies and files are available through a dedicated website, https://transparent-human-embryo.com/.

ADDITIONAL RESOURCES

Access to the 3D image datasets is available through https://transparent-human-embryo.com/. The database was created with the help of keen eye technologies.

KEY RESOURCES TABLE

KEY RESOURCES TABLE

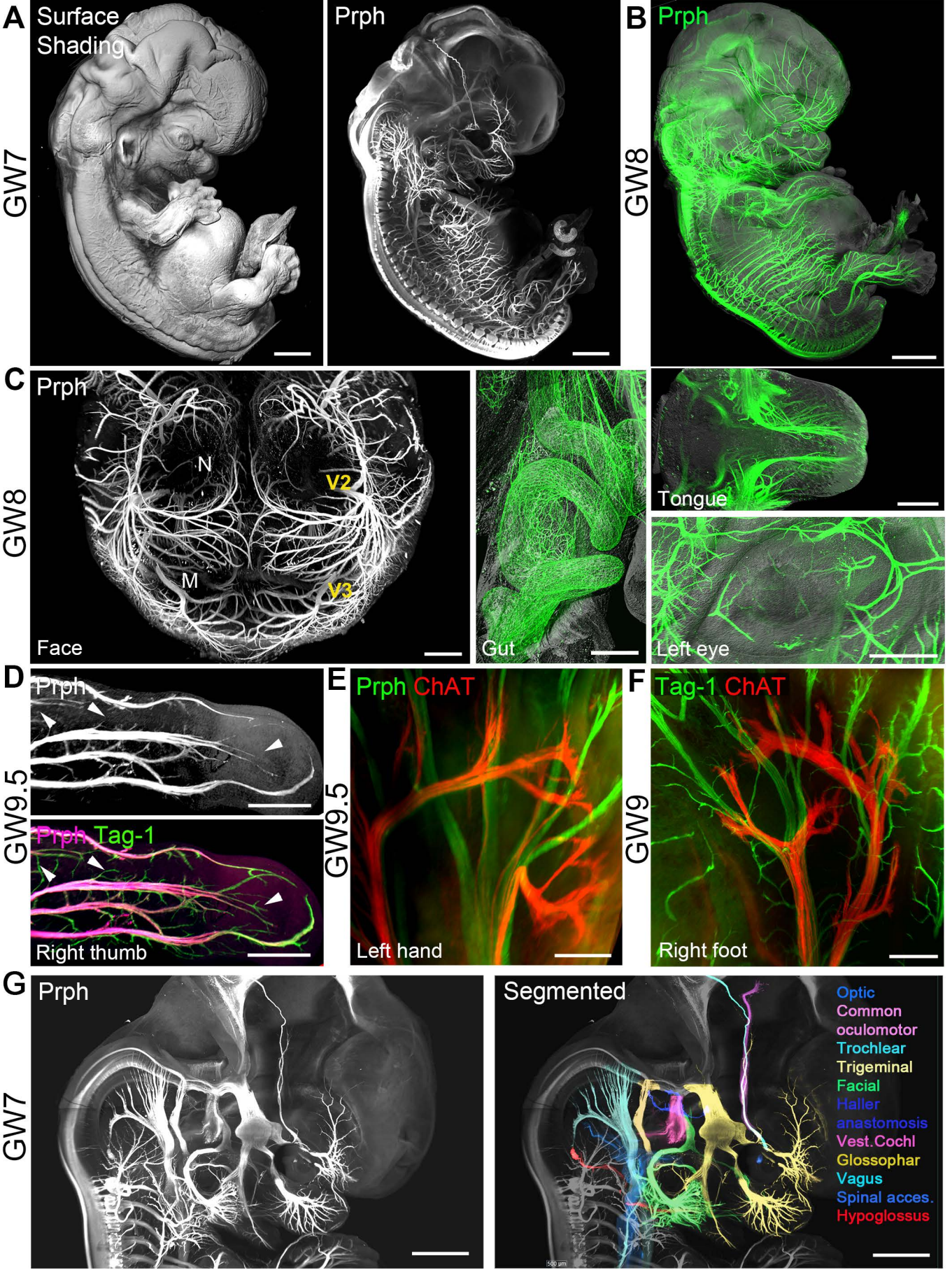
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-Barhl1	Sigma-Aldrich	Cat# HPA004809; RRID: AB_1078266
Mouse monoclonal anti-ALCAM/BEN (Clone F84.1)	Santa Cruz Biotechnology	Cat# sc 53980; RRID:AB_831079
Goat polyclonal anti-Choline Acetyltransferase	Millipore	Cat# AB144P; RRID:AB_2079751
Rabbit polyclonal anti-mouse collagen Type IV	MD Biosciences	Cat# MD20451; RRID:AB_10576569
Rat monoclonal anti-Ctip2	Abcam	Cat# ab18465; RRID:AB_2064130
Mouse monoclonal anti-D2-40 Endothelial marker	BioLegend	Cat# 916601; RRID:AB_2565182
Goat anti-Doublecortin (C18)	Santa Cruz Biotechnology	Cat# sc-8066; RRID:AB_2088494
Rabbit monoclonal anti-Doublecortin	Cell Signaling Technology	Cat# 4604S; RRID:AB_10693771
Mouse monoclonal anti-Foxp1 (Clone JC12)	Abcam	Cat# ab32010; RRID:AB_1141518
Goat polyclonal anti-Foxp2 (N16)	Santa Cruz Biotechnology	Cat# sc-21069; RRID:AB_2107124
Rabbit polyclonal anti-Foxp2	Abcam	Cat# ab16046; RRID:AB_2107107
Rabbit polyclonal anti-Phospho-Histone H3(Ser10)	Cell Signaling Technology	Cat# 9701S; RRID:AB_331534
Rabbit polyclonal anti-Islet1	Abcam	Cat# ab20670; RRID:AB_881306
Rabbit polyclonal anti-Ki67	Abcam	Cat# ab15580; RRID:AB_443209
Guinea-pig monoclonal anti-Lmx1b	Carmen Birchmeier, MaxDelbrück Center for Molecular Medicine;Berlin, Germany	Cat# Lmx1b; RRID:AB_2314752
Rabbit polyclonal anti-Gonadotropin releasing hormone	R. Benoit, McGill University; Montreal; Canada	Cat# LR5; RRID:AB_2314605
Mouse monoclonal anti-Myosin Heavy Chain (Clone A4.1025)	Millipore	Cat# 05-716; RRID:AB_309930
Mouse monoclonal anti-Myosin, Skeletal, Fast	Sigma-Aldrich	Cat# M4276; RRID:AB_477190
Mouse monoclonal anti-Myogenin	DSHB	Cat# F5D AB_2146602
Mouse monoclonal anti-Myosin (Smooth)	Sigma-Aldrich	Cat# M7786; RRID:AB_477239
Rabbit monoclonal anti-Olig-2	Millipore	Cat# AB9610 RRID:AB_10141047
Mouse monoclonal anti-Parvalbumine	Swant	Cat# 235 RRID:AB_10000343
Goat polyclonal anti-Human Pax2	R and D Systems	Cat# AF3364; RRID:AB_10889828
Rabbit polyclonal anti-Pax6	Millipore	Cat# AB5409; RRID:AB_2315065
Mouse Anti-Chicken PAX7 Monoclonal Antibody, Unconjugated	DSHB	Cat# pax7 RRID:AB_528428

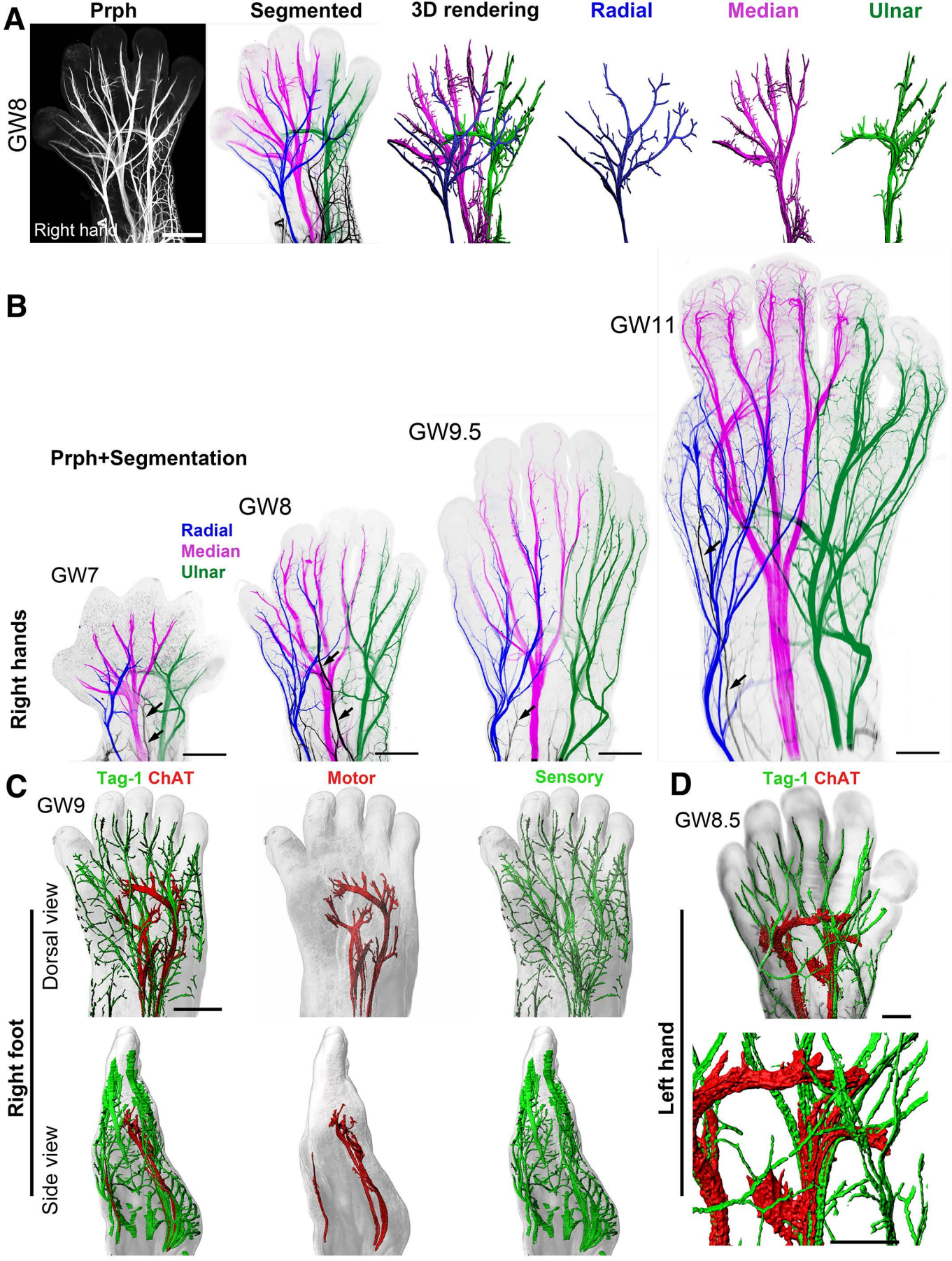
Mouse monoclonal anti-Peripherin (Clone 8G2)	Millipore	Cat# MAB1527; RRID:AB_2284441
Rabbit polyclonal anti-Peripherin	Millipore	Cat# AB1530; RRID:AB_90725
Rabbit polyclonal anti-Phox2b	J.-F. Brunet, Ecole Normale Superieure; Paris; France	Cat# Phox2b RRID:AB_2315160
Mouse monoclonal anti-PLVAP (Clone 174/2)	Abcam	Cat# ab81719; RRID:AB_1658370
Rabbit polyclonal anti-PROX1	Acris Antibodies GmBH	Cat# DP3501P; RRID:AB_1006742
Goat polyclonal anti-Human ROBO3	R and D Systems	Cat# AF3076; RRID:AB_2181865
Mouse monoclonal anti-SATB2 (Clone SATBA4B10)	Abcam	Cat# ab51502; RRID:AB_882455
Rabbit polyclonal anti-SIX2	Proteintech Group	Cat# 11562-1-AP; RRID:AB_2189084
Mouse monoclonal anti-Actin, alpha-Smooth Muscle	Sigma-Aldrich	Cat# A2547; RRID:AB_476701
Goat polyclonal anti-Sox10 (N-20)	Santa Cruz Biotechnology	Cat# sc-17342; RRID:AB_2195374
Rabbit polyclonal anti-SOX2	Abcam	Cat# ab97959; RRID:AB_2341193
Goat polyclonal anti-Sox2 (Y-17)	Santa Cruz Biotechnology	Cat# sc-17320; RRID:AB_2286684
Rabbit polyclonal anti-Sox9	Millipore	Cat# AB5535; RRID:AB_2239761
Goat polyclonal anti-Contactin-2/TAG-1	R and D Systems	Cat# AF4439; RRID:AB_2044647
Rabbit polyclonal anti-Contactin-2/TAG-1	Domna Karagogeos, University of Crete, Heraklion, Crete, Greece	DOI:10.1523/JNEUROSCI.2574-10.2010
Rabbit polyclonal anti-TBR1	Abcam	Cat# ab31940; RRID:AB_2200219
Rabbit polyclonal anti-Tlx3	Carmen Birchmeier, MaxDelbrück Center for Molecular Medicine;Berlin, Germany	Cat# Tlx3; RRID:AB_2532145
Mouse monoclonal anti-Neuronal Class III beta-Tubulin	Covance Research Products	Cat# MMS-435P; RRID:AB_2313773
Rabbit polyclonal anti-Tyrosine Hydroxylase	Millipore	Cat# AB152; RRID:AB_390204
Donkey Anti-Chicken IgG (H+L) Alexa Fluor 488 AffiniPure	Jackson ImmunoResearch Labs	Cat# 703-545-155; RRID:AB_2340375
Goat anti-Chicken IgY (H+L) Alexa Fluor 488 conjugate	Thermo Fischer Scientific	Cat# A-11039; RRID:AB_2534096
Alexa Fluor® 594 AffiniPure Donkey Anti-Guinea Pig IgG (H+L)	Jackson ImmunoResearch Labs	Cat# 706-585-148 RRID:AB_2340474
Donkey Anti-Guinea Pig IgG (H+L) Cy3 AffiniPure (min x Bov,Ck,Gt,Hms,Hrs,Hu,Ms,Rb,Rat,Shp,Sr Prot)	Jackson ImmunoResearch Labs	Cat# 706-165-148; RRID:AB_2340460
Donkey Anti-Guinea Pig IgG (H+L) Alexa Fluor 488 AffiniPure	Jackson ImmunoResearch Labs	Cat# 706-545-148; RRID:AB_2340472

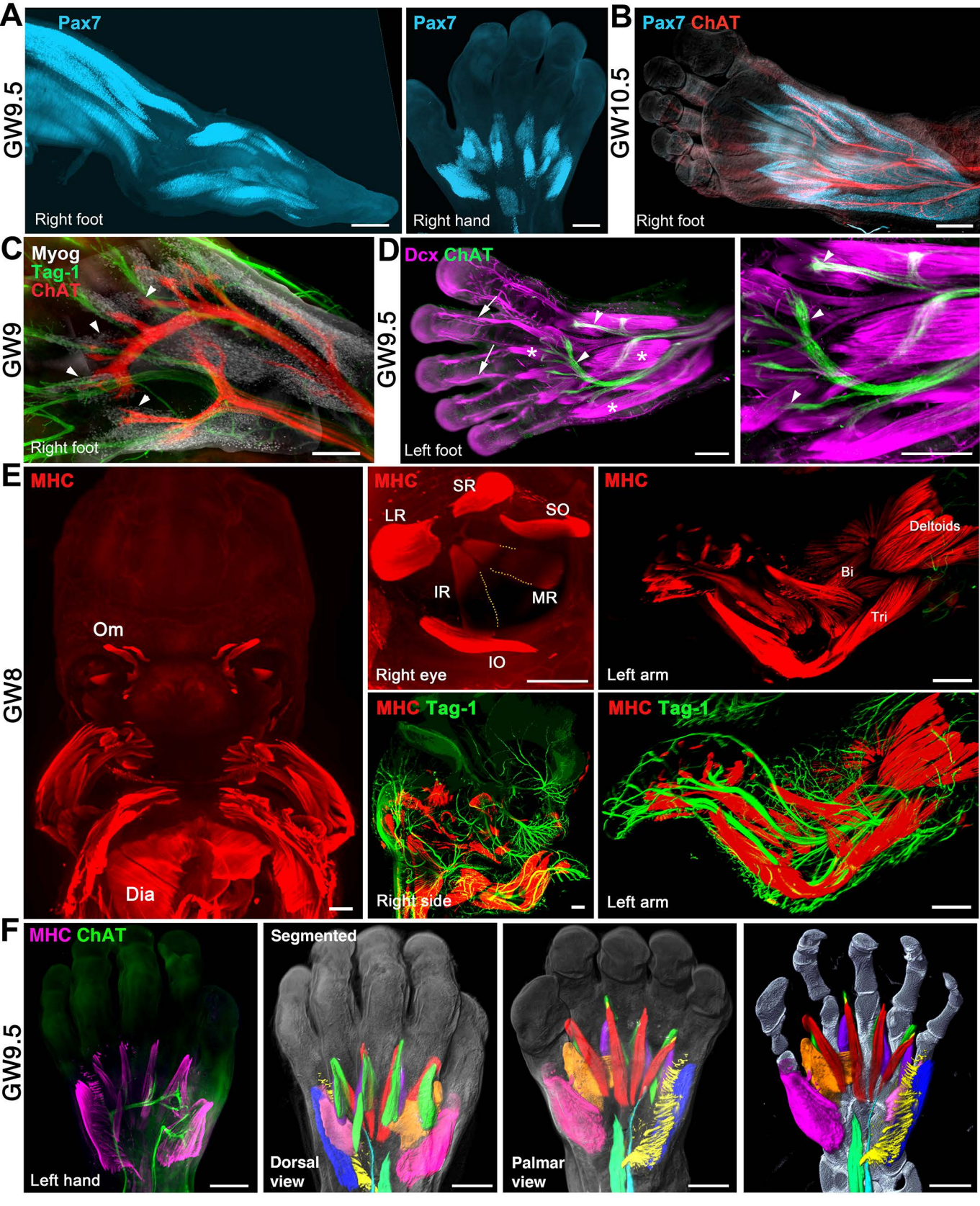
Donkey Anti-Rabbit IgG (H+L) Alexa Fluor 488 AffiniPure	Jackson ImmunoResearch Labs	Cat# 711-545-152; RRID:AB_2313584
Donkey Anti-Rabbit IgG (H+L) Alexa Fluor 647 AffiniPure	Jackson ImmunoResearch Labs	Cat# 711-605-152; RRID:AB_2492288
Donkey Anti-Rabbit IgG H&L (Alexa Fluor 488) preadsorbed antibody	Abcam	Cat# ab150061; RRID:AB_2571722
Donkey Anti-Rabbit IgG (H+L) Cy3 AffiniPure (min X Bov,Ck,Gt,GP,Sy Hms,Hrs,Hu,Ms,Rat,Shp Sr Prot)	Jackson ImmunoResearch Labs	Cat# 711-165-152; RRID:AB_2307443
Donkey Anti-Rat IgG (H+L) Cy3 AffiniPure (min x Bov,Ck,Gt,Sy Hms,Hrs,Hu,Ms,Rb, Shp,Sr Prot)	Jackson ImmunoResearch Labs	Cat# 712-165-153; RRID:AB_2340667
Donkey Anti-Rat IgG (H+L) Alexa Fluor 647 AffiniPure	Jackson ImmunoResearch Labs	Cat# 712-605-153; RRID:AB_2340694
Bovine Anti-Goat IgG (H+L) Alexa Fluor 488 AffiniPure	Jackson ImmunoResearch Labs	Cat# 805-545-180; RRID:AB_2340883
Bovine Anti-Goat IgG (H+L) Alexa Fluor 647 AffiniPure	Jackson ImmunoResearch Labs	Cat# 805-605-180 ;RRID:AB_2340885
Bovine Anti-Goat IgG (H+L) Cy3 AffiniPure (min x Bov,Ck,Gt,Sy Hms,Hrs,Hu,Ms,Rb, Rat Sr Prot)	Jackson ImmunoResearch Labs	Cat# 805-165-180; RRID:AB_2340880
Donkey Anti-Goat IgG H&L (Alexa Fluor 647) preadsorbed	Abcam	Cat# ab150135; RRID: N/A
Donkey Anti-Goat IgG (H+L) Cy™3 AffiniPure	Jackson ImmunoResearch Labs	Cat# 705-165-147 ; RRID:AB_2307351
Donkey Anti-Mouse IgG (H+L) Cy™3 AffiniPure	Jackson ImmunoResearch Labs	Cat# 715-165-150 RRID:AB_2340813
Donkey Anti-mouse IgG H&L (Alexa Fluor 555) preadsorbed	Abcam	Cat# ab150106; RRID: N/A
Donkey anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488	Molecular Probes	Cat# A-21202 RRID:AB_141607
Goat anti-Mouse IgM Heavy Chain Secondary Antibody, Alexa Fluor 488	Molecular Probes	Cat# A-21042 ; RRID:AB_141357
Donkey Anti-Mouse IgG (H+L) Alexa Fluor 647 AffiniPure	Jackson ImmunoResearch Labs	Cat# 715-605-150; RRID:AB_2340862
Donkey anti-Mouse IgG Secondary Antibody, Alexa Fluor 568	Termo Fisher Scientific	Cat# A10037; RRID:AB_2534013
Tested, non-working antibodies	Table S1	
Biological Samples		
Human tissue	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
Tetrahydrofuran Anhydre >99.9% (THF)	Sigma-Adrich	Cat# 186562 CAS Number 109-99-9

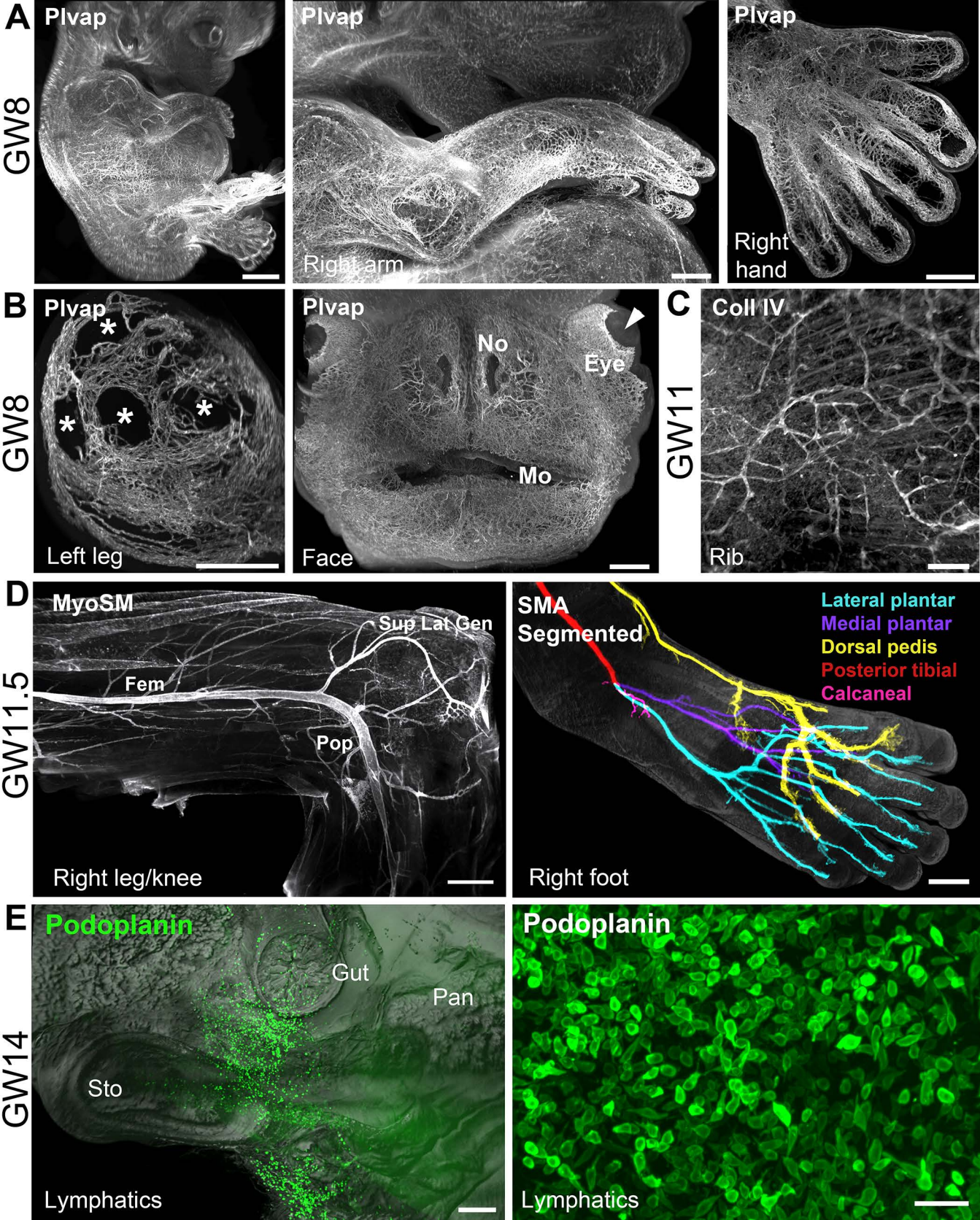
Dichloromethane (DCM)	Sigma-Adrich	Cat# 270997 CAS Number: 75-09-2
Dibenzyl ether (DBE)	Sigma-Adrich	Cat# 108014 CAS Number: 103-50-4
Methanol (MeOH)	VWR Chemicals	Cat# 20847.360 CAS Number: 67-56-1
Hydrogen peroxide solution (H_2O_2)	Sigma-Adrich	Cat# 216763 CAS Number: 7722-84-1
Agarose	Carl ROTH	Cat# 2267.4 CAS Number: 9012-36-6
Gelatin	VWR Chemicals	Cat# 24350.262 CAS Number: 9000-70-8
Thimerosal	Sigma-Adrich	Cat# T8784-5g CAS Number: 54-64-8
Triton X100	Sigma-Adrich	Cat# X100-500ml CAS Number: 9002-93-1
SYLGARD® 184 SILICONE ELASTOMER KIT	DOW CORNING	Cat# SYLGARD® 184 SILICONE ELASTOMER KIT CAS Number: N/A
Critical Commercial Assays		
Deposited Data		
Raw and analyzed data	This paper	www.transparent-human-embryo.com
Experimental Models: Cell Lines		
Experimental Models: Organisms/Strains		
Recombinant DNA		
Sequence-Based Reagents		
SRY sense 5'-AGCGATGATTACAGTCCAGC-3'	This paper	N/A
SRY antisense 5'-CCTACAGCTTTGTCCAGTGG-3'	This paper	N/A
FGF16 sense 5'- CGGGAGGGATACAGGACTAAAC-3'	This paper	N/A
FGF16 antisense 5'- CTGTAGGTAGCATCTGTGGC-3'	This paper	N/A
Software and Algorithms		
Imspector software	LaVision Biotec	http://www.lavisionbiotec.com/
Imaris x64 software (version 8.0.1)	Bitplane	http://www.bitplane.com/imaris/imaris; RRID:SCR_007370

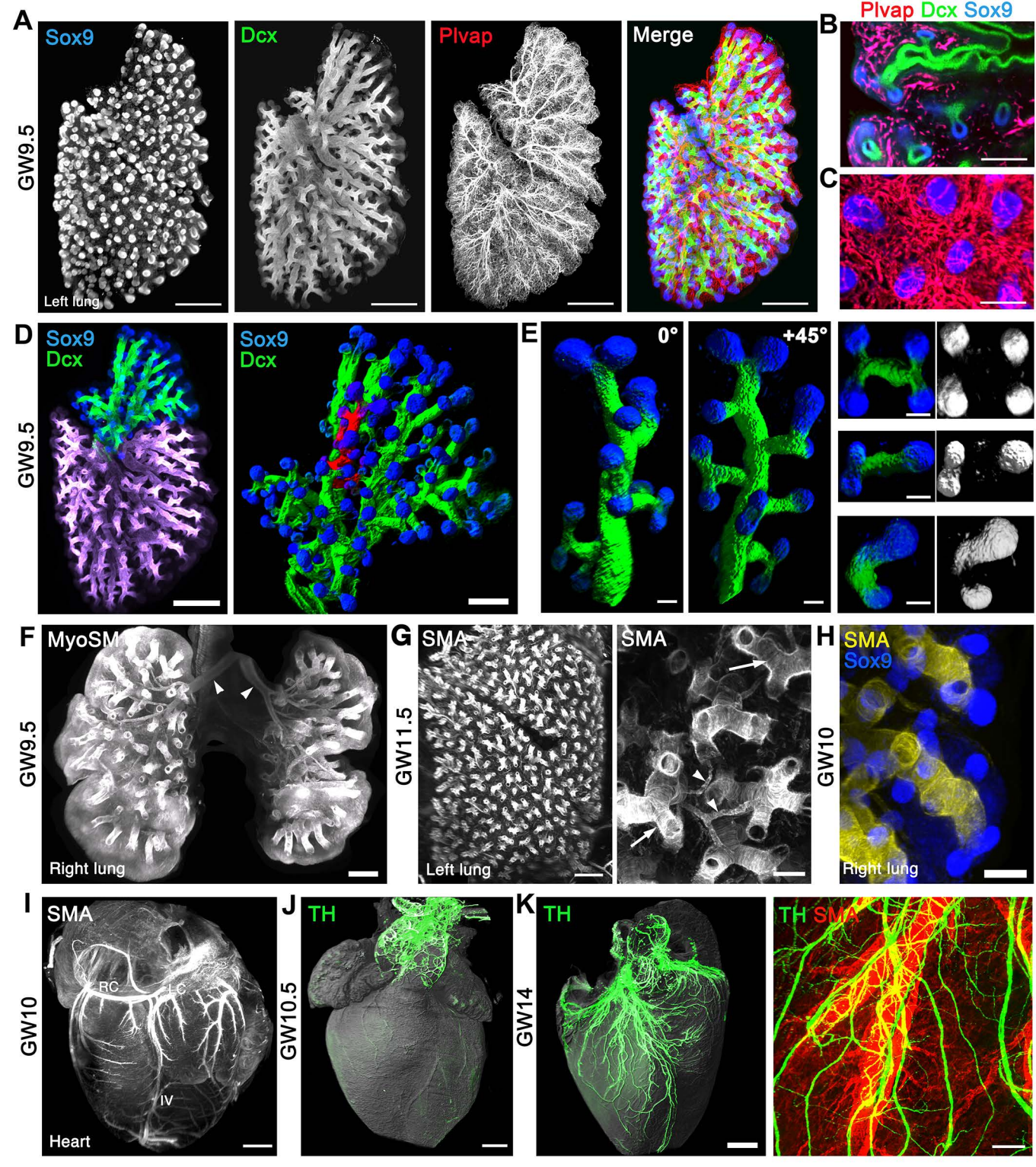
ImageJ (1.50e, Java 1.8.0_60, 64-bit)	NIH	http://imagej.nih.gov/ij/; RRID:SCR_003070
iMovie (version 10.1.1)	Apple	http://www.apple.com/fr/imovie/
Other		

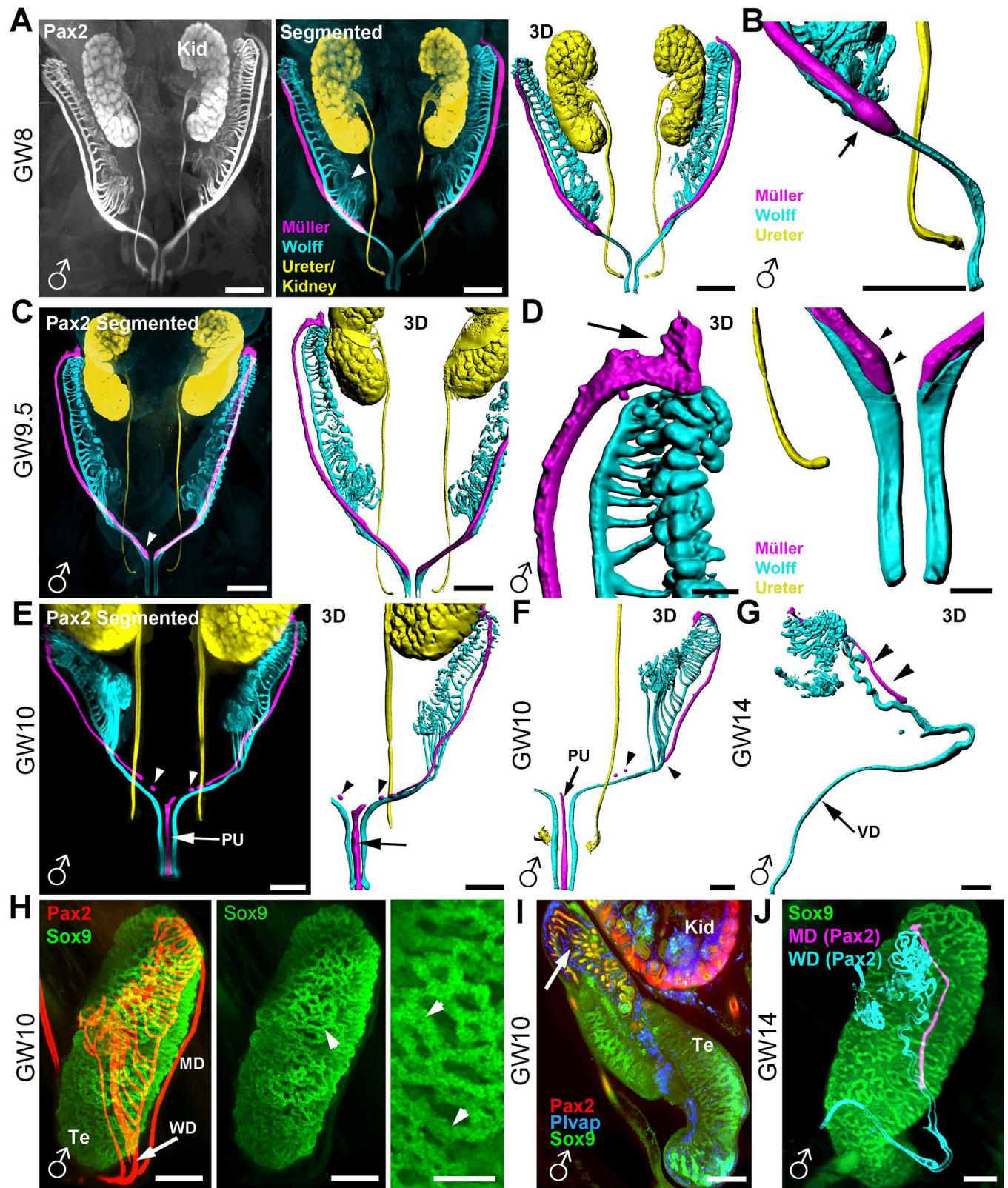

A
Surface Shading
Prph
GW7
B
Prph
GW8
C
Prph
N
V2
M
V3
Face
GW8
Gut
Tongue
Left eye
D
Prph
Prph Tag-1
Right thumb
GW9.5
E
Prph ChAT
Left hand
GW9.5
F
Tag-1 ChAT
Right foot
GW9
G
Prph
GW7
Segmented
Optic
Common oculomotor
Trochlear
Trigeminal
Facial
Haller anastomosis
Vest.Cochl
Glossophar
Vagus
Spinal acces.
Hypoglossus

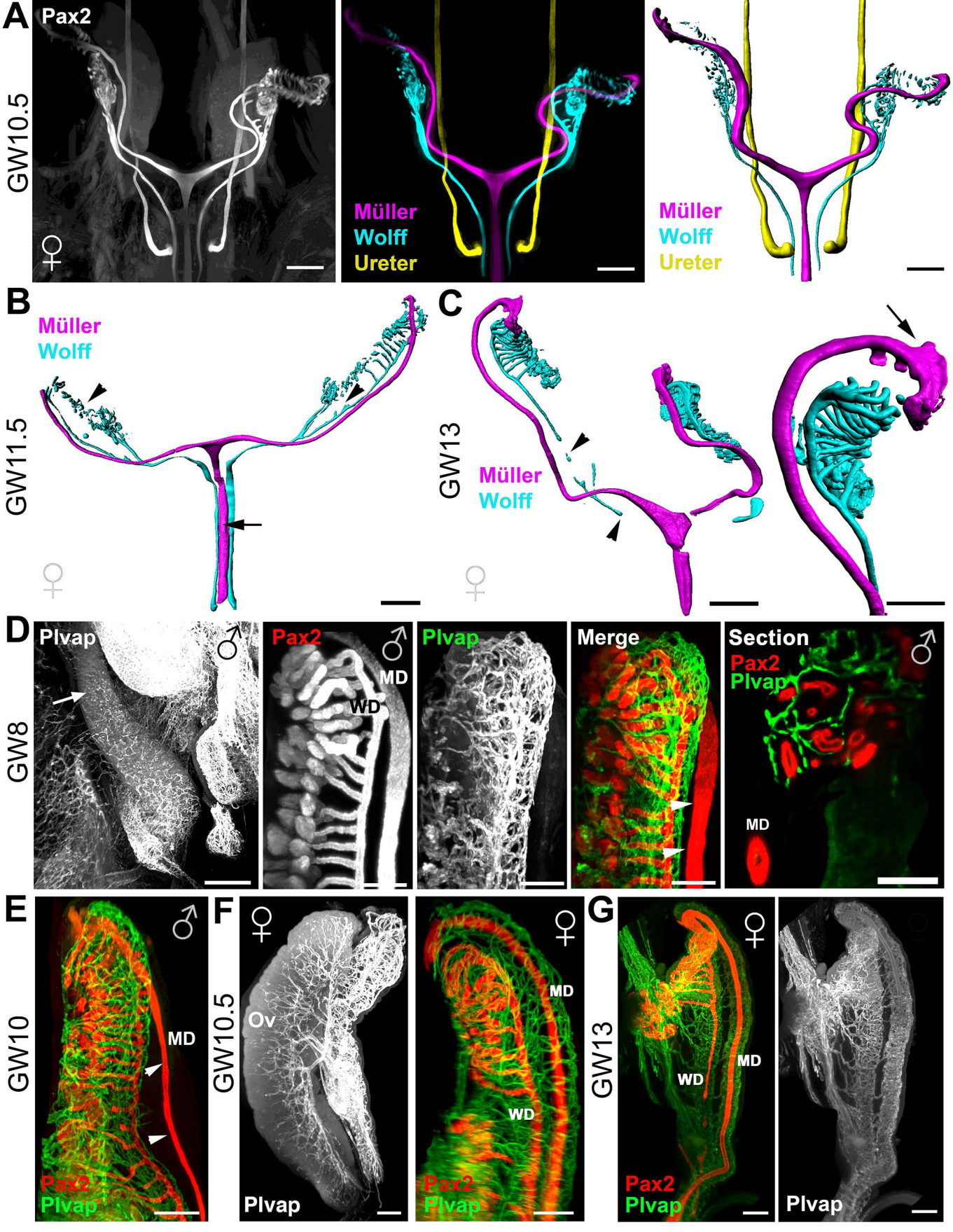

A
Prph
Segmented
3D rendering
Radial
Median
Ulnar
GW8
Right hand
B
GW11
GW9.5
Prph+Segmentation
GW8
Radial
Median
Ulnar
GW7
Right hands
C
Tag-1 ChAT
Motor
Sensory
GW9
Dorsal view
Right foot
Side view
D
Tag-1 ChAT
GW8.5
Left hand

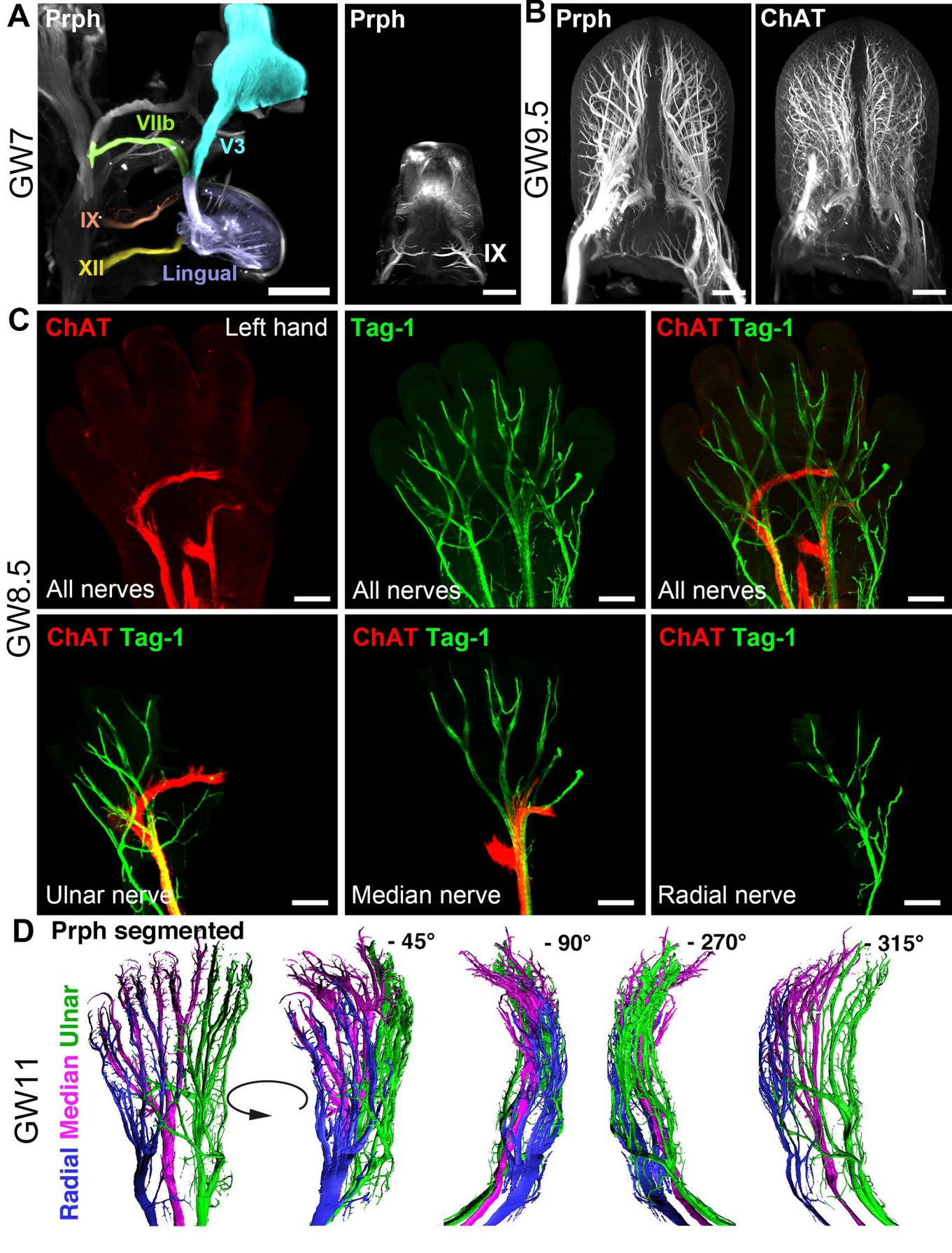

A
GW9.5
Pax7
Right foot
Pax7
Right hand
B
GW10.5
Pax7 ChAT
Right foot
C
GW9
Myog
Tag-1
ChAT
Right foot
D
GW9.5
Dcx ChAT
Left foot
E
GW8
MHC
Om
Dia
MHC
SR
LR
SO
IR
MR
IO
Right eye
MHC
Deltoids
Bi
Tri
Left arm
MHC Tag-1
Right side
MHC Tag-1
Left arm
F
GW9.5
MHC ChAT
Left hand
Segmented
Dorsal view
Palmar view

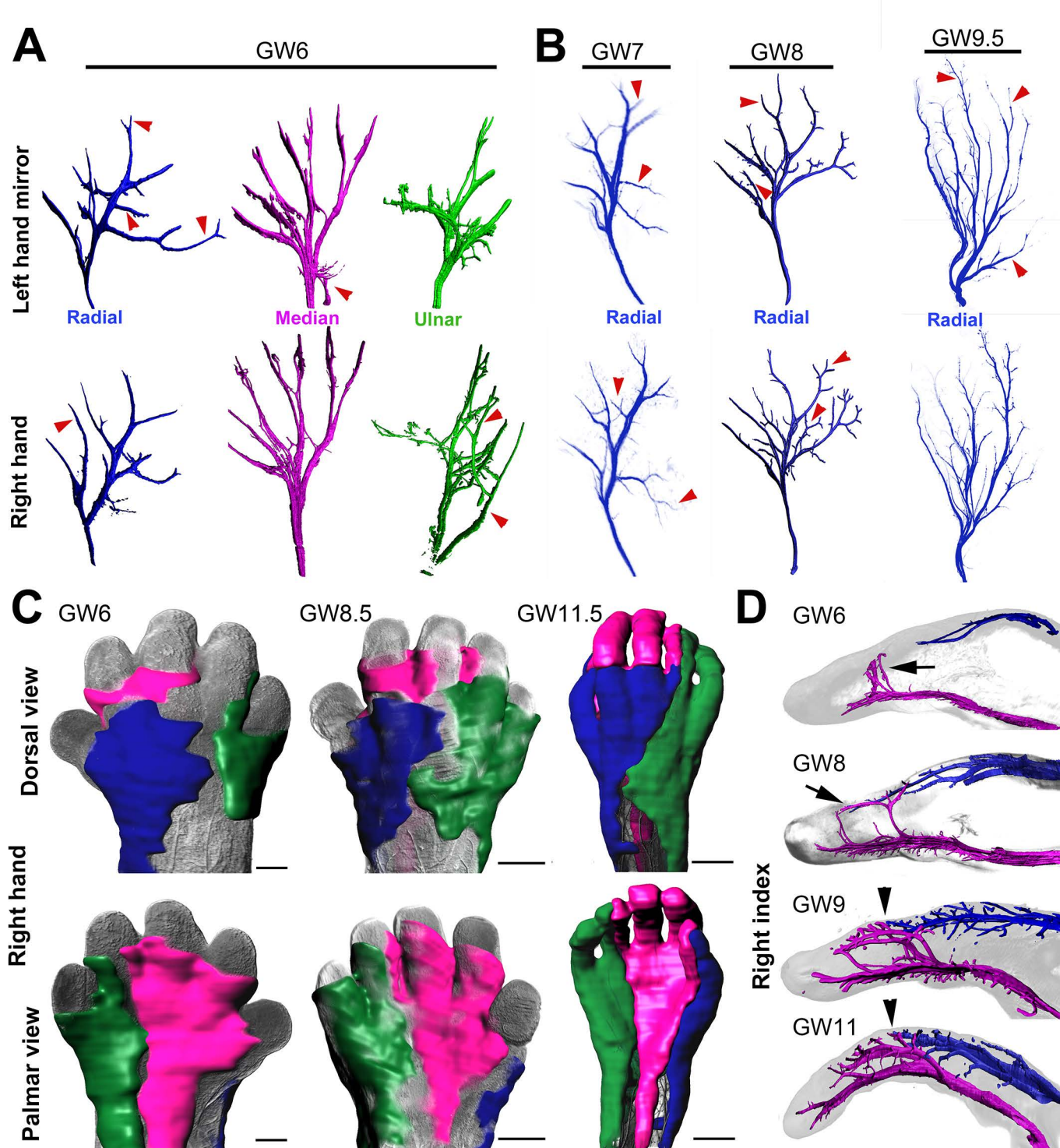

A
GW8
Plvap
Plvap
Right arm
Plvap
Right hand
B
GW8
Plvap
Left leg
Plvap
No
Eye
Mo
Face
C
GW11
Coll IV
Rib
D
GW11.5
MyoSM
Sup Lat Gen
Fem
Pop
Right leg/knee
SMA
Segmented
Lateral plantar
Medial plantar
Dorsal pedis
Posterior tibial
Calcaneal
Right foot
E
GW14
Podoplanin
Gut
Pan
Sto
Lymphatics
Podoplanin
Lymphatics

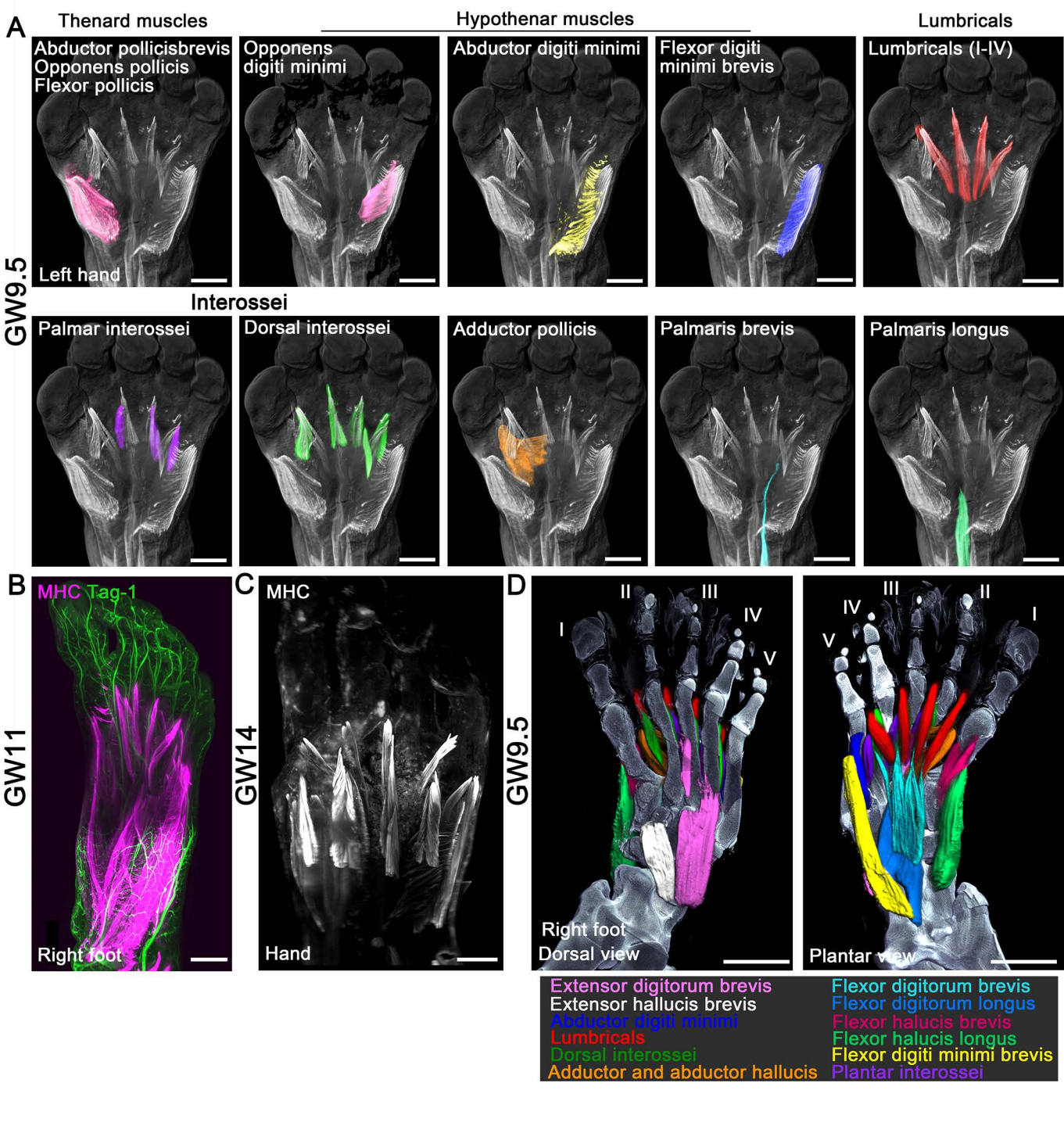

A
Sox9
Dcx
Plvap
Merge
GW9.5
Left lung
B
Plvap Dcx Sox9
C
D
Sox9
Dcx
GW9.5
E
0°
+45°
F
MyoSM
GW9.5
Right lung
G
SMA
GW11.5
Left lung
H
SMA
Sox9
GW10
Right lung
I
SMA
RC
LC
IV
GW10
Heart
J
TH
GW10.5
K
TH
GW14
TH SMA

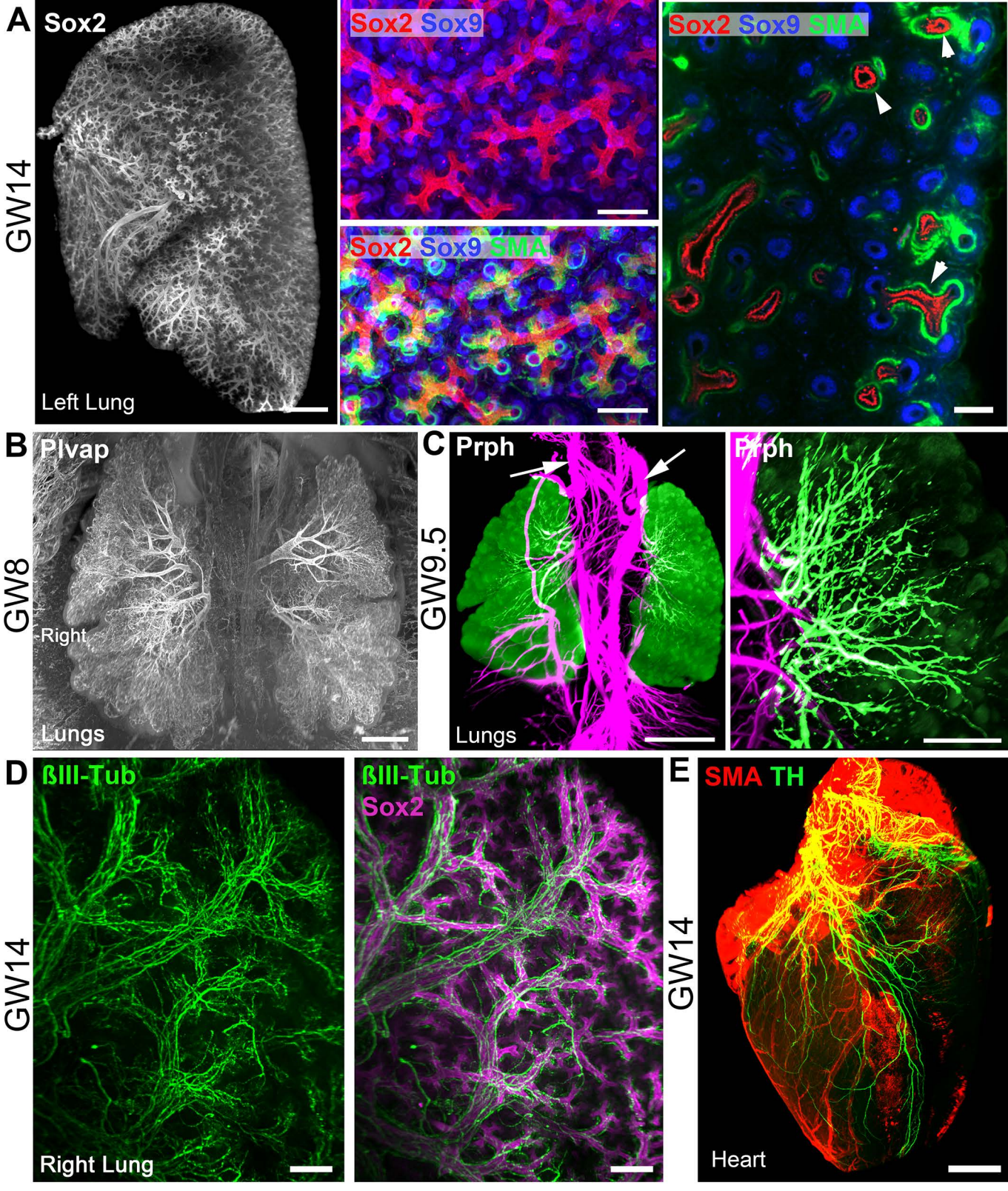

A
Pax2
Kid
Segmented
3D
GW8
Müller
Wolff
Ureter/
Kidney
B
Müller
Wolff
Ureter
C
Pax2 Segmented
3D
GW9.5
D
3D
Müller
Wolff
Ureter
E
Pax2 Segmented
3D
GW10
PU
F
3D
GW10
PU
G
3D
GW14
VD
H
Pax2
Sox9
Sox9
GW10
MD
Te
WD
I
Kid
Te
GW10
Pax2
Plvap
Sox9
J
Sox9
MD (Pax2)
WD (Pax2)
GW14

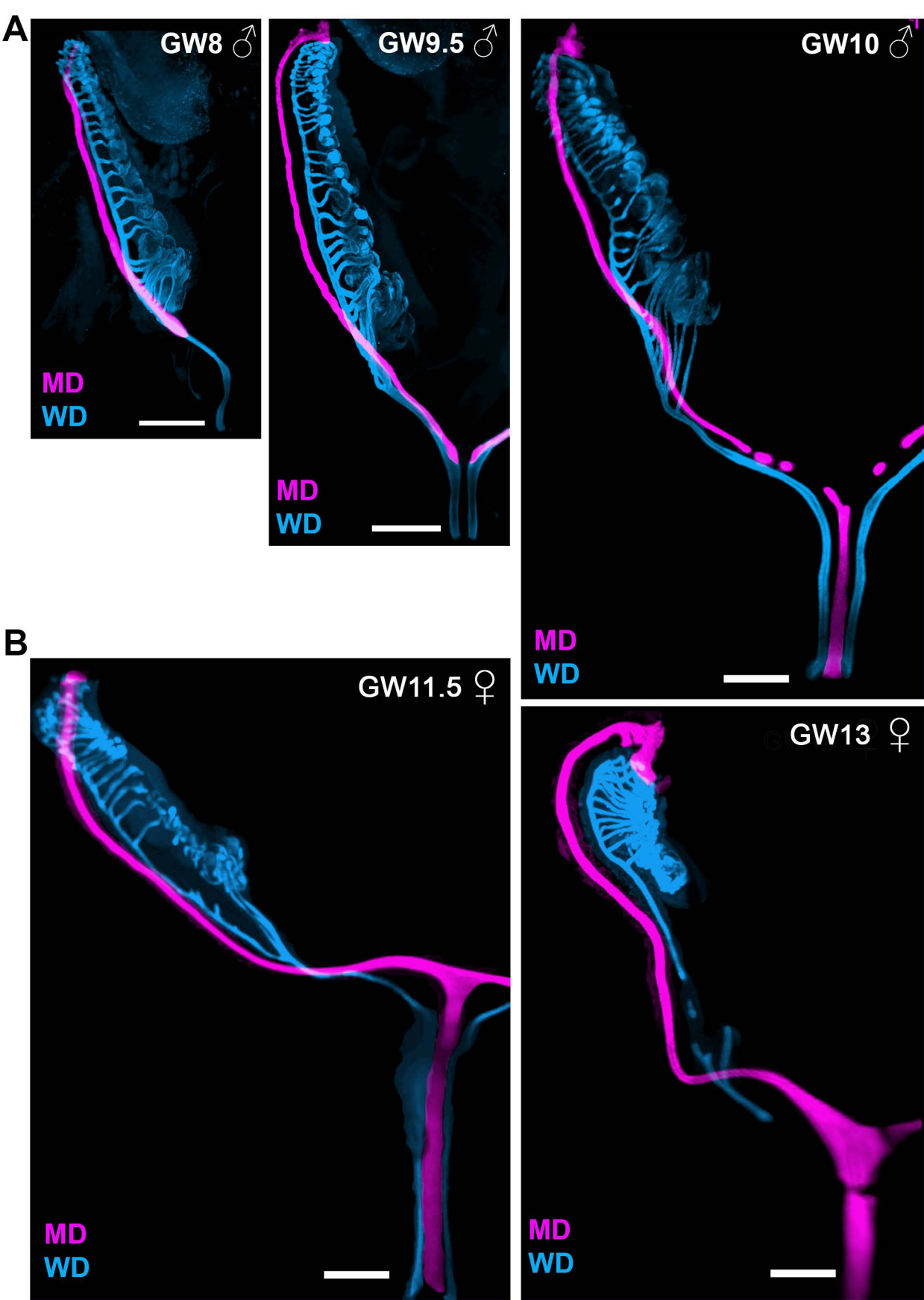

A
Pax2
GW10.5
♀
Müller
Wolff
Ureter
Müller
Wolff
Ureter
B
Müller
Wolff
GW11.5
♀
C
GW13
Müller
Wolff
♀
D
Plvap
♂
GW8
Pax2
♂
MD
WD
Plvap
Merge
Section
Pax2
Plvap
♂
MD
E
♂
GW10
MD
Pax2
Plvap
F
♀
GW10.5
Ov
Plvap
♀
MD
WD
Pax2
Plvap
G
♀
GW13
MD
WD
Pax2
Plvap
Plvap

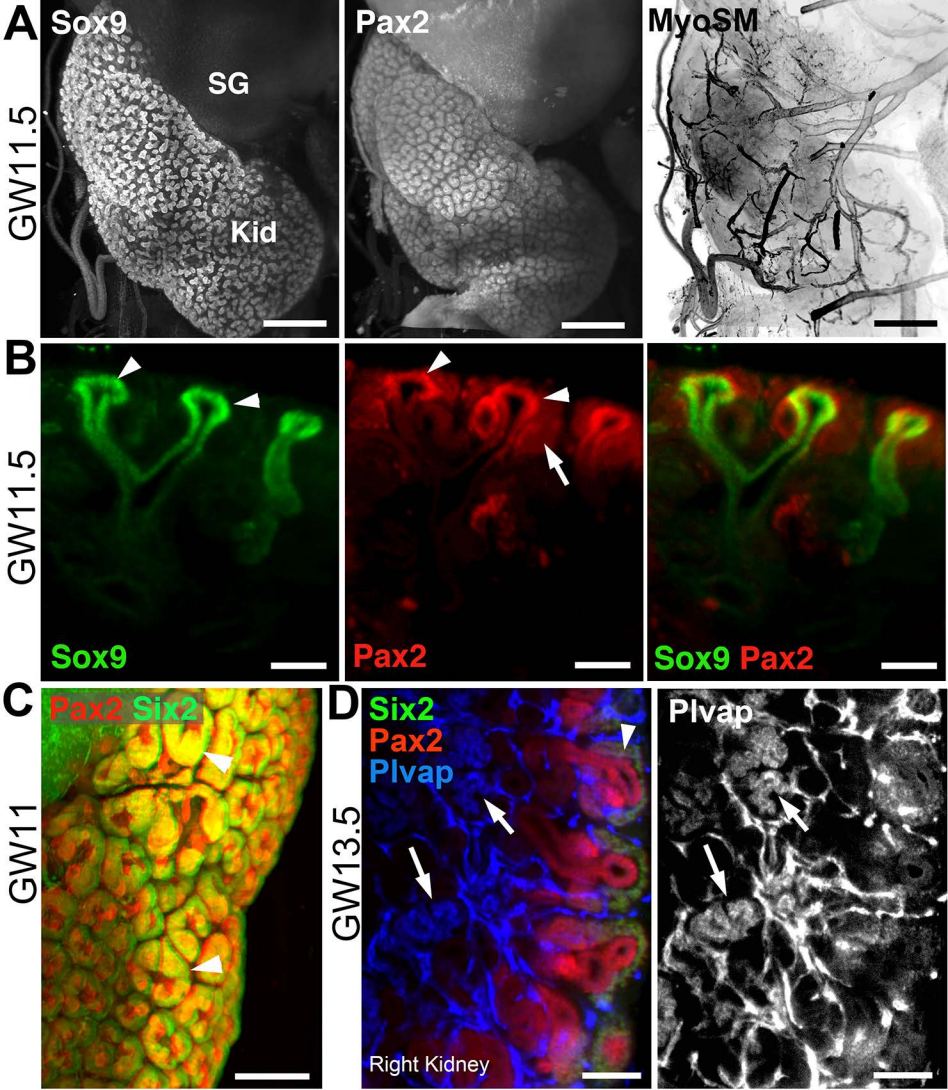

A
Prph
GW7
VIIb
V3
IX
XII
Lingual
Prph
IX
B
GW9.5
Prph
ChAT
C
GW8.5
ChAT
Left hand
All nerves
Tag-1
All nerves
ChAT Tag-1
All nerves
ChAT Tag-1
Ulnar nerve
ChAT Tag-1
Median nerve
ChAT Tag-1
Radial nerve
D
GW11
Prph segmented
Radial Median Ulnar
- 45°
- 90°
- 270°
- 315°

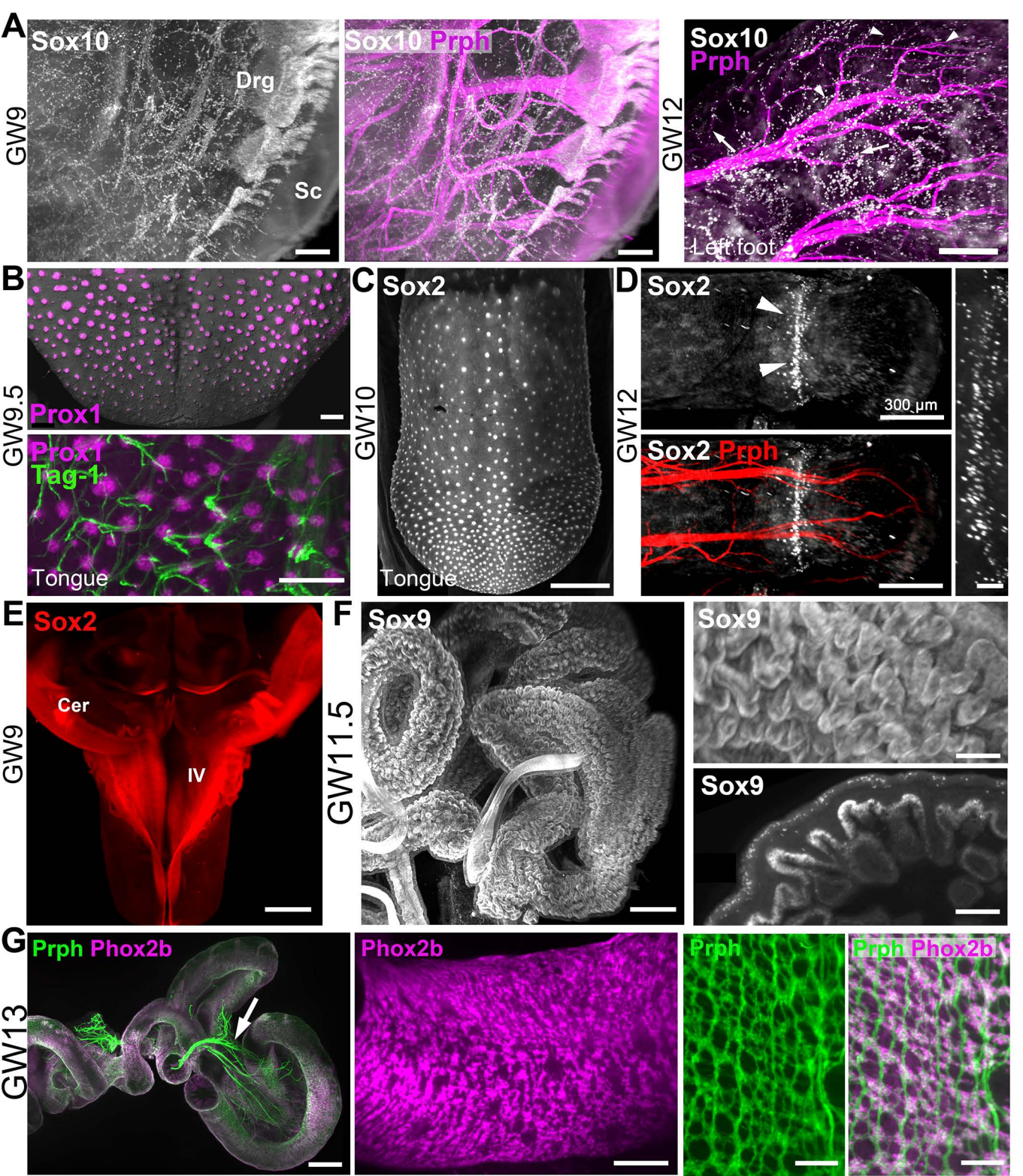

A
GW6
Left hand mirror
Radial
Median
Ulnar
Right hand
B
GW7
GW8
GW9.5
Radial
Radial
Radial
C
GW6
GW8.5
GW11.5
Dorsal view
Right hand
Palmar view
D
GW6
GW8
GW9
GW11
Right index


A
Thenard muscles
Hypothenar muscles
Lumbricals
GW9.5
Abductor pollicisbrevis
Opponens pollicis
Flexor pollicis
Left hand
Opponens digiti minimi
Abductor digiti minimi
Flexor digiti minimi brevis
Lumbricals (I-IV)
Interossei
Palmar interossei
Dorsal interossei
Adductor pollicis
Palmaris brevis
Palmaris longus
B
GW11
MHC Tag-1
Right foot
C
GW14
MHC
Hand
D
GW9.5
I
II
III
IV
V
Right foot
Dorsal view
Plantar view
Extensor digitorum brevis
Extensor hallucis brevis
Abductor digiti minimi
Lumbricals
Dorsal interossei
Adductor and abductor hallucis
Flexor digitorum brevis
Flexor digitorum longus
Flexor halucis brevis
Flexor halucis longus
Flexor digiti minimi brevis
Plantar interossei


A
Sox2
GW14
Left Lung
Sox2 Sox9
Sox2 Sox9 SMA
Sox2 Sox9 SMA
B
Plvap
GW8
Right
Lungs
C
Prph
GW9.5
Lungs
Prph
D
ßIII-Tub
GW14
Right Lung
ßIII-Tub
Sox2
E
SMA TH
GW14
Heart


A
GW8 ♂
MD
WD
GW9.5 ♂
MD
WD
GW10 ♂
MD
WD
B
GW11.5 ♀
MD
WD
GW13 ♀
MD
WD


A
Sox9
SG
Kid
GW11.5
Pax2
MyoSM
B
GW11.5
Sox9
Pax2
Sox9 Pax2
C
Pax2 Six2
GW11
D
GW13.5
Six2
Pax2
Plvap
Right Kidney
Plvap


A
Sox10
Drg
Sc
GW9
Sox10 Prph
Sox10
Prph
GW12
Left foot
B
GW9.5
Prox1
Prox1
Tag-1
Tongue
C
Sox2
GW10
Tongue
D
Sox2
GW12
300 μm
Sox2 Prph
E
Sox2
Cer
IV
GW9
F
Sox9
GW11.5
Sox9
Sox9
G
Prph Phox2b
GW13
Phox2b
Prph
Prph Phox2b