

Ex vivo time-lapse confocal imaging of the mouse embryo aorta

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Time-lapse confocal microscopy of mouse embryo slices was developed to access and image the living aorta. In this paper, we explain how to label all hematopoietic and endothelial cells inside the intact mouse aorta with fluorescent directly labeled antibodies. Then we describe the technique to cut nonfixed labeled embryos into thick slices that are further imaged by time-lapse confocal imaging. This approach allows direct observation of the dynamic cell behavior in the living aorta, which was previously inaccessible because of its location deep inside the opaque mouse embryo. In particular, this approach is sensitive enough to allow the experimenter to witness the transition from endothelial cells into hematopoietic stem/progenitor cells in the aorta, the first site of hematopoietic stem cell generation during development. The protocol can be applied to observe other embryonic sites throughout mouse development. A complete experiment requires ~2 d of practical work.

INTRODUCTION

Development of the protocol

Intact living organisms have been used for imaging approaches for a long time. Avian embryos have the advantage of being optically accessible when eggs are opened. Under appropriate *in vitro* conditions they can grow outside the shell for a couple of days. Direct observation is also possible with the zebrafish, which is transparent in embryonic stages^{1–3}. However, *in vivo* time-lapse imaging of developing mammalian embryos has always been challenging because of their intrauterine development and the fact that they are opaque. Furthermore, at embryonic day (E)8.5–E9.0 of development, mouse embryos are surrounded by extraembryonic tissues that are highly autofluorescent (e.g., yolk sac, amniotic sac). The removal of these tissues to optically access the embryo impairs further embryonic development. The lack of penetration in deeply located tissues by both confocal and two-photon microscopes (despite the higher z-range resolution) is a notable obstacle. Thus, direct observation of deeply located organs and tissues (in living mouse) has so far not been possible. To overcome these problems, we developed an embryo-slicing technique inspired from the field of neurobiology, wherein brain slices are used as alternatives to *in vivo* electrophysiology studies. The cytoarchitecture, mechanical stability and basic properties of the tissue are well conserved in nonfixed slices. Living embryonic tissues can thus be observed over relatively long periods of time. The methodology described here can be used to observe several nonfixed embryonic organs/tissues and to study various biological processes occurring in the mouse embryo between E9.0 and E11.5.

An important event, taking place in this period, is the production of blood stem cells or hematopoietic stem cells (HSCs)^{4,5}, which are first detected in the dorsal aorta of the aorta-gonad-mesonephros (AGM) region at E10.5 (refs. 6,7). HSCs most likely reside in cell clusters (referred to as intra-aortic hematopoietic clusters or IAHCs). IAHCs are attached to the endothelial layer of the main embryonic vessels (aorta, vitelline and umbilical arteries) in various

species⁸. HSCs are found to transit via the yolk sac, placenta and fetal liver before colonizing the bone marrow at E17 (refs. 6, 9–11). HSCs are responsible for constant blood cell replacement during the entire life of an adult organism. Although it has been established that HSCs are first detected in the AGM, the precise anatomical and cellular origins of these cells have long been uncertain. Pioneering experiments of interspecies grafts performed in the avian embryo model indicated that IAHCs, and therefore HSCs, derive from a specialized endothelial cell population of the aorta, referred to as ‘hemogenic’¹². However, several other cell types, for example, hemangioblasts and mesenchymal cells, have been proposed over the past decades as potent HSC precursors^{4,5}. A direct observation of the embryonic aorta was therefore necessary to determine whether the transition of the hemogenic endothelial cell into HSC actually occurs in the aorta.

We developed two techniques to bypass imaging problems associated with the opaqueness of the mouse embryo and the deep location of the aorta. In the first approach, nonfixed embryos, isolated from E9.0 to E11.5, were cut transversely (i.e., along the dorsal-ventral axis) into thick slices such that the aorta was visible and wide open in the middle of the slice. In the second approach, we first removed all dorsal tissues (somites and neural tube) from E9.0 to E11.5 embryos to access the full length of the aorta, which is located just behind the somites (anterior-posterior axis). Transgenic *Ly-6A-GFP* embryos were used because HSCs are GFP⁺ in these mice^{13,14}. *CD41-YFP* mice¹⁵ were also used to witness the birth of the first HSCs, as these are known to express CD41 (refs. 16–20). The aortic endothelium and IAHCs were stained before or after embryo preparation with specific fluorescent directly labeled antibodies. Subsequently, nonfixed embryos were dissected and immobilized into an agarose gel covered with myeloid long-term medium, and time-lapse imaged directly under a confocal microscope. By combining appropriate embryo dissection and intra-aortic staining procedures, we witnessed the genesis of hematopoietic stem/progenitor



cells (HSPCs) directly from ventral aortic endothelial hemogenic cells^{21–23}. Our results in mice have been confirmed by time-lapse confocal imaging of zebrafish embryos^{1,2,24}.

Applications of the method

Although we used the embryo-slicing technique to image the aorta, other embryonic tissues can also be observed at single-cell resolution (e.g., mesonephric tubules, gonads, somites, neural tube). It should therefore be possible to study other developmental processes taking place in the mid-gestation embryo. Two of these are the formation of the sympathetic nervous system by neural crest cells and the migration of primordial germ cells (PGCs) to the urogenital ridges. The slicing method is also suitable for observing extraembryonic tissues such as placenta (C.R., unpublished data). The protocol can also be used to examine the behavior of specific cell types in mutant embryos.

Different culture conditions and parameters can be tested on the cell types of interest during confocal imaging. For example, the level of oxygen (hypoxia versus normoxia) and the composition of the culture medium (supplemented with drugs, inhibitors or growth factors) can be varied. Although it is primarily designed for time-lapse imaging, our method can also be used to quickly snapshot live cells in their native three-dimensional (3D) embryonic microenvironment. It avoids any fixation steps and allows the use of directly labeled antibodies, which are normally used for flow cytometry analysis. The method is particularly interesting when used with fluorescent proteins such as GFP and YFP, as fixation procedures can cause the disappearance of the fluorescent signal. Multicolor staining can also be performed, both on nonfixed embryo slices (Supplementary Fig. 1) and on whole embryos (after removal of dorsal tissue; Supplementary Movie 1).

Comparisons with other methods

To study embryos, they must be removed from the maternal uterus and isolated from all surrounding extraembryonic tissues. This impairs further physiological embryo growth and survival. Thus, *ex vivo* mouse embryo culture systems have been developed. Specific parameters (e.g., high serum concentration, high O₂ level) allow the normal development of embryos outside of the uterus²⁵. Confocal imaging of live embryos, isolated from E7.5 to E9.5, is possible for up to 2 d using static *ex vivo* embryo cultures^{25,26}. However, observations are restricted to superficial embryonic tissues because of the opaqueness of the mouse embryo. Moreover, *in vitro* development of older mouse embryos has not been successful so far.

McIlwain developed brain slices to study the effects of hypoxia and hypoglycemia, to perform quantitative pharmacological analyses and to study synaptic plasticity²⁷. Various methods of tissue-slice cultures have since been developed to improve tissue viability and to increase the culture time period. For example, slices have been cultured on a rotating titanium insert; thereby ensuring an equal exposure to medium and gas on both slice surfaces. Alternatively, brain slices can be embedded in collagen gel and grown for short periods of time in stationary dishes. Such approaches are compatible with confocal imaging to study single synapses²⁸ and 3D morphometry using brain cortex or spinal cord slices²⁹. Later, the development of organotypic brain slice cultures became a very attractive tool for neurobiological studies. Slices prepared from various brain areas (from 7- to 8-d-old pups) can be maintained in culture for several weeks when placed on a semiporous membrane

at the air-medium interface. In such organotypic culture conditions, brain slices retain many essential architectural features, including neuronal connectivity, glial-neuronal interactions and physiological stoichiometry of the different cellular components within the tissue³⁰. Of note, organotypic cultures have also been used to culture embryonic hematopoietic tissues for 3 d (ref. 7). Under these conditions, the tissue remains viable but loses its architectural integrity. For example, the culture of AGM regions or subdissected aorta leads to the collapse of the aorta, which is obviously incompatible with further imaging of the aorta lumen (J.-C.B. and C.R., unpublished observations). Studies with brain slices show that the integrity of the tissue can be maintained *ex vivo*, even when only a part of the complete organ is used³⁰. It is sufficient to sustain the layered organization of the cortex and to support the migration of cortical neurons. It has also been possible to observe by time-lapse imaging the division and migration of neuronal progenitors in live fetal brain sections³¹. The slicing technique is applicable to several other organs, including kidney, liver, lung and heart, notably to explore toxicant-induced organ injury.

Molyneaux *et al.*³² used transverse slices from the hindgut regions of mouse embryos expressing enhanced GFP (EGFP) in PGCs. They were able to visualize and follow PGC-EGFP⁺ cell migration at E10.0–E10.5 from the gut to the genital ridges. However, this protocol is not suitable for embryonic aorta observation, as the aorta integrity is not preserved in the transverse slices (i.e., the aortic lumen closes when slices are placed into the medium for time-lapse imaging³²; Supplementary Movie 2). We overcame this problem by restraining the slices in a layer of low-melting-point agarose²¹.

Advantages and limitations of embryo-slicing techniques

Tissue-slicing methods fill the gap between *in vivo* and cell culture models. Indeed, the key attribute of these methods is that they preserve the multicellular complexity and 3D organization of the tissue of origin, thus maintaining the biologically relevant structural and functional features of the *in vivo* tissues. The embryo-slicing technique that we developed is particularly amenable to young embryonic tissues and does not require special molds for agarose embedding to hold tissue during the slicing procedure. Many slices can be obtained from a single embryo. It is possible to select specific embryo slices, as they remain together after slicing. The anterior-posterior orientation of the slice series is determined by the positioning of the embryo before slicing. A careful examination of the structures surrounding the aorta (e.g., urogenital ridges, vitelline connection, limb buds) can also help to indicate the slice position relative to the anterior-posterior axis. When the tissue chopper parameters are standardized, the embryo-slicing procedure is highly reproducible, producing slices of uniform thickness. This is a key parameter because of the relationship between slice thickness and gas and nutrient exchange by diffusion. In general, a slice thickness of < 200 μm allows oxygenation and nutrient exchange and optimal diffusion to the inner cell layers, and it avoids necrosis within the center of the tissue while allowing confocal imaging of the whole slice. The embryo-slicing method is very well suited to the study of the emergence of new cell types, patterning and cell polarization as well as regional differences.

The main limitations of embryo-slicing techniques are the absence of blood flow and the limited viability of the slices (in the range of 2 d), which impair truly long-term studies. Increasing the slice thickness might pose problems for antibody penetration and

optical detection. For our particular studies, many slices needed to be examined in order to observe HSPC emergence, as this is a rare event.

Experimental design

Mice and embryos. The use of mice and embryos for experiments requires compliance with standards for care and use of laboratory animals and approval from the local ethical review board. Embryo slicing and imaging can be applied to different mouse backgrounds. However, the stages of development may slightly differ between wild-type mouse strains, and between wild-type and mutant mice. The most reliable method for staging embryos between E9 and E11 is to count the number of somite pairs and to observe the eye and limb bud development (for E11). Staging the embryo must be precise and can be very crucial for particular studies. We have observed that only embryos older than 33 somite pairs (s.p.) are able to generate HSPCs from the ventral aortic endothelium. The use of transgenic or knock-in embryos carrying a fluorescent reporter is very useful for confocal microscopy observation and requires no antibody staining. For example, some endothelial cells and IAHCs express GFP in the aorta of E10.5 *Ly-6A-GFP* transgenic embryos¹⁴. These embryos represent a nice model for observing the transition of hemogenic endothelium (GFP⁺) into HSPCs (GFP⁺).

Dissection and sample preparation for imaging. All dissection procedures should be performed on a black background and done as quickly as possible to increase the viability of the tissues (for example, with several people dissecting at the same time). The count of somite pairs is more accurate when the amniotic sac is removed from the embryo. Counting is facilitated when light is coming from only one side of the dissection microscope. This creates

a shadow that improves the visibility of the somites. When embryos are cut transversely, both ventral and dorsal parts of the aorta are visible. The aorta is surrounded by an intact 3D microenvironment, including the urogenital ridges, gut, neural tube, notochord and body walls. With this cut orientation, the emergence of hematopoietic cells from the endothelium toward the lumen of the aorta will appear in the *xy* plane, which gives a better resolution than the *z* plane. However, the live sections required for such imaging are 200 μm thick and represent only a small portion of the complete aorta. Imaging of multiple successive embryo slices is required for a complete perception of events occurring in the aorta. Removing the dorsal tissue and part of the roof of the aorta allows observation of the aorta from a dorsal perspective; this allows a proper and sharp visualization of the floor and sides of large portions of noninterrupted aorta, but the roof of the aorta can obviously not be imaged. Moreover, only cells emerging from the sides of the aortic endothelium will appear in the *xy* plane. Emergence from the ventral endothelium will be observed in the *z* plane, but with less resolution.

Fluorescent labeling. Staining of nonfixed embryos (transversely cut or without dorsal tissue) is possible with the use of antibodies that are directly conjugated to fluorochromes and that are classically used for flow cytometry analyses. The choice of fluorochrome used for the antibody staining is crucial. The staining is performed either before or after the cutting procedure. We obtained similar results for various surface markers tested (Table 1). Each antibody must be titrated to obtain a specific staining with reduced background, and an IgG control antibody coupled to the same fluorochrome must be used and recorded with the same parameters on the confocal microscope.

TABLE 1 | Antibodies and fluorophores for embryo slice or embryo caudal part staining.

Antigen	Conjugate	Clone	Company	Cat. no.	Dilution
CD41	PE	MW Reg 30	Santa Cruz Biotechnology	sc-19963 PE	1/50
CD4	PE	GK1.5	BD Pharmingen	557308	1/100
CD31	PE	MEC 13.3	BD Pharmingen	553373	1/100
	Alexa Fluor 647		BioLegend	102516	1/100
c-kit	PE	2B8	BD Pharmingen	553355	1/100
	APC			553356	1/100
CD45	FITC	30-F11	BD Pharmingen	553080	1/200
	PE			553081	1/200
	APC			559864	1/200
CD34	Alexa Fluor 647	RAM34	eBioscience	51-0341-82	1/100
Flk-1	PE	Avas 12α1	BD Pharmingen	555308	1/50
Tie-2	PE	TEK4	eBioscience	12-5987-82	1/100
CD11b	FITC	M1/70	BD Pharmingen	553310	1/100
	PE			553311	1/100
F4/80	PE	CI:A3-1	AbD Serotec	MCA497PE	1/100
VE-Cadherin	Alexa Fluor 647	eBioBV13 (BV13)	eBioscience	51-1441-82	1/50



The rationale for injecting antibodies directly inside the aorta is to label the cells facing the aortic lumen (endothelial cells) and not those inside the mesenchyme (tissue surrounding the aorta). The new HSPCs generated in the aortic lumen are labeled, thus proving their endothelial origin¹⁴. To mark cells outside the aorta, one can stain after embryo slicing. Although it is possible to stain directly on the caudal embryo part that has been freed of dorsal tissue, we have found it more purposeful to perform such staining procedures on transverse sections.

We preferentially chose dyes with an infrared excitation/emission wavelength. First, this allowed simultaneous imaging with GFP or YFP (or green fluorochromes as fluorescein isothiocyanate). Second, in this spectral region photon scattering is reduced, which allows greater optical penetration and image sharpness. We preferentially used antibodies coupled to Alexa Fluor 647 dye, as this dye is bright and photostable. In comparison, phycoerythrin (PE) is much more sensitive to photobleaching. We used a long-distance working lens of $\times 20$ or $\times 40$ magnification and the laser and filter combinations described in **Table 2**.

Image acquisition. Choosing the right microscope setup is of great importance for time-lapse imaging. Different criteria need to be taken into account. First, it is crucial to limit photodamage to cells and fluorescent proteins/dyes. Second, the microscope setup should provide a maximum resolution in each *xyzt* dimensions without affecting cell viability. For example, we have imaged live sections over a *z* range of 125 μm with an $\times 40$ dry lens, because a dry lens offers a superior working distance (in comparison to an oil-immersion lens). Furthermore, we increased the pinhole aperture to 2 Airy units in order to have a confocal plane of about 5 μm instead of 1 μm . Such a setup decreases the *z* resolution and captures out-of-focus light. At the same time, it allows lower laser exposure and intensity. In this regard, we do not recommend two-photon or spinning-disk microscopes, despite their obvious advantages, because the pinhole cannot be increased, thereby precluding the previous strategy. We also note that the dyes we used for conjugation to antibodies required low light exposure in order to prevent the formation of toxic substances such as free radicals. In summary, the frequency and the intensity with which the lasers excite the fluorescent proteins/dyes should be kept as low as possible; this is made possible by increasing the light going to the detectors, although it results in compromised resolution.

Image reconstruction. Lowering the laser power also has consequences for the signal-to-noise ratio. During acquisition, it is possible to reduce noise by acquiring four sequential images.

TABLE 2 | Confocal lasers and emission filters.

Fluorophores and fluorescent proteins	Excitation laser/wavelength (nm)	Emission filter (nm)
GFP	Argon gas laser 488	BP 500–550
FITC	Argon gas laser 488	BP 500–550
PE	Argon gas laser 488 and/or Dpss 561 laser 561	BP 570–620
Alexa Fluor 647	HeNe laser 633	BP 650–720 or LP 650
APC	HeNe laser 633	BP 650–720 or LP 650

After acquisition, contrast can be improved by using deconvolution software. With a proper algorithm, the convolved objects will theoretically be translated back to the actual structures from which they were derived. The end result is an image with a slightly better resolution and a better signal-to-noise ratio. We used the Huygens deconvolution software, which can work both with a measured point spread function (PSF) or a theoretical PSF (derived from the optical parameters of the microscope). With thick live specimens, the real PSF will change during deeper imaging. We thus applied a theoretical PSF.

Image registration and analysis. Emergence of HSPCs from the aortic endothelium is a very rare event (we estimated the event at < 2 per embryonic aorta), which means that a large number of images must be acquired and analyzed. In typical experiments, we could image seven different positions (or embryo slices, or *xyz* volumes), during periods of up to 15 h, with recurring cycles of 15 min (approximately the time required to image seven volumes). The size of the data generated (3–6 GB per position, depending on the number of channels and resolution used) can be quite large and must be considered for obvious storage and analysis reasons. The raw *xyzt* data were edited using ImageJ, either by selecting a single plane or by making a maximal projection of multiple planes. During acquisition, some movements of the specimen can occur as a result of stage heating, systematic errors in stage movement and/or shrinkage of the aorta. To correct for any drifting, we used ImageJ and the StackReg plug-in using the ‘rigid body’ option³³. The 3D renderings were performed from the raw data with Volocity software.

MATERIALS

REAGENTS

- Wild-type or transgenic mice **! CAUTION** Mice must be housed according to the institutional guidelines, and all animal procedures must be carried out in compliance with the relevant standards for humane care and use of laboratory animals.
- Embryos (see REAGENT SETUP)
- Dulbecco’s phosphate-buffered saline (DPBS without Ca and Mg, Lonza, cat. no. BE17-512F)
- CO₂ independent medium (CO₂IM, Invitrogen, cat. no. 18045-054)

- Myeloid long-term culture medium (MyeloCult M5300, StemCell Technologies, cat. no. 5350)
- Fetal bovine serum (FBS, Hyclone/Perbio, cat. no. CH30160.03)
- Low-melting-point agarose (Sigma-Aldrich, cat. no. A9414)
- Absolute ethanol (Sigma-Aldrich, cat. no. BCR656)
- Milli-Q water (house-made with a Millipore water system)
- Hydrocortisone succinate (Sigma-Aldrich, cat. no. H2270)
- Penicillin-streptomycin mixture 100 UI ml⁻¹ final (PS, Lonza, cat. no. DE17-602E)



PROTOCOL

- Recombinant murine interleukin-3 (rmIL-3, BD Pharmingen, cat. no. 554579)
- Monoclonal antibodies (**Table 1**) ▲ **CRITICAL** Some fluorophores are incompatible with overtime time-lapse imaging because of rapid photo-bleaching. When available, we recommend using antibodies labeled with Alexa Fluor 647 (Invitrogen) (more stable than fluorescein isothiocyanate or PE).

EQUIPMENT

- Leica SP5 AOBs DMI600 inverse microscope (Leica Microsystems). The microscope is equipped with three fluorescent channels and a transmission channel; high-power (200 mW) argon laser, 561 diode and 633 HeNe laser; automated xy stage; objective HCX PL Fluotar L $\times 40.0$ dry; numerical aperture 0.6, working distance 1.9–3.3 mm (depending on the cover slip correction ring settings). Alternatively, experiments can also be performed with a Zeiss LSM510 confocal microscope (Carl Zeiss, <http://www.zeiss.com/lsm>).
- Stereo dissection microscopes SZX12 and SZH10 (Olympus) with a magnification range from $\times 7$ to $\times 40$ with a black background stage and cold light source
- Incubator at 37 °C (Sanyo Electric, cat. no. MCO-5AC)
- McIlwain tissue chopper (The Mickle Laboratory Engineering; the manual for setting up the tissue chopper can be found at http://www.stoeltingeurope.com/stoelting/stores/stoelting/products/docs/1760_mcilwain_tissue_chopper.pdf).
- Micropipette puller (Narishige, <http://products.narishige-group.com/group1/PC-10/injection/english.html>, Model PC-10)
- Dissection tools (Needles, sharpened tungsten wire of 0.375-mm diameter attached to metal holders or insulin syringes; Dumont fine forceps no. 5; Dumont fine forceps 5/45; curved fine point forceps; and spring scissors (Roboz, cat. no. RS-5630). All dissection instruments are sterilized in 70% (vol/vol) ethanol and dried before and after use.)
- Plastic tissue culture dishes (35 \times 10 mm; Greiner Bio-One, cat. no. 627160)
- Plastic tissue culture dishes (60 \times 15 mm; Greiner Bio-One, cat. no. 628960)
- Image analysis software (We recommend ImageJ (Rasband W.S., ImageJ, U.S. National Institutes of Health, <http://rsb.info.nih.gov/ij/>, 1997–2009) and Volocity, <http://www.perkinelmer.com/nl/pages/020/cellularimaging/products/volocity.xhtml>.)
- Huygens deconvolution software (Scientific Volume Imaging, <http://www.svi.nl>)
- Borosilicate glass capillaries (outer diameter 1.9 mm, inner diameter 0.78 mm; Harvard Apparatus, cat. no. GC100T-15)
- Round glass cover slip (diameter: 24 mm, Menzel Glaser, cat. no. CB00240RA1)
- Chamber ring (Attolfluor cell chamber, Invitrogen, <http://products.invitrogen.com/ivgn/product/A7816>, cat. no. A-7816)
- Plastic bucket (500 ml, Vitlab, cat. no. 441081)
- Black round plastic disk (custom-made) ▲ **CRITICAL** The commercially available supports are white. It is crucial to have them in black (noncommercialized) to see the embryo slices after cutting. To make black supports, cut a black cylinder of vinyl chloride polymer (diameter: 5 cm) into disks (thickness: 1.5 mm).
- Gilson pipettes and corresponding filter tips (Greiner Bio-One)
- Short Pasteur capillary pipettes (Wu Mainz)
- Double-edge stainless steel razor blade (Electron Microscopy Sciences, cat. no. 72000)
- Puradisc 30 syringe filter (Cellulose acetate, 0.45 μ m, sterile, Whatman, cat. no. 10462100)
- BD Multifit glass syringe (50 cc, BD Biosciences, cat. no. 512435)
- 295 Bottle top filters (0.2 μ m, 500-ml capacity, MF75 Series, Nalgene, cat. no. 295-4545)
- Premium facial tissue, extra soft (Tork, cat. no. 140270)
- PeCon insert P (PeCon)
- Microcentrifuge tubes

REAGENT SETUP

Embryos Generate embryos either from crosses of transgenic males (heterozygous or homozygous) and wild-type females (from the same background) or from crosses of wild-type males and females. If one is interested in imaging HSPCs, the Ly-6A(Sca-1) GFP reporter transgenic mice can be used^{13,14}. Mate mice in the late afternoon and consider the day of vaginal plug observation as embryonic day (E)0. Select transgenic embryos (expressing a fluorescent reporter) on the basis of fluorescent expression after microscopic observation by using an Olympus IX70 fluorescent microscope. Perform genotyping of embryos from nonfluorescent transgenic lines by PCR.

Dissection medium Supplement PBS with 10% FBS (1:10, vol/vol) and a penicillin-streptomycin mixture (1:100, vol/vol; PBS/FBS/PS). The medium can be stored at 4 °C for 1 month. For CO₂-independent medium, supplement with 10% FBS (1:10, vol/vol) and penicillin-streptomycin mixture (1:100, vol/vol; CO₂IM/FBS/PS) and store at 4 °C for up to 1 month. ▲ **CRITICAL** The FBS must be heat-inactivated for 30 min in a water bath at 56 °C. ▲ **CRITICAL** All reagents and solutions should be prepared under sterile conditions. Filter all medium using a 0.2- μ m filter.

Confocal culture medium Supplement myeloid long-term medium with hydrocortisone (final concentration: 10⁻⁶ M) and rmIL-3 (final concentration: 200 ng ml⁻¹). Unsupplemented myeloid long-term medium can be aliquotted and stored at -20 °C (it can be stored until the expiration date indicated on the product label). ▲ **CRITICAL** The myeloid long-term medium supplemented with hydrocortisone and rmIL-3 is best freshly prepared just before use. ▲ **CRITICAL** All reagents and solutions should be prepared under sterile conditions. Filter all medium using a 0.2- μ m filter.

Hydrocortisone stock solution Dissolve 100 mg of hydrocortisone powder in 2 ml of sterile PBS. After complete dissolution, aliquot the solution (50 μ l per microcentrifuge tube). Store tubes at -20 °C (they can be stored for at least 6 months). Use the hydrocortisone at 0.5 mg ml⁻¹ after dilution in confocal culture medium (1:100, vol/vol). ▲ **CRITICAL** All reagents and solutions should be prepared under sterile conditions.

Recombinant murine interleukin-3 Predilute the recombinant murine interleukin-3 (rmIL-3) stock solution (200 μ g ml⁻¹) in PBS/FBS/PS (1:20, vol/vol). Store tubes at -80 °C (they can be stored for at least 3 months). Use the predilution at a final concentration of 200 ng ml⁻¹ in confocal culture medium (1:50, vol/vol). ▲ **CRITICAL** All reagents and solutions should be prepared under sterile conditions.

Ethanol, 70% (vol/vol) Dilute absolute ethanol into Milli-Q water (7:3, vol/vol).

Low-melting-point agarose gel at 1% (wt/vol) For 100 ml of low-melting-point agarose, weigh 1 g of agarose powder and dissolve it in 100 ml of PBS (1:100, wt/vol).

EQUIPMENT SETUP

Preparation of micropipettes for intra-aortic injection Pull glass capillary tubes using a micropipette puller. ▲ **CRITICAL** After elongation, break the extremity of the capillary and make sure that the capillary tip is sharp enough and not too large at its extremity, so that the embryo body to the aorta is penetrated without breaking the capillary. Prepare several elongated capillaries in advance because they break easily.

Tissue chopper Adjust the thickness of embryo slices by means of the micrometer head (maximum thickness: 1 mm; each division on the thimble represents 1 μ m, each small division on the barrel represents 25 μ m). Adjust the force of the blade by turning the blade force knob and adjust the speed with the speed control knob. ▲ **CRITICAL** The position of the razor blade and the cutting speed and force are crucial for perfect embryo cutting to prevent damage to the embryo. First, make a trial cut on the support (with no embryo). The mark of the total length of the razor should be visible on the support. This indicates that the razor is perfectly parallel to the support. Test the speed and force of the razor blade on one embryo to optimize the cutting and to make sure that the cut goes through the entire embryo body without crushing it.

Confocal microscope On the Leica SP5 microscope, we use the following image parameters, although they can be varied: 1,024 \times 1,024 \times 25 pixels; 298.8 \times 298.8 \times 120 μ m stack; z increment: 5 μ m; and x and y pixel size: 292.1 μ m. To image the embryonic aorta, we use the following: a scan speed 400 Hz and a pinhole of 2 Airy units; emission bands (Leica spectral detectors) of BP 500–550, BP 570–620 and BP 650–750 (**Table 2** and Step 20); frame average: 4; and time steps: 15 min. We heat the stage to 37 °C with a Pecon insert P. A stream of compressed air premixed with 5% CO₂ is passed through a bubble flask with distilled water before blowing over the cells.

Table 2 summarizes the combination of lasers and filters. The combinations may vary depending on the fluorophores used. It is crucial to choose fluorophores that present a minimum overlap between the collected signals.

▲ **CRITICAL** The specificity of fluorescent staining must be validated by staining embryo slices with isotype antibody controls (and/or with the use of antibodies against molecules that are not expressed in the tissue of interest). In the case of multicolor staining, embryo slices must be stained with each individual antibody to verify that fluorescent signals are only detected in the appropriate channel and there is no 'bleed-through' to another channel.

PROCEDURE

Isolation of embryos ● TIMING 1–1.5 h

1| Euthanize the pregnant female mouse. See **Figure 1**, **Figure 2** and see **Supplementary Movie 3** for a demonstration of embryo isolation.

▲ **CRITICAL STEP** The euthanasia method chosen must be consistent with the all relevant institutional regulations.

The technique must result in the quick and painless death of the animal. We perform cervical dislocation but the mouse can also be euthanized after carbon dioxide inhalation.

2| Clean the mouse abdomen with 70% (vol/vol) ethanol.

3| Make a transverse incision on the abdomen (skin and muscle layers).

4| Carefully pull the complete uterus out of the abdominal cavity with fine forceps (**Fig. 2a**) and place it in a dish containing 5 ml of PBS/FBS/PS.

▲ **CRITICAL STEP** Be careful not to squeeze the embryos between the tips of the forceps.

5| Clean the uterus from surrounding blood by passaging it in a clean dish containing 5 ml of PBS/FBS/PS.

6| Open the uterus with forceps to access the yolk sac layer.

▲ **CRITICAL STEP** Be careful not to pierce the yolk sac. There is a risk that the embryo will pop out and be damaged, particularly with early stage embryos.

▲ **CRITICAL STEP** The embryo dissection must be performed as fast as possible to improve the viability of embryonic cells that are particularly fragile.

7| Remove the embryo conceptus with its yolk sac and placenta intact and attached, and place it in a 60-mm dish containing CO₂IM/FBS/PS (**Fig. 2b**).

8| Separate each embryo with yolk sac from the placenta (**Fig. 2c,d**) and then remove the yolk sac (**Fig. 2e,f**).

▲ **CRITICAL STEP** If transgenic mothers are used, carefully wash the embryos after they are removed from the uterus to avoid any contamination of the embryonic tissues by the mother's blood.

9| Remove the thin amniotic sac surrounding the embryo (**Fig. 2f**).

▲ **CRITICAL STEP** It is important to remove this membrane before the somite pair count or any further dissection procedures.

10| Stage each embryo by counting the somite pairs.

11| In transgenic litters, transfer each embryo into the well of a 24-well plate and genotype them directly under a fluorescent microscope. For nonfluorescent transgenes, perform the genotyping by PCR using a small piece of the head.

12| Cut the head and heart with needles just before the forelimbs, and cut the tail just after the hind limbs (**Fig. 2g**). Use the embryo trunk for the rest of the procedure.

Staining of the embryo aorta ● TIMING 1–1.5 h

13| If you are staining with antibodies, inject antibodies to stain the aorta before (option A) or after (option B) slicing of the embryos. If you are following option B and staining after slicing of the embryos, first perform Step 14A. See **Supplementary Movie 4** for a demonstration of the injection. Injection of antibodies into the aorta flushes away the

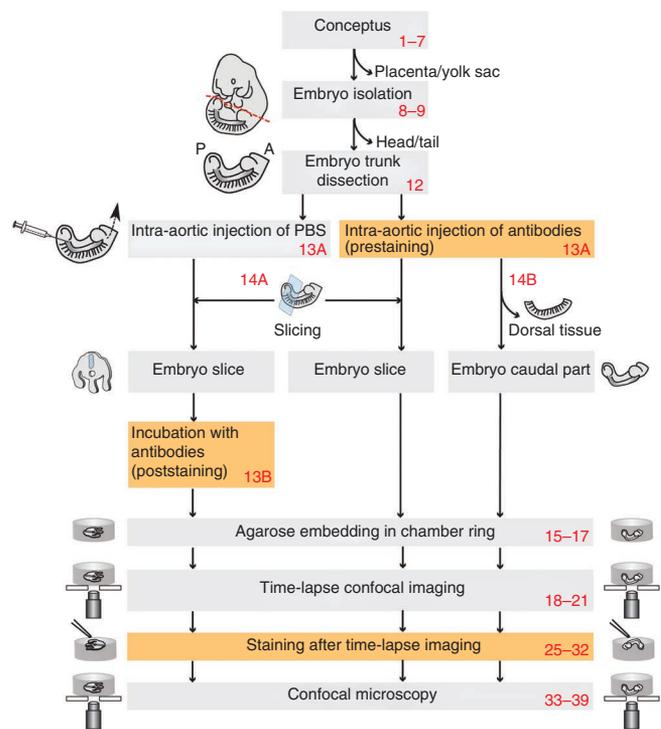


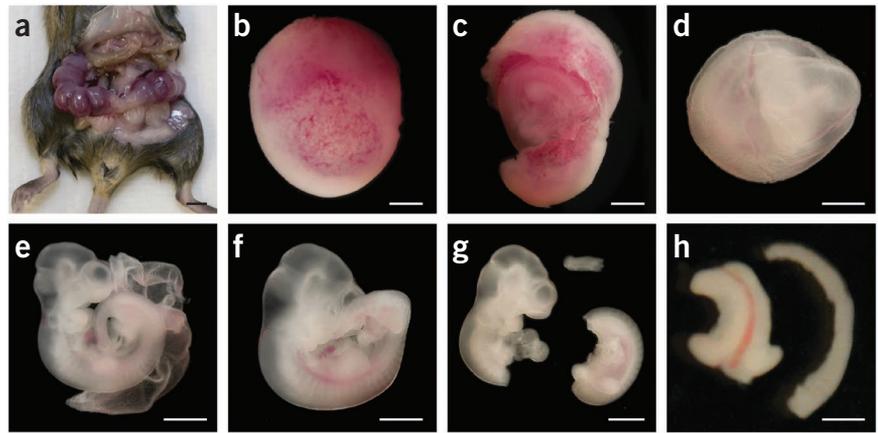
Figure 1 | Workflow for mouse embryo confocal imaging. Steps of the protocol are indicated in red. Steps of antibody staining are indicated in orange. A, anterior; P, posterior.



PROTOCOL

Figure 2 | Dissection procedure of E10 embryo.

(a) The uterus is carefully pulled out with forceps after transverse incision of the abdomen with scissors. Scale bar, 0.5 cm. (b) The embryo is removed from the uterus with the placenta and yolk sac attached and intact. (c,d) While gently grasping the fine forceps, open the embryo conceptus, remove the Reichert's membrane surrounding the yolk sac, cut the umbilical vessels with fine scissors and separate the embryo from the placenta with the yolk sac intact. (e) To separate the embryo, first grasp and tear open the yolk sac with fine forceps and then tear off the yolk sac, which is now connected to the embryo proper via the vitelline vessels. (f) Cut the vitelline vessels with fine scissors and remove the thin amniotic sac that is surrounding the embryo. (g) After placing the embryo on the side, use needles to cut off the upper part of the embryo including the head and heart (up to the upper limbs) and the tail (down to the lower limbs). (h) The dorsal tissue is cut off from the trunk of the embryo with needles. The blood in the aorta was not flushed away to show that all dorsal tissue must be properly cut to clearly visualize the aorta localized just behind it. Scale bars in b–h, 1 mm.



blood inside the aorta at the same time. Be aware that the staining procedure before embryo slicing allows only the staining of cells that are inside the aorta (aortic endothelial layer and hematopoietic cells). The staining procedure after embryo slicing allows the staining of cells that are also outside the aorta (e.g., in the surrounding mesenchyme).

(A) Prestaining (before embryo cutting) ● TIMING 1–1.5 h

- (i) Prepare the antibody dilution (Table 1) PBS/FBS/PS and place the solution in the cap of a microcentrifuge tube (cut off from the microcentrifuge tube).

? TROUBLESHOOTING

- (ii) Prepare the micropipette for intra-aorta injection by breaking the extremity of the capillary carefully by pushing the tip of the capillary on the side of a plastic dish. Make sure that the extremity of the glass capillary is sufficiently open (by aspirating and flushing away some PBS/FBS/PS and air).

▲ **CRITICAL STEP** Make sure that the capillary tip is sharp enough and not too large at its end, such that the embryo body can be penetrated to the aorta without breaking the needle.

- (iii) Transfer the embryo with a curved forceps to the inside lid of a 60-mm dish containing a thin layer of PBS/FBS/PS.

▲ **CRITICAL STEP** Several embryos can be placed in the dish lid at the same time and injected successively. However, the injection procedure must be done quickly to avoid drying out the embryos because the embryo surface is exposed to the air during the procedure.

- (iv) Aspirate the antibody dilution with an elongated glass capillary connected with a 50-cc syringe.

- (v) Place the capillary at the posterior extremity of the embryo and go through the embryo to reach the inside of the aorta (Fig. 3a–c).

- (vi) Inject the antibody dilution slowly in the aorta, taking a few seconds by applying a constant pressure to the syringe plunger.

▲ **CRITICAL STEP** Make sure that the capillary is inside the aorta and not in the surrounding tissues. All the blood must disappear from the aorta, which becomes transparent. The blood exits via the anterior part of the embryo. Owing to the dissection, the aorta might not contain much blood anymore, making the injection inside the transparent aorta more difficult. If the injection is performed outside the aorta, a deformation of the surrounding tissue will appear.

- (vii) Replace the injected embryo in a 60-mm dish containing fresh PBS/FBS/PS.

- (viii) Place the dish at 4 °C for 30 min.

(B) Poststaining (after embryo cutting) ● TIMING 1–1.5 h

- (i) Transfer the embryo slices (produced as described in Step 14A) into the well of a 24-well plate.

- (ii) Remove the PBS/FBS/PS with a P-200 pipette.

▲ **CRITICAL STEP** Be careful not to aspirate the embryo slices.

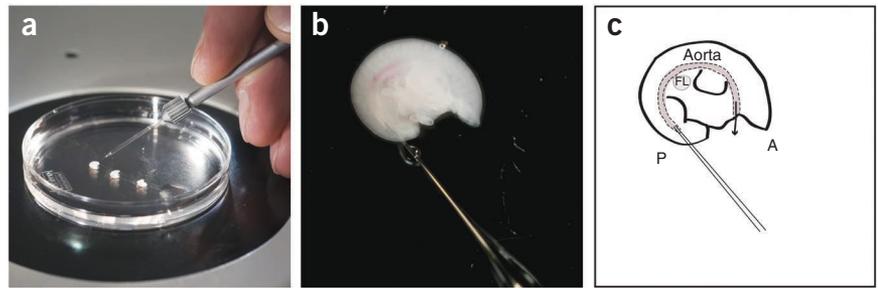
- (iii) Immediately add both 300 µl of PBS/FBS/PS per well and the antibody (the volume depends on the antibody dilution; Table 1).

- (iv) Place the dish at 4 °C for 30 min (to allow the antibody staining).

- (v) Remove the antibody solution with a P-200 pipette.

- (vi) Add 1 ml of PBS/FBS/PS into the well.

Figure 3 | Intra-aortic injection procedure. Embryos are placed with a curved forceps in the lid of a 60-mm culture dish (the lid contains a thin layer of PBS/FBS/PS). The embryos are positioned on their sides. (a) After filling the glass capillary with antibody dilution, inject the solution inside the aorta by piercing through the tissues (posterior side of the embryo) to access the aorta. The blood will be pushed away from the aorta and released in the plate lid. (b) Close-up view of the intra-aortic injection. (c) Schematic representation of b. A, anterior; FL, fetal liver; P, posterior.



- (vii) Wash for 10 min at room temperature (~20 °C).
- (viii) Remove the PBS/FBS/PS from the well and repeat Step 13B(vi–viii) once.
- (ix) Add 1 ml of PBS/FBS/PS to the embryo slices.

Preparation of embryo specimens for live imaging ● **TIMING 1.5–2 h**

14 | To observe the aorta under the confocal microscope, cut the embryos into transverse thick slices (option A; see also **Supplementary Movie 5**). In an alternative approach, the caudal part of the embryo can be used after removing the somites (option B).

(A) Preparation of embryo slices ● **TIMING 1.5 h**

- (i) Prepare the chamber ring by closing the bottom with a round glass cover slip.
- (ii) Place the embryo with a curved forceps on the top of the round plastic disk.
- (iii) Carefully stretch the embryos with needles and place them on the same line (**Fig. 4a**).
 - ▲ **CRITICAL STEP** To ensure that embryos do not dry out, a maximum of three embryos can be cut in a row.
- (iv) Place the disk on the tissue chopper’s circular stainless steel table.
 - ▲ **CRITICAL STEP** The embryos must be oriented perpendicularly to the razor blade.
 - ? **TROUBLESHOOTING**
- (v) Cut the embryo into transverse slices of 200 μm (**Fig. 4b**).
 - ? **TROUBLESHOOTING**
- (vi) After cutting, cover the sliced embryo with a few drops of PBS/FBS/PS (**Fig. 4c**).
- (vii) Place the disk under the microscope and separate the embryo slices with needles (**Fig. 4d**).
 - ▲ **CRITICAL STEP** Separate properly cut embryo slices from debris and incomplete or destroyed slices.
- (viii) Remove all debris and incomplete or destroyed slices with the corner of a thin paper towel (**Fig. 4e**).
 - ▲ **CRITICAL STEP** Be very careful not to remove the correct embryo slices.
- (ix) Add a few drops of PBS/FBS/PS on the top of the embryo slices.
- (x) Aspirate the sections with a P-1000 pipette and a blue tip cut at the extremity with a razor blade.
 - ▲ **CRITICAL STEP** The extremity of the tip must be enlarged (to avoid damaging the slices) and cut clean (to prevent slices from sticking to the tip).
- (xi) Place the slices directly in the chamber ring or in the well of a 24-well plate (to perform antibody staining, see Step 13B).
 - ? **TROUBLESHOOTING**
- (xii) Prepare 100 ml of low-melting-point agarose. Weigh 1 g of agarose powder in a 500-ml bucket, add 100 ml of PBS and dissolve the suspension in the microwave.
 - ▲ **CRITICAL STEP** Make sure that the agarose is completely dissolved.
- (xiii) Place the bucket of melted agarose in an incubator at 37 °C.
- (xiv) Remove the PBS/FBS/PS in the chamber ring with a P-1000 pipette and a cut blue tip.
- (xv) Use the P-1000 pipette and a cut blue tip to aspirate 1 ml of the agarose gel. Pour the gel immediately on the top of the slices.
 - ▲ **CRITICAL STEP** The agarose gel must be at ~55 °C when aspirated (still liquid). The gel is at ~37 °C after transfer into the chamber ring (to avoid cell damage).
- (xvi) Place the chamber ring under the stereo dissection microscope and push all embryo slices very delicately to the bottom and center of the chamber (**Fig. 4f**).
 - ▲ **CRITICAL STEP** After pouring the gel, the slices must be positioned as quickly as possible at the bottom of the chamber, against the cover slip, before the agarose gel solidifies (**Fig. 4f–i**).
 - ? **TROUBLESHOOTING**

PROTOCOL

Figure 4 | Preparation of embryo slices.

(a) The embryos are placed on a round black cutting board and slightly stretched with needles. (b) The cutting board is placed on the stage of the tissue chopper. The axis of the embryos must be perpendicular to the razor blade. (c) After cutting, a few drops of PBS/FBS/PS are added to recover the embryo slices. Scale bar, 1 mm. (d) The cutting board is placed under a microscope and then all embryo slices are separated with needles. (e) All debris and incomplete or destroyed slices are removed with the corner of a piece of soft tissue paper. PBS/FBS/PS is added on the top of the embryo slices and then all embryo sections are transferred into the culture chamber. (f–h) After removing the PBS/FBS/PS, the agarose gel is poured on the top of the embryo slices that are immediately positioned as closely as possible on the cover slip and in the center of the culture chamber with needle before the agarose gel solidifies. (h) Scale bar, 500 μm . (i) Close-up showing an embryo slice. Scale bar, 100 μm .



(B) Preparation of embryo caudal parts (separated from the dorsal tissue) ● TIMING 30 min to 1 h

- (i) Prepare the chamber ring by closing the bottom with a round glass cover slip.
- (ii) Place the embryo on its side in a dish containing 5 ml of PBS/FBS/PS.
- (iii) Cut the dorsal tissue off with needles (**Fig. 2h**).
 - ▲ **CRITICAL STEP** Be careful to remove all dorsal tissue as well as part of the roof of the aorta to access the rest of the aorta (sides and floor) over its full length.
 - ? **TROUBLESHOOTING**
- (iv) Transfer the tissue with a curved forceps into the chamber ring containing 1 ml of PBS/FBS/PS.
- (v) Remove the PBS/FBS/PS with a P-1000 pipette and a cut blue tip.
- (vi) Prepare the agarose gel (see Step 14A(xii, xiii)).
- (vii) Use a P-1000 pipette and a cut blue tip to aspirate 1 ml of the agarose gel. Pour the gel immediately on the top of the embryo caudal part.
 - ▲ **CRITICAL STEP** The agarose gel must be at $\sim 55^\circ\text{C}$ when aspirated (still liquid). The gel is at $\sim 37^\circ\text{C}$ after transfer into the chamber ring (to avoid cell damage).
- (viii) Place the chamber ring under the stereo dissection microscope and very delicately push all embryo caudal parts to the bottom and center of the chamber, with the dorsal part facing the cover slip.
 - ▲ **CRITICAL STEP** After pouring the gel, embryo caudal parts must be positioned as quickly as possible to the bottom of the chamber, against the cover slip, before the agarose gel solidifies. Position the embryos with the posterior/anterior axis parallel to the cover slip and maintain the position while the agarose gel starts to solidify to prevent the embryos from falling on their sides. The posterior part of each embryo must be facing down (against the cover slip) and the anterior part must be facing up.

15| Place the culture chamber at 4°C in the refrigerator for few minutes until the gel solidifies.

16| Prepare fresh myeloid long-term medium supplemented with hydrocortisone and IL-3 (see REAGENT SETUP).

17| Add 1 ml of myeloid long-term medium on the top of the solidified agarose gel in the chamber ring.

Time-lapse confocal imaging of the embryo slices and embryo caudal parts ● **TIMING** Up to 24 h

18| Place the chamber ring on the heated stage of the confocal microscope (**Supplementary Fig. 2a**).

▲ **CRITICAL STEP** This step varies depending on the microscope model, stage and culture chamber system. The chamber ring must be at 37 °C and supplied with oxygen (5% CO₂/air) and humidified atmosphere during the entire imaging session. The culture chamber must be tightly closed to avoid evaporation of the medium. We used the lid of a 30-mm dish, along with a transparent lid fitted for the heated plate chamber to maintain a constant gas supply (**Supplementary Fig. 2b**).

? **TROUBLESHOOTING**

19| Check the specimens (embryo sections or embryo caudal parts) with the transmitted light (to verify their quality and integrity). Observe each successive field of the chamber ring in a zigzag fashion in order to avoid missing any specimens. An average of seven embryo slices or four embryo caudal parts can be imaged sequentially. In the case of embryo caudal part imaging, select several positions along the aorta where the aortic roof is properly removed (see Experimental design). Because of their round and shiny appearance under the transmitted light, it is possible to recognize IAHCs in E10.5 embryo sections or embryo caudal parts through the microscope's eyepieces.

20| Set up the parameters on the confocal microscope.

▲ **CRITICAL STEP** The parameters can vary, depending on the apparatus used and the purpose of each experiment. As an example, to observe the emergence of HSPCs, choose the *xyzt* mode and set the resolution of *xy* acquisition to 1,024 × 1,024, with bidirectional scanning, at a 400-Hz frequency. Make the final images from the line average of four scans. When using the ×40 dry lens, increase the pinhole to 2 Airy units. Set the gain for the different channels to the maximum and adapt the laser intensities accordingly, but without oversaturation. This will keep cell photobleaching/damage to a minimum (see Experimental design). Do not modify the background threshold or offset as it could eliminate useful fluorescent signals. Set the position at the central *z* position, and calculate the mean of the observable extreme *z*. Depending on the microscope, the same *z* range (± 60 μm) should be applied from this reference point to all positions. Set the time separating two scanning cycles to 15 min and the experiment time to 15 h, although these parameters can be changed depending on the aim of the experiment.

? **TROUBLESHOOTING**

21| Launch the imaging experiment.

? **TROUBLESHOOTING**

22| At the end of time-lapse imaging, save the data for further analysis. The experiment can either be stopped at this point or further staining can be performed (e.g., to characterize the cells that have newly emerged in the lumen of the aorta).

? **TROUBLESHOOTING**

Staining of the embryo specimens after time-lapse imaging ● **TIMING** 1.5–2 h

23| Save the position coordinates of each specimen.

24| Remove the chamber ring from the microscope stage.

▲ **CRITICAL STEP** To return to the same positions after staining, mark the chamber ring with a pen in reference to either obvious static points on the incubation chamber or any other points that will allow you to place the chamber ring back in the exact same position. Then make incisions in the agarose gel in reference to the previous marks that will allow you to place the gel containing the specimens back in the chamber ring in the exact same position.

25| Prepare 100 μl of antibody dilution in PBS/FBS/PS.

▲ **CRITICAL STEP** The concentration depends on each antibody and should be titrated (**Table 1**). The fluorochrome used must not overlap with the previous colors used during the time-lapse imaging. We routinely use c-kit antibodies conjugated to R-PE to identify the newly emerged HSPCs.

26| Remove the myeloid long-term medium from the top of the agarose gel.

27| Free the gel from the chamber ring and use a curved forceps to place it on a 60-mm dish lid (the bottom of the gel, where the sections are, is placed up).

28| Carefully dispense the antibody dilution on the top of the agarose gel (**Supplementary Fig. 2c**).

29| Incubate the gel for 30 min at 4 °C in the dark.

PROTOCOL

- 30| Dispense a large volume (sufficient to cover the gel) of PBS/FBS/PS on the top of the gel.
- 31| Incubate the gel for 5 min at room temperature in the dark.
- 32| Remove the PBS/FBS/PS and repeat Steps 30–32 twice.
- 33| Place the gel back in the original chamber ring containing a new cover slip.
▲ **CRITICAL STEP** Remove bubbles that can form between the gel and the cover slip by pushing them carefully toward the location reference incisions. The incisions can be slightly increased to facilitate bubble exclusion. However, the structure of the gel must remain as intact as possible.
- 34| Adjust the position of the agarose gel to the references marked on the chamber ring.
▲ **CRITICAL STEP** It is crucial that the gel keeps its original structure in order to find the original xyz positions and cells of interest within the specimens.
- 35| By using the reference marks, place the chamber ring on the confocal microscope stage at the exact same position that was used during imaging.
- 36| For settings, excite the fluorochrome with the appropriate laser and corresponding filter for acquisition, depending on the new fluorochrome used.
- 37| In a xyz mode, open the saved position coordinates.
▲ **CRITICAL STEP** Verify whether the positions are identical to those used during the time-lapse imaging.
- 38| After opening one position coordinate, delete the other ones and image the single position over a maximal z range.
- 39| Repeat Step 38 for all positions.

Image analysis ● TIMING 2–5 h

40| If you are studying HSCs, examine the fluorescent channel for stained endothelial cells (e.g., CD31 conjugated to Alexa Fluor 647) in combination with the channel corresponding to the hematopoietic marker (e.g., Ly-6A-GFP). Inspect each z section separately at every time point of the movie. Look for cells emerging from the aortic endothelium. If such an event is visible, verify that the cell was not already present in the lumen at an adjacent z section. Indeed, some cells can enter the present z section and give the impression of emerging from the endothelium. Confirm the emergence by looking at the transmitted light channel separately. A real newly generated IAHC should give enough contrast to be discernible in this channel during emergence.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
13A(i)	Background after antibody staining	Antibodies are too concentrated	Each antibody must be titrated before use; IgG control coupled with the same fluorochrome must be tested as a background control
	No cell movement or cell death	Use of blocking antibody	If the goal of the experiment is to follow cells without interfering with their behavior, preferably use a nonblocking antibody
	No staining in the aorta	The antibody did not go through the aorta	For a proper intra-aorta injection, the pipette must be sharp enough to go through the embryo to reach the aorta. The thickness of the pipette is also important; if too thin it will break and too thick it will not enter the aorta. The average diameter of the pipette tip should be ~0.1 mm

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		The antibody concentration is too low	All antibodies must be titrated before use. Note that antibodies are usually twice as concentrated compared with the concentrations used for flow cytometry
14A(iv,v)	Poor image of the aorta	The sections are cut crookedly	It is important that the cut is performed perpendicular to the axis of the aorta. Thus, embryos must be placed as straight as possible before cutting
14A(v)	Embryo slices are damaged during cutting procedure	Parameters of the tissue chopper not are optimal	For proper cutting, the razor blade must be perfectly perpendicular to the cutting plane. The force of cutting must be tested on the support (no embryo) and the blade should leave a mark over its full length. The speed of the cutting must also be tested. The embryo must be cut throughout its entire body in order for each section to be easily detachable
14A(xi)	Loss of embryo slices during the procedure	Handling of the embryo slices not careful enough	Embryo cutting must be done on a black support to easily see the slices. The embryo slices are small, fragile and sticky. When transferred with a blue tip, make sure that the tip is cut with a smooth end to avoid retaining the slices. Be careful that the sections do not stick to the inside border edge of the culture chamber
14A(xvi)	Embryo slices are damaged during the immobilization procedure	Solidification of the agarose gel before positioning all embryo slices	Minimize the time between pouring the gel into the culture chamber and positioning of the embryo slices. To slow down the process of agarose solidification outside of the incubator, put the needle and the culture chamber in the incubator at 37 °C before placing the embryo sections
	Embryo slices move and/or the aortic lumen closes	Embryo slices are placed in medium for confocal imaging	Embryo slices must be covered with a layer of 1% (wt/vol) low-melting-point agarose before adding the medium. The agarose restrains and preserves the integrity of the slices without impairing cell movement
14B(iii)	No fluorescent signal in intact embryos	Partial removal of dorsal tissue	If the dorsal tissue is not cut off as close as possible to the aorta, the fluorescence in the aorta will not be detectable
		No opening of the aorta	To see the fluorescence inside the aorta, the roof of the aorta must be partially removed
18, 20	Poor sample cell viability	Problem with the microscope parameters	It is important that the sample stays overnight at 37 °C with constant air/5% CO ₂ supply. The chamber must be closed properly to avoid any evaporation of the medium. The laser power(s) should be kept as low as possible
21	Loss of focus during confocal imaging	Temperature variation of the agarose gel or focus drift in the microscope system	The room needs to be properly climate controlled to keep the temperature changes during the experiment to <1 °C h ⁻¹ and <2 °C for the whole experiment. The microscope setup needs to be placed at 37 °C for at least 1 h before starting the time-lapse imaging. Before starting the imaging, the culture chamber containing the agarose gel and the medium on top must be stabilized at 37 °C to avoid any density changes in the gel
22	No fluorescent signal in embryo slices after time-lapse imaging	Photobleaching	The laser intensity must be tested in order to get the best fluorescent signal without photobleaching
	Problems storing the movie files	Files are too big to be stored or analyzed	Imaging for up to 15 h generates very large files that are difficult to store and analyze Some space can be saved by imaging with a smaller resolution (512 × 512 instead of 1,024 × 1,024). A 64-bit operating system and a large amount of internal memory (≥8 GB) is recommended

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● TIMING

Steps 1–12, Isolation of embryos:
1–1.5 h

Step 13, Staining of embryo aorta:
1–1.5 h

Steps 14–17, Preparation of embryo
specimen for live imaging: 1.5–2 h

Steps 18–22, Time-lapse confocal
imaging of the embryo slices and
embryo caudal parts: up to 24 h

Steps 23–39, Staining of the embryo
specimens after time-lapse imaging:
1.5–2 h

Step 40, Image analysis: 2–5 h

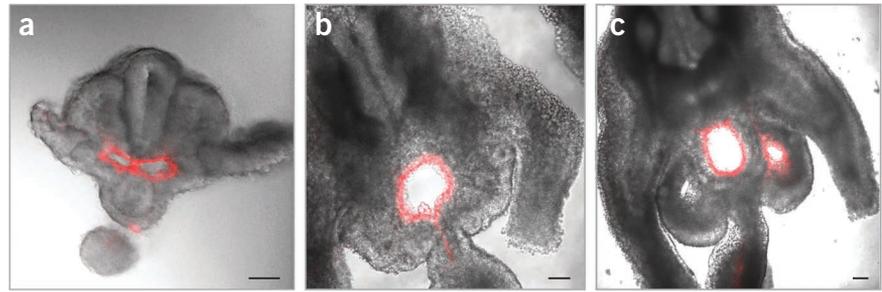


Figure 5 | Examples of embryo slices. (a–c) Slices from E9.5 (a), early E10 (b) and E11 embryos (c) are shown. The embryos were injected directly in the aorta with Alexa Fluor 647–anti-CD31 antibodies before slicing and imaging. The Alexa Fluor 647 signal (in red) is merged with the transmitted light image. Note that at E9.5 the aorta is still paired. Only the aortic endothelium and the hematopoietic clusters are labeled with Alexa Fluor 647–anti-CD31 antibodies. The vitelline vessels (b) and cardinal veins (c) are sometimes also stained if they are part of the embryo slice. Scale bars, 50 μ m.

ANTICIPATED RESULTS

Slices can be obtained from E9, E10 and E11 embryos (**Fig. 5**). However, E10 embryos have the optimal size for sectioning (around 9 sections per embryo). E9 embryos provide only few sections (around 4 per embryo), and many embryos are needed for a complete confocal microscopy experiment. E11 embryos are the most difficult to cut because of their large size. The speed and force of the razor blade need to be optimized to obtain nondamaged sections at this stage of embryo development.

The rare event of hematopoietic cell formation from the mouse aortic endothelium can be clearly visualized by performing time-lapse confocal microscopy on the embryo sections or embryo caudal parts²¹. Because the emergence event is so rare (<2 per embryo), many slices need to be observed and carefully analyzed. We limited the time-lapse imaging to a maximum of 15 h because we never observed a higher rate of hematopoietic emergence events over a longer period of observation. However, confocal imaging can be extended to 24 h without obvious cell or tissue mortality. For longer periods, we cannot exclude the possibility that the extended exposure time to lasers will compromise the integrity of the tissue or cell behavior. The disintegration of the aortic endothelium is a good indication that the slices have undergone deleterious changes. This is a phenomenon typically observed after long imaging periods; when it occurs, the data should not be used. Importantly, existing HSPCs can, in principle, be distinguished from newly emerging HSPCs by carefully analyzing the optical planes adjacent to the plane in which the emergence is observed. However, we strongly advise to re-stain the slice where an emergence event has been observed with specific fluorescent antibodies to ascertain the identity of the newly generated cell. We have found that new HSPCs express c-kit, a reliable marker. In addition, c-kit is highly expressed on the surface of the HIACs, which facilitates visualization of these cells.

Note: Supplementary information is available via the HTML version of this article.

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AUTHOR CONTRIBUTIONS C.R. and C.A.-S. developed the nonfixed embryo slicing. C.R., J.-C.B. and W.A.v.C. developed the time-lapse confocal imaging procedure of nonfixed embryo slices/embryo caudal parts. C.R. and J.-C.B. developed and performed the described protocol. All authors wrote the paper. T.C. filmed and edited the three movies showing the experimental procedure.

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- Bertrand, J.Y. *et al.* Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* **464**, 108–111 (2010).
- Kissa, K. & Herbomel, P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* **464**, 112–115 (2010).

- Kissa, K. *et al.* Live imaging of emerging hematopoietic stem cells and early thymus colonization. *Blood* **111**, 1147–1156 (2008).
- Cumano, A. & Godin, I. Ontogeny of the hematopoietic system. *Annu. Rev. Immunol.* **25**, 745–785 (2007).
- Dzierzak, E. & Speck, N.A. Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nat. Immunol.* **9**, 129–136 (2008).
- Muller, A.M., Medvinsky, A., Strouboulis, J., Grosfeld, F. & Dzierzak, E. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1**, 291–301 (1994).
- Medvinsky, A. & Dzierzak, E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897–906 (1996).
- Dieterlen-Lievre, F., Pouget, C., Bollerot, K. & Jaffredo, T. Are intra-aortic hemopoietic cells derived from endothelial cells during ontogeny? *Trends Cardiovasc. Med.* **16**, 128–139 (2006).
- Gekas, C., Dieterlen-Lievre, F., Orkin, S.H. & Mikkola, H.K. The placenta is a niche for hematopoietic stem cells. *Dev. Cell* **8**, 365–375 (2005).
- Ottersbach, K. & Dzierzak, E. The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev. Cell* **8**, 377–387 (2005).
- Christensen, J.L., Wright, D.E., Wagers, A.J. & Weissman, I.L. Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biol.* **2**, E75 (2004).
- Jaffredo, T., Gautier, R., Eichmann, A. & Dieterlen-Lievre, F. Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development* **125**, 4575–4583 (1998).

13. Ma, X., Robin, C., Ottersbach, K. & Dzierzak, E. The Ly-6A (Sca-1) GFP transgene is expressed in all adult mouse hematopoietic stem cells. *Stem Cells* **20**, 514–521 (2002).
14. de Bruijn, M.F. *et al.* Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity* **16**, 673–683 (2002).
15. Zhang, J. *et al.* CD41-YFP mice allow *in vivo* labeling of megakaryocytic cells and reveal a subset of platelets hyperreactive to thrombin stimulation. *Exp. Hematol.* **35**, 490–499 (2007).
16. Emambokus, N.R. & Frampton, J. The glycoprotein IIb molecule is expressed on early murine hematopoietic progenitors and regulates their numbers in sites of hematopoiesis. *Immunity* **19**, 33–45 (2003).
17. Ferkowicz, M.J. *et al.* CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development* **130**, 4393–4403 (2003).
18. Matsubara, A. *et al.* Endomucin, a CD34-like sialomucin, marks hematopoietic stem cells throughout development. *J. Exp. Med.* **202**, 1483–1492 (2005).
19. Mikkola, H.K., Fujiwara, Y., Schlaeger, T.M., Traver, D. & Orkin, S.H. Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. *Blood* **101**, 508–516 (2003).
20. Robin, C., Ottersbach, K., Boisset, J.C., Oziemlak, A. & Dzierzak, E. CD41 is developmentally regulated and differentially expressed on mouse hematopoietic stem cells. *Blood* **117**, 5088–5091 (2011).
21. Boisset, J.C. *et al.* *In vivo* imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* **464**, 116–120 (2010).
22. Boisset, J.C. & Robin, C. Imaging the founder of adult hematopoiesis in the mouse embryo aorta. *Cell Cycle* **9**, 2487–2488 (2010).
23. Swiers, G., Speck, N.A. & de Bruijn, M.F. Visualizing blood cell emergence from aortic endothelium. *Cell Stem Cell* **6**, 289–290 (2010).
24. Lam, E.Y., Hall, C.J., Crosier, P.S., Crosier, K.E. & Flores, M.V. Live imaging of Runx1 expression in the dorsal aorta tracks the emergence of blood progenitors from endothelial cells. *Blood* **116**, 909–914 (2010).
25. Jones, E.A. *et al.* Dynamic *in vivo* imaging of postimplantation mammalian embryos using whole embryo culture. *Genesis* **34**, 228–235 (2002).
26. Yamanaka, Y., Tamplin, O.J., Beckers, A., Gossler, A. & Rossant, J. Live imaging and genetic analysis of mouse notochord formation reveals regional morphogenetic mechanisms. *Dev. Cell* **13**, 884–896 (2007).
27. Yamamoto, C. & McIlwain, H. Electrical activities in thin sections from the mammalian brain maintained in chemically-defined media *in vitro*. *J. Neurochem.* **13**, 1333–1343 (1966).
28. Oertner, T.G. Functional imaging of single synapses in brain slices. *Exp. Physiol.* **87**, 733–736 (2002).
29. Chvatal, A. *et al.* Three-dimensional confocal morphometry reveals structural changes in astrocyte morphology *in situ*. *J. Neurosci. Res.* **85**, 260–271 (2007).
30. Gahwiler, B.H., Capogna, M., Debanne, D., McKinney, R.A. & Thompson, S.M. Organotypic slice cultures: a technique has come of age. *Trends Neurosci.* **20**, 471–477 (1997).
31. Wang, X. *et al.* Asymmetric centrosome inheritance maintains neural progenitors in the neocortex. *Nature* **461**, 947–955 (2009).
32. Molyneaux, K.A., Stallock, J., Schaible, K. & Wylie, C. Time-lapse analysis of living mouse germ cell migration. *Dev. Biol.* **240**, 488–498 (2001).
33. Thevenaz, P., Ruttimann, U.E. & Unser, M. A pyramid approach to subpixel registration based on intensity. *IEEE Trans. Image Process* **7**, 27–41 (1998).

