

Live imaging and quantitative analysis of gastrulation in mouse embryos using light-sheet microscopy and 3D tracking tools

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This protocol describes how to observe gastrulation in living mouse embryos by using light-sheet microscopy and computational tools to analyze the resulting image data at the single-cell level. We describe a series of techniques needed to image the embryos under physiological conditions, including how to hold mouse embryos without agarose embedding, how to transfer embryos without air exposure and how to construct environmental chambers for live imaging by digital scanned light-sheet microscopy (DSLM). Computational tools include manual and semiautomatic tracking programs that are developed for analyzing the large 4D data sets acquired with this system. Note that this protocol does not include details of how to build the light-sheet microscope itself. Time-lapse imaging ends within 12 h, with subsequent tracking analysis requiring 3–6 d. Other than some mouse-handling skills, this protocol requires no advanced skills or knowledge. Light-sheet microscopes are becoming more widely available, and thus the techniques outlined in this paper should be helpful for investigating mouse embryogenesis.

INTRODUCTION

During mouse embryo gastrulation, epiblast cells traverse the primitive streak at the posterior end and then differentiate into mesodermal cells and migrate anteriorly. These movements are essential for subsequent embryonic morphogenesis, yet they are still not fully understood. To enable single-cell tracking in mouse embryos during gastrulation, the imaging system requires both a high imaging speed (to track single cells in dense tissue) and deep optical penetration (for imaging inside the cylindrical-shaped embryo, which has a diameter of 20–100 μm). Various techniques have attempted to overcome these issues, but they have been unsuccessful. Conventional wide-field fluorescence microscopes suffer from low contrast, whereas confocal fluorescence microscopy permits only superficial visualization of the embryo. Multiphoton microscopy allows imaging to a greater depth, but the temporal resolution is not sufficient for cell tracking^{1,2}.

Light-sheet fluorescence microscopy (LSFM), which uses planar illumination perpendicular to the detection axis, is suited for time-lapse imaging of developmental processes because it offers the advantages of high signal-to-noise ratio (S/N), high speed and deep tissue penetration^{3–7} compared with other wide-field, confocal and multiphoton microscopy (see **Table 1**, or ref. 8 or the online supplementary material of ref. 9 for a more detailed comparison of these microscopy methods). LSFM has been used to observe the development of zebrafish, *Drosophila* and *C. elegans*^{9–13}. Until our recent publication¹⁴, there were no reports in the literature of its application to mammalian embryos, presumably because of the difficulties of holding and culturing mammalian embryos in an LSFM instrument. Here we describe how to image gastrulation in living mouse embryos by using DSLM, a widely used type of LSFM, based on the methods used in our previous publication¹⁴. Techniques described include holding, transferring and culturing mouse embryos during gastrulation, as well as the

software tools we developed for analyzing single-cell migration from large 4D image data sets.

An overview of the protocol and an estimated time frame is shown in **Figure 1**. It is roughly divided into three parts: preparation, measurement and analysis. Preparation takes place up to 1 week before the experiment (microscope installation, equipment setup and mice mating) in addition to the day of the experiment itself (media preparation and microscope calibration). Measurement includes the techniques for handling mouse embryos, from dissection to live imaging in LSFM. Analysis includes pre-processing, two types of tracking methods and representing trajectories. Users can choose either manual or semiautomatic tracking methods, depending on data and requirements.

TABLE 1 | Comparison of fluorescence microscopies.

	DSLM ^a	Confocal ^a	Two-photon ^a
Lateral resolution ^b	r	$r/\sqrt{2}$	$r\sqrt{2}$
Deep tissue imaging ^c	++	+	++
Temporal resolution	63 Mvoxel/s	2–6 Mvoxel/s	1 Mvoxel/s
Illumination intensity ^b	E	$10^3 \cdot E$	$10^6 \cdot E$
Signal-to-noise ratio	1,000:1	60:1	10:1
Cost efficiency	≥50,000 €	>100,000 €	>200,000 €

Table modified from ref. 8.

^aIllumination wavelengths are different for two-photon versus DSLM or confocal. ^bRelative factors depend on the optical configuration and sample. ^cPlus signs (+) indicate the relative quality of axial resolution.

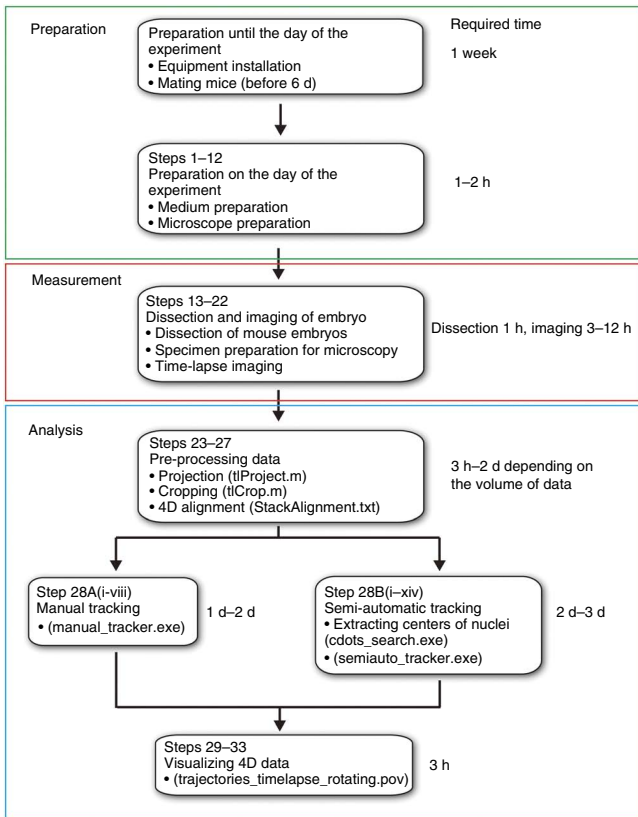


Figure 1 | Experimental flowchart of key steps describing the three main parts of the protocol. An approximate timeline is provided for each step.

Applications and limitations

This protocol breaks through the existing barriers to imaging whole mouse embryos during gastrulation. There are many possible applications of this methodology; for instance, direct observation of distal visceral endoderm migration may reveal the origin of anterior-posterior asymmetry^{15,16}, or these methods could reveal whether the nodal cells derive from definitive ectoderm^{17–19}. Imaging with DSLM may also reveal the origin and migration mechanism of primordial germ cells, which are thought to emerge during gastrulation^{20–22}, and it could be used to dissect the role of collective migration during gastrulation^{23,24}. This protocol may even be applicable beyond the study of gastrulation, e.g., in the study of the role of cancer genes in epithelial-mesenchymal transition, thereby assisting in the discovery and development of novel drugs²⁵.

The main limitation of the current protocol is the effect of phototoxicity on embryonic development. In our previous study using a fusion of histone 2B (H2B) to GFP, we showed that 3 h of laser light illumination did not adversely affect development¹⁴. However after 10 h, laser-illuminated embryos developed abnormally (compared with embryos in the same chamber that were not exposed to laser light and which typically developed normally). There are a number of parameters that potentially could be refined or adapted to increase the imaging window:

- **Excitation wavelength.** Fusions to mCherry allow excitation at longer wavelengths, which permits up to 12 h of illumination without the occurrence of developmental defects (Supplementary Fig. 1).

- **Light intensity.** The use of better cameras for fluorescence detection may reduce the required amount of laser light, thus protecting the sample from overexposure. For example, by using an Orca-AG (Hamamatsu) camera for fluorescence detection, ~23 mJ of illuminating laser light is required per stack in our DSLM. Higher-sensitivity cameras, such as an EM-CCD camera, would also allow lower laser light intensities to be used.

Although embryos acquire better resistance against phototoxicity after gastrulation (later than embryonic day (E)7.5), the spatial resolution is reduced in deeper regions of the embryo. The maximum depth at which adequate-quality images can be obtained is 140 μm at a 488-nm illumination wavelength¹⁴ and 400 μm at 568 nm (T.I. and S.N., unpublished data). Thus, it should be possible to observe whole mouse embryos until the late bud stage (E7.5), but not later stages, by using this protocol.

Experimental design

Immobilization of embryos. In most LSM setups, samples are typically immobilized by embedding them in agarose. Although this method does not affect the development, e.g., of *Drosophila* embryos²⁶, mouse embryos show abnormal development (T.I. and S.N., unpublished data). Therefore, we use a holder made from an acrylic resin rod (Fig. 2 and Supplementary Fig. 2), as described in our previous study¹⁴, which has a pocket with several holes of different diameters to hold the embryos. The embryos are held in place by the Reichert's membrane (Fig. 2); a portion of this membrane should be retained at the extraembryonic region when dissecting the embryo.

Embryo transfer. After the embryos are dissected in a Petri dish under a stereomicroscope, they must be transferred into the DSLM chamber without exposing the embryos to air; embryos are subject to deformation when exposed to air, and this deformation causes abnormal development. The system that we developed¹⁴ to achieve this is shown in Figure 3.

Temperature and atmosphere control. To culture isolated whole mouse embryos for up to 36 h (from postimplantation to gastrulation stage), strict culture conditions of 37 °C and 5% CO₂ and 5% O₂ must be maintained²⁷. To achieve this, we developed a custom-made temperature-regulated specimen chamber with an atmospheric control system (Fig. 4 and Equipment Setup).

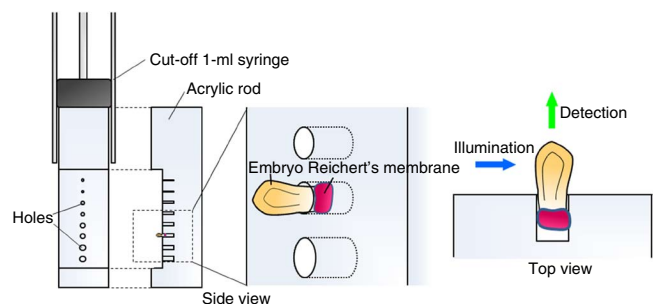


Figure 2 | Schematic of the method used to hold mouse embryos for DSLM imaging. The embryos are placed into the holes of an acrylic rod attached to the piston of a tip-truncated 1-ml syringe. The embryo is held stably in the hole via the Reichert's membrane at the extraembryonic region. Top and side views are shown.

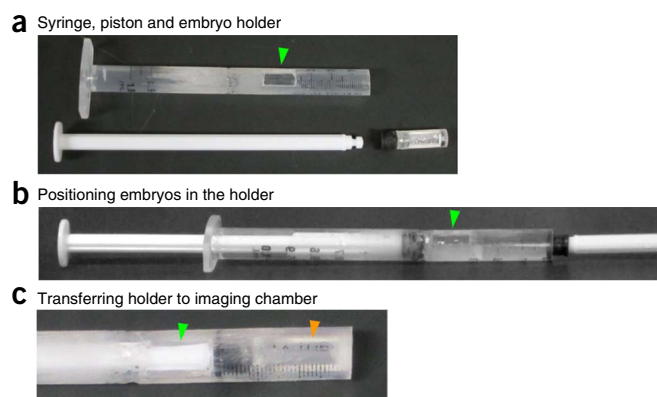


Figure 3 | Embryo transfer method. (a) For transferring embryos from the dish to the chamber of the DSLM, a cut-off 1-ml syringe with a window (indicated by green arrowhead) is used. (b) Embryos are moved to the pocket of the holder filled with culture medium under a wide-field microscope. (c) After placing the embryo in the holder, the holder is positioned to the end of the syringe, such that it is located above the window (the position of the holder is indicated by orange arrowhead). At this position, the holder can be transported to the microscope without exposing the embryo to air. The holder is inserted into the culture medium in the DSLM imaging chamber.

To create a 5% CO₂ and 5% O₂ atmosphere, we used a gas mixer produced by Tokai Hit, but other commercially available gas mixers can be used (minimum flow under 100 ml/min is desirable). The gas inlet was fabricated from an acrylic plate by using a milling machine, as detailed in the design drawing in **Supplementary Figure 3**. For temperature control, we custom-ordered a specific chamber fitted with a water-cooled Peltier device. Instead of the Peltier device, a chamber embedded with a heating foil could be used to keep the temperature at 37 °C (ref. 9).

Handling 4D image data sets. The amount of data acquired with DSLM is on the order of 100–300 GB (1,344 pixel × 1,024 pixel

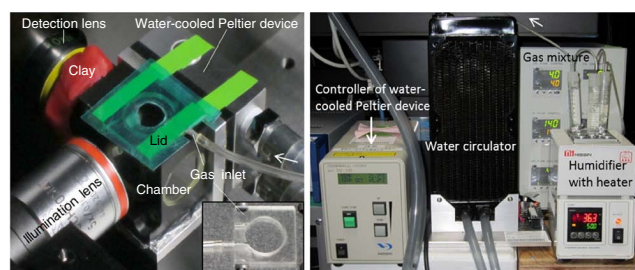


Figure 4 | Photograph of the assembled system around the chamber (left) and humidifier, gas mixture and temperature controller (right). The gas is mixed by using a gas mixer and injected at the top of the chamber through the gas inlet and humidifier with heater (white arrows). To prevent evaporation of the culture medium, a lid is placed on the inlet. Inserted figure at the lower right of the left panel indicates the gas inlet.

images, 16-bit image depth, 100 planes per stack, every 5 min, for a total of 10 h) per experiment. To eliminate lengthy electronic file transfer, we recommend using internal hard disks in removable cases (Century). After data acquisition, the hard disk can be easily removed from the acquisition computer and physically transferred to the processing computer for data analysis.

Analytical tools. As a key step of the data analysis, each individual nucleus must be tracked over time. We tested several commercial and noncommercial software packages, but none of them were suitable for the analysis of our data sets; either they could not handle the large amount of 4D image data or they did not properly distinguish between neighboring nuclei. To address this issue, we developed new software¹⁴, which are provided here as **Supplementary Data 1** and **2** (see **Table 2** for a list of software provided in **Supplementary Data 1**; source code is provided in **Supplementary Data 2**). We developed two types of tracking tools: a semiautomatic tracking tool for mesoderm and ectoderm

TABLE 2 | Description of the customized programs contained in **Supplementary Data 1**.

Name of program	Platform	Description
tProjection.m	MATLAB	Make maximum projection at each time point
tCrop.m	MATLAB	Crop images according to the indicated ROI
StackAlignment.txt	Macro of ImageJ	Align images four-dimensionally. This macro needs plug-ins, 'StackReg', 'MaltStackReg' and 'TransformJ'
manual_tracker.exe	C++ and C#	Support four-dimensional manual tracking (Fig. 5)
cdots_search.exe	C++ and C#	Extract nuclear centers for semi-automatic tracking. This program requires 'ichi_srcx.exe' in the same folder (Fig. 6)
ichi_srcx.exe	C++	Required program for processing 'cdots_search.exe'
semiauto_tracer.exe	C++ and C#	Track indicated centers of nuclei-extracted 'cdots_search.exe' (Fig. 7). This program requires both 'cc_trace.exe' and 'cc_reduce.exe'
cc_trace.exe	C++	Required program for processing 'semiauto_tracer.exe'
cc_reduce.exe	C++	Required program for processing 'semiauto_tracer.exe'
trajectories_timelapse_rotating.pov	POV-Ray	Represent tracked trajectories

nuclei (semi-auto_tracer.exe) and a manually assisted tracking tool for epiblast nuclei (manual_tracker.exe), which tend to be very crowded and therefore difficult to track automatically.

As both manual and semiautomatic tracking tools require users to verify the tracking, it is guaranteed that no trace will show critical errors.

MATERIALS

REAGENTS

- Male nuclear-labeled transgenic mice, 2–12 months old (e.g., *H2B-GFP*, *H2B-mCherry*)²⁸ **! CAUTION** Please note that all experiments should be conducted in accordance with relevant ethics guidelines and regulations.
- Female ICR mice, 6–8 weeks of age (Japan SLC)
- Phenol red–free DMEM (Invitrogen, cat. no. 21063-029)
- FBS (Invitrogen, cat. no. 10438-026)
- Rat serum (prepared from retired male rats of Japan SLC)
- Trolox (Cayman, cat. no. 53188-07-1)
- Penicillin-streptomycin (Invitrogen, cat. no. 15070-063)

EQUIPMENT

- DSLM instrument (see Equipment Setup)
- Petri dishes, 35 × 10 mm (BD Falcon, cat. no. 351008)
- Micro-forceps, no. 5 (Fontax, cat. no. 7-162-33) **▲ CRITICAL** Sharpen the tip of the forceps with sandpaper; use grade no. 1,000 for sharpening and grade no. 6,000 for polishing.
- Syringe, 1 ml (Terumo, cat. no. SS-01T)
- Acryl rod, 5-mm diameter (**Supplementary Fig. 2**)
- Gas inlet made from acryl plate, 25 × 25 × 2 mm (**Supplementary Fig. 3**)
- Plastic lid with a 5-mm-diameter hole, 25 × 25 × 0.2 mm
- Cyanoacrylate adhesive (Toagosei, cat. no. 30115)
- Half-cut 15-ml tube (BD Falcon, cat. no. 352196)
- Tapered glass capillary connected to a silicone tube with mouthpiece
- Color tape (Asone, cat. no. 6-691-01)
- Sterile 20-ml syringe (Terumo, cat. no. SS-20ESZ)
- Sterile syringe filter, 0.2 μm (Corning, cat. no. 431218)
- CO₂ incubator, 37 °C (Sanyo, cat. no. MCO-5ACUV)
- Thermotolerant clay (Pelikan, Nakiplast)
- Kimwipes (Crecia, cat. no. 62011)
- Distilled water
- Tungsten needles (Equipment Setup)
- ImageJ (<http://rsbweb.nih.gov/ij/>) with StackReg²⁹ (<http://bigwww.epfl.ch/thevenaz/stackreg/>), MultiStackReg³⁰ (<http://bradbusse.net/downloads.html>) and TransformJ³¹ (<http://www.imagescience.org/meijering/software/transformj/>) plug-ins
- MATLAB R2012a (The MathWorks)
- POV-Ray (<http://www.povray.org/>)

REAGENT SETUP

Dissection medium 1 Combine 10% (vol/vol) FBS, 1% (vol/vol) Trolox and 1% (vol/vol) penicillin-streptomycin in DMEM. This medium is used for dissecting the uterus. For a total of 3 ml, use 2.64 ml of DMEM, 0.3 ml of FBS, 0.03 ml of Trolox and 0.03 ml of penicillin-streptomycin.

▲ CRITICAL Freshly make the medium on the day of the experiment.

Dissection medium 2 Combine 10% (vol/vol) FBS, 1% (vol/vol) Trolox, 1% (vol/vol) penicillin-streptomycin and 50% (vol/vol) serum in DMEM. This medium is used for dissecting decidua. For a total of 3 ml, use 1.14 ml of DMEM, 0.3 ml of FBS, 0.03 ml of Trolox, 0.03 ml of penicillin-streptomycin and 1.5 ml of serum. **▲ CRITICAL** Freshly make the medium on the day of the experiment. **▲ CRITICAL** Dissection medium 2, control medium, transfer medium and culture medium have the same composition, and thus it is helpful to prepare these at the same time.

Control medium Mix 10% (vol/vol) FBS, 1% (vol/vol) Trolox, 1% (vol/vol) penicillin-streptomycin and 50% (vol/vol) serum in DMEM; this medium is used for embryos that are not used for the observation. For a total of 3 ml, use 1.14 ml of DMEM, 0.3 ml of FBS, 0.03 ml of Trolox, 0.03 ml of penicillin-streptomycin and 1.5 ml of serum.

▲ CRITICAL Freshly make the medium on the day of the experiment.

▲ CRITICAL Dissection medium 2, control medium, transfer medium and culture medium have the same composition, and thus it is helpful to prepare these at the same time.

Transfer medium Mix 10% (vol/vol) FBS, 1% (vol/vol) Trolox, 1% (vol/vol) penicillin-streptomycin and 50% (vol/vol) serum in DMEM; this medium is used for transferring embryos from the stereoscope to the DSLM chamber.

For a total of 3 ml, use 1.14 ml of DMEM, 0.3 ml of FBS, 0.03 ml of Trolox, 0.03 ml of penicillin-streptomycin and 1.5 ml of serum. **▲ CRITICAL** Freshly make the medium on the day of the experiment. **▲ CRITICAL** Dissection medium 2, control medium, transfer medium and culture medium have the same composition, and thus it is helpful to prepare these at the same time.

Washing medium Washing medium contains 10% (vol/vol) FBS, 1% (vol/vol) Trolox and 1% (vol/vol) penicillin-streptomycin in DMEM; this medium is used for washing inside the chamber before filling the culture medium. For a total of 10 ml, use 8.8 ml of DMEM, 1 ml of FBS, 1 ml of Trolox and 1 ml of penicillin-streptomycin. **▲ CRITICAL** Freshly make the medium on the day of the experiment.

Culture medium Culture medium contains 10% (vol/vol) FBS, 1% (vol/vol) Trolox, 1% (vol/vol) penicillin-streptomycin and 50% (vol/vol) serum in DMEM; this medium is used for observing embryos. For a total of 10 ml, use 3.8 ml of DMEM, 1 ml of FBS, 1 ml of Trolox, 1 ml of penicillin-streptomycin and 5 ml of serum. **▲ CRITICAL** Freshly make the medium on the day of the experiment. **▲ CRITICAL** Dissection medium 2, control medium, transfer medium and culture medium have the same composition, and thus it is helpful to prepare these at the same time.

EQUIPMENT SETUP

Light-sheet microscopy We used a DSLM instrument as previously reported¹⁴ (**Supplementary Fig. 4**). LSFM instruments generally have relatively similar sample chambers, and thus our protocol is also applicable to other LSFM implementations. In our case, an argon-krypton laser (Melles Griot, cat. no. 35 LTL 835-200) was used as the light source. The wavelength of the laser beam (488 nm or 568 nm) was selected by using an acousto-optical tunable filter (AA Opto-Electronic, cat. no. AA.AOTF.nC-400-650nm-PV-TN) and scanned through the sample by using a two-axis high-speed galvanometer scanner (GSI Lumonics VM500+). The scanned light sheet was created with an f-theta lens (Sill Optics, cat. no. S4LFT0061/065) and a low-numerical-aperture (NA) illumination objective (Carl Zeiss, Plan-Apochromat ×5/0.16). Fluorescence emitted by the specimen was detected by using a water-immersion detection objective (Carl Zeiss, Achromat ×20/0.5 or ×10/0.3) and recorded with a CCD camera (Hamamatsu, Orca AG) through a long-pass filter (Semrock, RazorEdge RU 488 or 568). The image data were recorded with our custom DSLM control software, which was developed in the Microsoft .NET framework⁹. The sample stage comprises a set of three linear translation stages (Physik Instrumente, M-111K028) and one micro-rotation stage (Physik Instrumente, M-116.DG). The data were acquired with an acquisition computer (3.0 GHz dual-core processor, 4 GB RAM) fitted with removable cases for internal hard-disk drives (Century). For more detail, refer to the supplementary material of ref. 9 and to ref. 32. Two additional resources for constructing light-sheet microscopes are the OpenSPIM wiki (<http://openspim.org/>) and an open-spin microscopy³³ Google site (<https://sites.google.com/site/openspinmicroscopy/>).

Embryo holder The embryo holder is made from a 5-mm-diameter acrylic rod, as detailed in **Supplementary Figure 2**, by using a mini lathe and a micro-milling machine. The holder is glued to the tip of a 1-ml syringe piston with cyanoacrylate adhesive. A 1-ml syringe is cut off at the tip and a 3 × 9 mm window (same size as the bottom face of the embryo holder pocket) is cut out at a distance of 10 mm from the tip (**Fig. 3**). This window is used for transferring embryos from the dissection dish to the holder.

These holder systems can be reused after washing and sonication for 10 min.

Temperature and atmosphere control The chamber uses a water-cooled Peltier device–mounted chamber (Hayashi Watch-Works) for temperature control. A 5% O₂ and 5% CO₂ gas mixture is made with a gas mixer (Tokai Hit,

cat. no. GM-6000), and it is injected via a gas inlet into the specimen chamber. Mixed gas is humidified by using 37 °C water before injection. The gas inlet is made from a 2-mm-thick acrylic plate, as detailed in **Supplementary Figure 3**. A stainless steel pipe (1.2-mm internal diameter, 5 mm length) is attached to one channel of the adaptor and the silicone rubber tube from the gas mixer is connected to this pipe (**Fig. 4**). **Tungsten needles** Tungsten needles are made according to ref. 34. Briefly, a tungsten wire is cut into 40–50-mm sections. One side of the tungsten wire

is tapered by using an electric transformer in 1 N NaOH. Finally, the tapered tungsten piece is stuck into the tip of a wooden chopstick.

Software preparation Manual and semiautomatic tracking programs have been tested only on 64-bit Windows XP and Windows 7 computers. Execution of the semiautomatic tracking program requires installation of 'Microsoft Visual C++ 2010 Redistributable Package', 'Microsoft .NET Framework 4' and 'OpenCV version 2.4.0'. The path to the directory of OpenCV needs to be set in the operating system's environmental variables.

PROCEDURE

Medium preparation ● TIMING 30–40 min

- 1| Prepare the media described in the Reagent Setup section. Pour dissection medium 1, dissection medium 2 and control medium into 35-mm Petri dishes and label them. Place all media into a 5% CO₂ at 37 °C incubator for at least 10 min before use.
- 2| Assemble the embryo holder except for the plugged piston at the end of the syringe in **Figure 3b**. Suck the transfer medium with a piston in a half-cut 15-ml tube filled with medium. Then, plug the end of syringe with another piston from the opposite direction.
- 3| Remove the bubbles in the holes of the holder by using a tapered glass capillary connected to a silicone tube with a mouthpiece.
▲ **CRITICAL STEP** If bubbles are not removed, they will swell in the warmed chamber and disrupt images during observation.
- 4| Place the assembled holder in a 5% CO₂ incubator at 37 °C for at least 10 min before use.

Microscope calibration ● TIMING 30 min

- 5| Turn on all devices.
- 6| Seal the interface between the chamber and detection lens with thermotolerant clay if you are using a water-immersion lens (**Fig. 4**), and fill the chamber with distilled water.
▲ **CRITICAL STEP** Confirm that the water does not leak from the chamber. If a leak is found, disassemble and reassemble the setup.
- 7| Tape the gas inlet to the top of the chamber and place a lid on the gas inlet (**Fig. 4**).
- 8| Attach the stage-positioning system to the imaging chamber and insert a mirror for calibration. Next, adjust the light-sheet position and the focal plane of the detection system to be co-planar: First, tilt the mirror such that it is located at a 45-degree angle to both the illumination source and the detection axis, and so that it reflects the light sheet directly onto the camera. Adjust the position of the detection lens to focus on the light reflected off the mirror surface, and then adjust the position of the illumination lens such that the thinnest section of the light sheet is located in the center of the field of view.

? TROUBLESHOOTING

- 9| Remove the mirror and stage-positioning system. Remove the water in the chamber. Add ~9 ml of washing medium by using a 20-ml syringe with a 0.2-μm syringe filter attached.
- 10| Pipet the medium a few times to thoroughly wash the chamber. Leave the medium in the chamber for ~10 min.
- 11| Remove the medium and replace it with fresh culture medium by using a 20-ml syringe with a 0.2-μm syringe filter attached.
- 12| Reattach the stage-positioning system.

Dissection and imaging ● TIMING dissection 1 h, imaging 3–12 h

▲ **CRITICAL** Before starting embryo dissection, check that: all media are located in the incubator, the DSLM instrument is ready to use, 5% CO₂ and 5% O₂ are provided, the chamber temperature has stabilized at 37 °C, the chamber is loaded with

culture medium and the embryo holder is assembled. Any delay after beginning dissection could be detrimental to the health of the embryo.

13| Euthanize an E6.5-pregnant mouse. Remove the uterus and place it on a Kimwipe to remove blood. Then transfer it to dissection medium 1 and dissect the uterus. For a detailed dissection method, refer to refs. 34,35.

! CAUTION Please note that all experiments should be conducted in accordance with relevant ethics guidelines and regulations.

14| Move the decidua to dissection medium 2 with a tip-truncated pipette, and then dissect them.

▲ CRITICAL STEP Leave some Reichert's membrane at the extracellular region. It is required for proper adhesion of the embryos to the specimen holder.

? TROUBLESHOOTING

15| Put the sample holder under the stereomicroscope (**Fig. 3b**). Fix the holder's position with clay so that it does not rotate.

16| Transfer the embryos from the dish to the pocket of the holder through the window of the syringe by using a tip-truncated pipette.

17| Under a stereomicroscope, set the embryos into the holes by using tungsten needles (a detailed method is described in ref. 36).

18| Remove the piston that plugs the end of the syringe. Push the other piston (with the holder attached) until the bottom of the holder reaches the end of the syringe.

▲ CRITICAL STEP To prevent deformation and abnormal embryo development, be careful to avoid getting air in the pocket of the holder.

? TROUBLESHOOTING

19| Transfer the embryo holder to the microscope stage and push the holder into the medium in the chamber.

20| Rotate the embryo holder to face the detection lens.

21| Adjust the focus of the detection lens while viewing the fluorescence of the embryo.

▲ CRITICAL STEP This step is needed even though the foci of both the illumination and the detection lenses were adjusted in Step 8. The optimal alignment typically changes slightly once the specimen is inserted in the imaging chamber.

22| Set up time-lapse recording in the microscope control software and start imaging. We usually set a 300-ms exposure time, 100–150 planes per stack with 2.58 μm of z space and a 3-min time interval.

? TROUBLESHOOTING

Data pre-processing ● **TIMING 3 h–2 d, depending on the volume of data**

23| Transfer the data disk from the acquisition computer to the processing computer.

24| Generate maximum projections at each time point by using the MATLAB script 'tlProject.m' in **Supplementary Data 1**. Adjust the file name, directory, and so on and run the script.

25| Crop the images with 'tlCrop.m' (**Supplementary Data 1**) to the minimum required volume according to the maximum-intensity projections generated in Step 24.

▲ CRITICAL STEP The processing time of alignment and tracking depends on the size of the data set. It may also be helpful to reduce the number of data points.

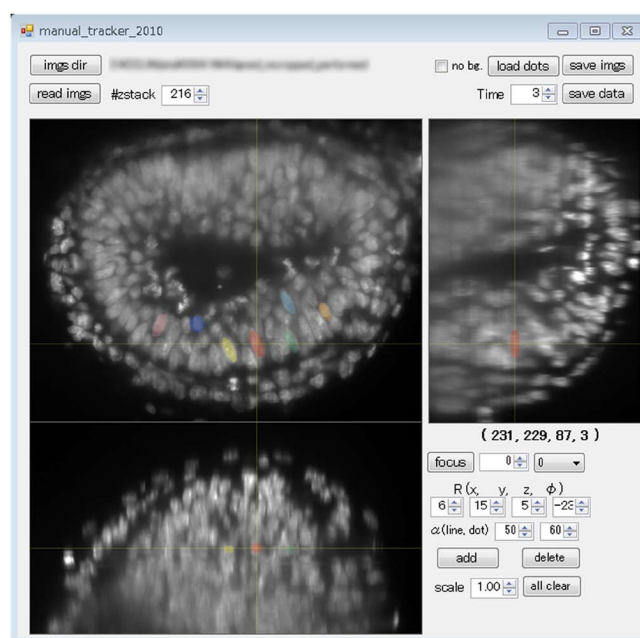
26| If images drift with time, align the images with the ImageJ macro, 'StackAlignment.txt' in **Supplementary Data 1**.

▲ CRITICAL STEP This macro requires the ImageJ plug-ins StackReg²⁹, MultiStackReg³⁰ and TransformJ³¹.

? TROUBLESHOOTING

27| Repeat Steps 24 and 25 to generate maximum-intensity projections for the aligned data set and crop again.

Figure 5 | Graphical user interface of the manual tracking program. This program enables manual tracking of 4D data. The centers of the xy , yz and xz sections are locked. In order to add a tracking marker, click on the 'add' button and then right-click. The color of the marker is adjusted automatically. The shape and transparency of the marker can be changed with ' $R(x, y, z, \phi)$ ' and ' α ' button.



Tracking

28 | Follow option A for manual tracking, or use option B for semiautomatic tracking.

(A) Manual tracking ● TIMING 1–2 d

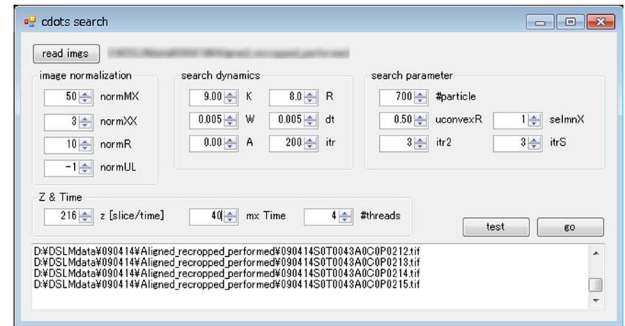
- (i) Start 'manual_tracker.exe' (**Supplementary Data 1**).
▲ CRITICAL STEP If the data format differs from the TIFF format, convert the data file to TIFF format (using ImageJ or other software) before initiating the manual tracking program. The manual tracking program supports only single-channel data.
- (ii) Set the number of files per stack at '#zstack' and indicate the folder containing the TIFF files after clicking on the 'imgs dir' button. Next, load the data by clicking on the 'read imgs' button (**Fig. 5**).
- (iii) After the 'read images' (Step 28A(ii)), the 'xy', 'yz' and 'xz' panels are locked to the mouse position. Left-clicking switches the mode from 'images locked to mouse position' to 'static images'. Click on the 'add' button and decide on the position with a right click. The size, angle and transparency of the marker can be changed by using the ' $R(x, y, z, \phi)$ ' or ' α ' panel.
- (iv) Press 'x' to proceed to the next time point. If you need to go back, press 'z'. Rotating the scroll wheel of the computer mouse shifts the z coordinate. Next, indicate the position with a right-click again.
- (v) When the trace ends, press 'add' and start the next track.
- (vi) To save data, press the 'save' button and indicate the desired location for the file. A color information text file 'cols.txt' is automatically created in the selected folder as well. The columns in the data file are as follows (from left to right): track number, time point, x coordinate, y coordinate, slice number (z coordinate), x length of the marker, y length of the marker, z length of the marker and tilt in the xy plane of the marker.
- (vii) To save images with markers, click on 'save imgs' button. The folder, including images with markers, is created in the input directory. If you want to save only markers (without images), check 'no bg'.
- (viii) To load previously saved marker positions, click on 'load dots' and then select the saved data. The file 'cols.txt' is required to be in the same folder.

(B) Semiautomatic tracking ● TIMING 2–3 d

- (i) For semiautomatic tracking, the centers of nuclei need to be extracted (Step 28B(i–iv)) before starting the tracking program (Step 28B(v–xiv)). Run the program 'cdots_search.exe'. This program also requires 'ichi_srcx.exe' to be in the same folder. These programs are included in **Supplementary Data 1**.
 - (ii) Click on the 'read imgs' button and select the folder containing TIFF or PNG image data (**Fig. 6**).
 - (iii) Set the slice number per stack, the number of time points and the parallel thread number of the computer in 'Z & Time'. To reduce processing time, the 'itr*' parameters (which indicate the number of iterations) can be reduced. For all other parameters, the default setting is usually appropriate.
 - (iv) Click on the 'go' button to start. Two folders, 'cdots' containing position files and 'imgs' containing overlay images with extracted nuclei positions, are created in the input folder. Note that command prompt windows are opened during processing. It takes ~2–4 h to process 1 GB of TIFF data.
▲ CRITICAL STEP If extraction fails, use the 'test' button for diagnosis. This mode shows the overlaid image with temporary centers during processing. 'Test' mode does not output results. To exit this mode, close all command-prompt windows.
 - (v) Run 'semiauto_tracer.exe'. This program requires both 'cc_trace.exe' and 'cc_reduce.exe' to be in the same folder.
- ? TROUBLESHOOTING**
- (vi) Click on the 'imgs dir' button and select the 'imgs' folder with the PNG images containing the dots created in the pre-processing steps (Step 28B(i–iv)).
 - (vii) Set the number of slices per stack at '#zstack' (**Fig. 7**). In order to reduce memory usage while handling large amounts of data, set the range of displayed images with '#tstack'. Set the desired number of time points. The tracking process is

PROTOCOL

Figure 6 | Graphical user interface of the program for nuclei center extraction before semiautomatic tracking. This program extracts the centers of nuclei for semiautomatic tracking. Open image data by clicking on the 'read imgs' button. Enter the number of slices per stack, time point and parallel processing threads. Click on 'go' to start.



performed for the time range in the input folder. Thus, even if only a subset of time points were read, a user can move beyond the initial time segment, by using the 'read fw' or 'read bw' buttons.

- (viii) Click on the 'read imgs' button to start reading the data. This step usually takes 3–12 min.
- (ix) Indicate the positions of the nuclei at the first time point with a right-click, and then choose a unique color to mark each individual nucleus. Left-clicking switches the mode from 'images locked to mouse position' to 'static images'. After selecting the initial positions, press 'init_opt' to start the detection of nuclei positions. The detection range can be changed with '(R r)', where R means z length and r means the radius in the xy plane.
- (x) After finishing detection at the first time point, start semiautomatic tracking with the 'trace' button. If 'reverse' is checked, the program tracks backward in time.
- (xi) After finishing the tracking, press the 'fix_overlap' button. This deletes overlapping tracks.
- (xii) Check all tracks manually. Focus on one track by using the pull-down menu, and confirm the markers at all time points. Use the 'x' and 'z' buttons to change the current time point. If a mistake is found, use the 'delete' button to delete the wrong data point at the first time point at which the mistake is visible.
- (xiii) After deleting all incorrect tracks, indicate the correct position at the first time point of the deleted tracks and click on 'init_opt' again. The algorithm may have multiple options for a new track. First, browse the candidates with 'nn_search' button. Then, select the correct track and connect to the previous segment by using the 'list connec' button. **▲ CRITICAL STEP** The trajectory data are automatically saved when the 'init_opt' button is pressed. The data will be in four '.dat' files and one '.log' file in the input folder. 'ttd.dat' records the center position of tracked traces. When you are updating saved data, click on the 'save data' button.
- (xiv) To save images, click on 'save imgs'. A 'pics' folder, including images with overlaid markers, is then created in the input directory. If only marker data are needed, check 'bg. off'.

Rendering 4D mouse embryo data with POV-Ray ● TIMING 3 h

29 | Download and install POV-Ray from <http://www.povray.org/>. POV-Ray is a free 3D-rendering software (full download and installation instructions are on the website).

30 | Generate 'ttd.pov.dat' from 'ttd.dat' and 'cols.pov.dat' from 'cols.dat' of the tracking results from Step 28A(vi) or 28B(xiii). Replace the spaces with commas. Make sure not to put a comma at the end of the file.

31 | Run 'trajectories_timelapse_rotating.pov'. Next, indicate the location of 'ini' file with the 'Ini' button. Write the number of rows of 'ttd.pov.dat' at 'TTTN' in line 100 and the number of rows of 'cols.pov.dat' at 'TRACKN' in line 101.

32 | Start by clicking on the 'Run' button.

33 | To change the viewpoint, manipulate the camera and light settings. Make sure that the simulated embryo boundary is matched to the collected data set by adjusting the script according to your particular data.

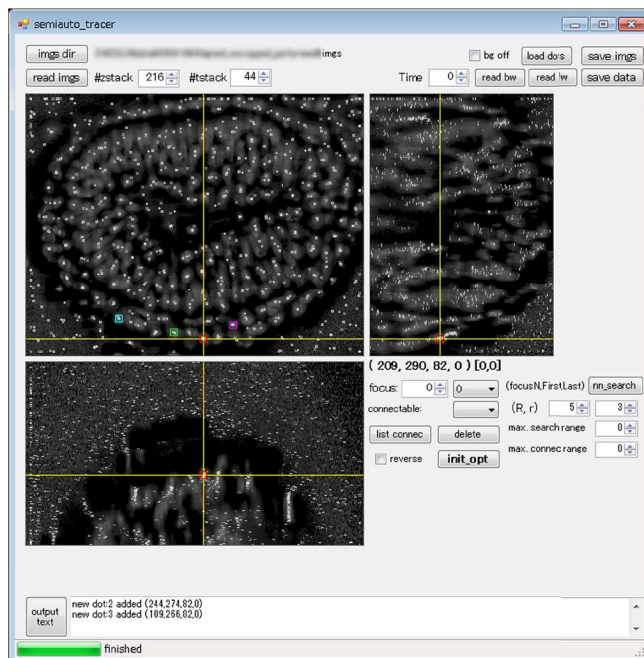


Figure 7 | Graphical user interface of the semiautomatic tracking program. This program automatically tracks the extracted nuclei centers. Select the positions at the initial time point and then start tracking. Check tracks manually and delete the wrong tracks from the first time point that an error occurs. Then retrace and connect to the previous segment.

? TROUBLESHOOTING

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

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
8	Laser light is missing	The mirror is not set at 45° correctly Observing different position	Reset the mirror Find the brighter direction of light by increasing illumination intensity
14	Embryo is injured during the peeling of Reichert's membrane	The tip of the forceps is not sharp enough	Sharpen the tip of the forceps using sandpaper no. 1,000 (for sharpening) and no. 6,000 (for polishing)
18	The pocket of the embryo holder includes air when the holder is pushed to the end of syringe	The volume of medium is short or the holder is not tilted	Add transfer medium before pushing the piston and tilt the end of the syringe up while pushing the piston
22	Image is defocused after 1 or 2 h of observation	Detection lens has drifted	Readjust the focus of the detection lens periodically
	Level of culture medium is markedly reduced	Mixed gas is not adequately humidified	Check whether the humidifier has enough water
26	The macro does not work	Required plug-ins are not installed	Install StackReg, MaltiStackReg, and TransformJ. StackReg needs TurboReg and TransformJ needs imagescience.jar
	The macro does not work	Incorrect input information	Be sure to use a double backslash to indicate separating directory
28B(v)	The program does not start	Required software is not installed	Install 'Microsoft Visual C++ 2010 Redistributable Package', 'Microsoft .NET Framework 4' and 'OpenCV version 2.4.0'. The path to the directory of OpenCV is needed to be set in the environmental variables of the operating system

● TIMING

Steps 1–4, medium preparation: 30–40 min

Steps 5–12, microscope calibration: 30 min

Steps 13–22, dissection and imaging: dissection 1 h, imaging 3–12 h

Steps 23–27, data pre-processing: 3 h–2 d, depending on the volume of data

Step 28A, manual tracking: 1–2 d

Step 28B, semiautomatic tracking: 2–3 d

Steps 29–33, rendering 4D data of mouse embryo with POV-Ray: 3 h

ANTICIPATED RESULTS

The protocol described here enables the observation of entire living mouse embryos during gastrulation and the analysis of single-cell movements from 4D image data. An example of an imaging result is shown in **Figure 8**, which shows optical

sections of H2B-GFP mouse embryos at E6.5. The upper left image shows a section at 78 μ m from the distal end, and the upper right image shows the same image with annotation.

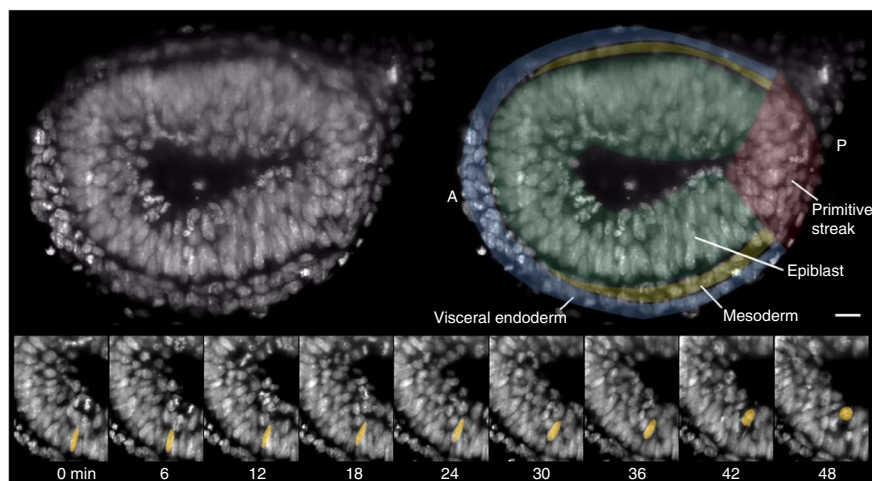
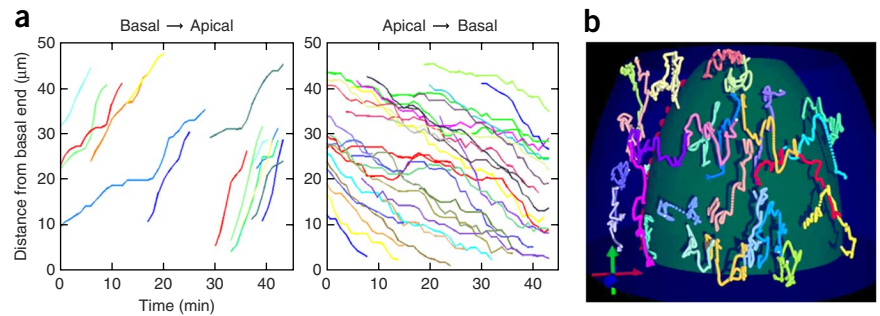


Figure 8 | Example of live imaging of whole mouse embryos at embryonic day 6.5. Top left image shows an optical slice 78 μ m from the distal end of the embryo. Top right image shows the annotated slice. Blue, visceral endoderm; green, epiblast; yellow, mesoderm; red, primitive streak. Anterior (A) is to the left, posterior (P) to the right. Scale bar, 20 μ m. Bottom images show a time series of interkinetic nuclear migration in the epiblast. Adapted from ref. 14.

Figure 9 | Example of tracking analyses for epiblast and mesodermal nuclei. (a) Kymographs of apical and basal migration of epiblast nuclei. (b) Computationally reconstructed trajectories of mesodermal nuclei. The embryo consists of two surfaces: the outer visceral endoderm (blue) and a boundary surface between the visceral endoderm and mesoderm (green). A white marker at the end of each trajectory indicates the last point. Red, blue and green arrows at the lower left indicate the anterior-posterior axis, right-left axis and proximal-distal axis, respectively. Adapted from ref. 14.



The bottom images show a time-lapse sequence of interkinetic nuclear migration in epiblast. Other sections of nuclear- and membrane-stained embryos at E5.5, 6.5 and 7.5 are shown in **Supplementary Figure 5** (time-lapse movie at E6.5: **Supplementary Video 1**). Results of analysis are shown in **Figure 9**. **Figure 9a** shows the kymographs of nuclear migration in the epiblast and **Figure 9b** shows the trajectories of mesodermal cells. These results indicate that mesodermal cells migrate not collectively but individually, which was unknown before the application of the methods outlined in this protocol.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS T.I. developed most of the protocols described here and performed the experiments with the help of H.K.-K. under the supervision of S.N. K.N. developed the analysis tools under the supervision of A.M. P.J.K. and E.H.K.S. developed DSLM. T.I. and P.J.K. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Erratum: Live imaging and quantitative analysis of gastrulation in mouse embryos using light-sheet microscopy and 3D tracking tools

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In the version of this article initially published, Table 1 contained a number of errors: lateral resolution for two-photon was erroneously stated as ' r divided by the square root of 2' instead of ' r multiplied by the square root of 2'; the illumination intensity for two-photon microscopy was given as ' 10^3 times E ' instead of ' 10^6 times E '; 'lateral resolution' was assigned the incorrect footnote; and reference 11 was incorrectly cited as a source for the table. An additional footnote (a) has also been added to the version that was originally published. These errors have been corrected in the HTML and PDF versions of the article.