Live Imaging of Whole Mouse Embryos during Gastrulation: Migration Analyses of Epiblast and Mesodermal Cells

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Abstract

During gastrulation in the mouse embryo, dynamic cell movements including epiblast invagination and mesodermal layer expansion lead to the establishment of the three-layered body plan. The precise details of these movements, however, are sometimes elusive, because of the limitations in live imaging. To overcome this problem, we developed techniques to enable observation of living mouse embryos with digital scanned light sheet microscope (DSLM). The achieved deep and high time-resolution images of GFP-expressing nuclei and following 3D tracking analysis revealed the following findings: (i) Interkinetic nuclear migration (INM) occurs in the epiblast at embryonic day (E)6 and 6.5. (ii) INM-like migration occurs in the E5.5 embryo, when the epiblast is a monolayer and not yet pseudostratified. (iii) Primary driving force for INM at E6.5 is not pressure from neighboring nuclei. (iv) Mesodermal cells migrate not as a sheet but as individual cells without coordination.

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Introduction

Establishment of the three germ layers during gastrulation occurs via a highly orchestrated set of morphogenetic events that shape the early embryo [1–5]. In mouse embryos, this process begins as epiblast cells traverse the primitive streak at the posterior end. These cells then differentiate into mesodermal cells and migrate anteriorly. Although these events are essential for subsequent embryonic morphogenesis, live imaging of these processes has been strictly limited to a part of the embryo [6,7].

Live imaging analysis of whole mouse embryos during gastrulation requires deep optical penetration of the specimen and high time resolution. Conventional wide-field fluorescence microscopes suffer from low contrast, and confocal fluorescence microscopy permits only superficial visualization of the embryo. Multi-photon microscopy allows imaging to a greater depth, but the temporal resolution is not sufficient for cell tracking. In order to overcome these limitations, we used digital scanned light-sheet microscopy (DSLM) [8,9], a type of light sheet-based fluorescence microscopy (LSFM) that uses planar illumination perpendicular to the detection axis (Figure S1). This method offers the advantages of a high signal-to-noise ratio (S/N), high speed, and good optical penetration [10,11]. In this study, we used DSLM in combination with a mouse embryo culture system to perform time-lapse imaging of whole mouse embryos during gastrulation. We also developed software tools for tracking of the nuclei, and report cell migration properties of the epiblast and the mesodermal cells.

Results

Mouse embryo culture system for DSLM

In order to observe a whole mouse embryo during gastrulation, we developed a series of techniques for mouse embryo culture in the chamber of DSLM. These techniques include a specific embryo holder for mouse embryos, transfer methods of embryos without exposing to the air, temperature regulation and an atmosphere control (Figure 1). Since mouse embryos cannot be cultured in the sort of conventional agarose holder typically used for samples in LSFM, we made a specimen holder for mouse embryos (Figure 1A and B). The embryos are placed onto 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 sticky Reichert’s membrane at the ectoplacental cone. The embryo is transferred to the holder via a window on the cylinder (Figure 1C), and then set to the stage of the DSLM. This procedure prevents the embryos getting damaged by exposure to the air. For temperature control, we developed water-cooled Peltier device-mounted chamber (Figure 1D, HAYASHI WATCH-WORKS CO., LTD). The 5% CO₂ and 5% O₂ gas mixture is injected via a...
gas adopter into the specimen chamber, over the surface of culture medium (Figure 1E). A lid is put at the top of the chamber to prevent drying of culture medium.

Live imaging of the whole mouse embryo at E6.5 by DSLM

We then performed time-lapse imaging of Histone H2B-GFP–expressing mouse embryos at embryonic day 6.5 (E6.5) (Figure 2A–C and Movie S1). We obtained images from the distal end to the end of embryonic body with 1.5 minute interval, which is difficult with the other kind of microscopy. At this nearly maximum time resolution, the embryos exhibited abnormal development after ten hours of illumination (total energy, 46 mJ) although DSLM illuminates lower energy to the sample than the conventional microscopy, suggesting high fragility to phototoxicity of mouse embryos at this stage. Until three hours they looked normal without major developmental defects (Figure S2).

We observed nuclei in the epiblast migrate along the apical-basal axis and divide near the apical surface (Figure 2C). This phenomenon is known as interkinetic nuclear migration (INM) that occur in the neuroepithelium at later stages in mouse, chick, zebrafish, etc. [12–14] with cell cycle-dependent manner [15]. In E6.5 mouse epiblast, nuclei in S phase are reported to localize at basal side in fixed embryo [16]. INM has been observed in pseudostratified epithelium of the spinal cord, cerebral cortex, and retina [17–20], and in other pseudostratified tissues [21–23]. We confirmed the epiblast in this stage is pseudostratified by membrane staining of a fixed sample (Figure 2D), i.e. the INM in E6.5 epiblast appeared to be a common feature in pseudostratified epithelia.

Live imaging of an E5.5 and E6 mouse embryos

Next we examined earlier stages, E5.5 and E6 (Figure 3). At E6, the structure of the epiblast is pseudostratified (Figure 3D), and exhibits INM as at E6.5 (Figure 3C and Movie S2). On the other hand, at E5.5 the epiblast does not yet appear to have a pseudostratified structure (Figure 3H) whereas the nuclei exhibit an INM-like movement (Figure 3G and Movie S3). This result was unexpected because INM is reported to be a conserved feature in pseudostratified structures [24]. Although the role of this INM-like movement is still unknown, we infer that there might be the

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**Figure 1. Mouse embryo culture system for DSLM.** (A) Side and top views of the embryo holder. 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 ectoplacental cone. (B) Photograph of the embryo holder. (C) The embryo transfer method. For embryo transfer from dish to the chamber of the DSLM, we use the cylinder with window (window is indicated by green arrowhead). Embryos are moved to the space of the holder filled with culture medium under wide-field microscope (middle). After setting embryo into the holder, the holder is pushed until the end of the cylinder and stayed at the position without window (bottom, the position of the holder is indicated by orange arrowhead). At this position we can move the holder without loss of the culture medium. The holder is pushed into culture medium in the chamber of DSLM. (D) Photograph of the gas adopter. (E) Photograph of the assembled system around the chamber. The gas mixture is injected at the top of the chamber though the gas adopter. To prevent evaporation of the culture medium, a lid is set at the top of the adopter.

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relationship between INM and the transformation from a monolayer to a pseudostratified structure.

Tracking the epiblast nuclei and characterization of INM

To characterize INM, we tracked the three-dimensional (3D) positions of all nuclei in a portion of the epiblast at E6.5 (n = 103; 83 (anterior–posterior) ×42 (left–right) ×77 (proximal–distal) μm, Movie S4 and 5). Tracking was performed manually by the aid of a custom-made 3D tracking assistance software. Kymographs of both apical and basal nuclear migrations are shown in Figure 4A. We measured average speeds of 1.09±0.34 (mean ± SD) μm/min from the basal to the apical surface (n = 29) and 0.54±0.14 μm/min from the apical to the basal surface (n = 28). These results are consistent with the values measured in other tissues and species [25–27].

A recent study suggested that basal migration in mouse brain is not the result of a cell-autonomous force derived from intrinsic motor proteins, but rather of a passive pushing force caused by the apical migration of neighboring nuclei [28]. To evaluate the contribution of such force for the INM of mouse epiblast, we first measured the nuclear density at the apical, middle, and basal levels (Figure 4B). The density at the basal level is higher than that at the apical level (1.6±, n = 18), so it seems unlikely that the basal migration is caused passively by a pushing force from apically positioned nuclei. On the other hand, it is possible that basally positioned nuclei may create a force that pushes another nucleus apically. We next measured the correlation between the velocity of a nucleus and its relative position to neighboring nuclei. The latter was represented as the difference between the distance of a nucleus from its forwardly-positioned neighbor (i.e., the nucleus ahead of the cell in the direction of migration) and the distance from its backwardly-positioned neighbor (Figure 4C). If the migration is caused by a pushing force from backward-neighbor nuclei, the velocity should become greater as the difference in distances increases, indicating contact between the backward neighbor and the free forward space. The velocity of neither orientation was not correlated with the difference in distance (apical, R² = 0.0232; basal, R² = 0.0011). Therefore, in the epiblast of mouse embryo, the main driving force for both apical and basal nuclear migrations would be caused by cell-autonomous mechanisms, not by a passive force, although additional analysis is necessary for conclusion.

In order to further characterize INM in the epiblast at E6.5, we next evaluated the 3D orientation of cell division (Figure S3). The distribution of orientation of cell division had no apparent bias within the epiblast (Figure S3A). And nuclear divisions perpendicular to the apical surface occurred more often than those with...
oblique or parallel orientations (41% of total divisions, Figure S3B). This result is consistent with results obtained in fixed samples [16].

Three-dimensional tracking of mesodermal cell migrations

Imaging by DSLM also allowed visualization of migrating mesodermal cells. In mouse embryo, time-lapse imaging of mesodermal cell has not been previously performed with high temporal and spatial resolution, hence the precise details of
mesodermal cell migration have remained unknown [3]. In contrast to epiblast cells with elongated shape, mesodermal cells are compact so that movement the nuclei can be regarded as that of cells themselves. Migration of mesodermal nuclei was tracked using automatic tracking tools we developed. Reconstructed 3D trajectories are shown in Figure 5A and Movie S6. This result indicates that the paths followed by mesodermal cells are not straightforward. In order to elucidate these paths, we measured the distributions of the one-step velocities (top row in Figure 5B) and the ratio of the actual migration path length to the linear displacement (bottom row); this ratio indicates the degree of zigzag movement. Previous reports predict that axial mesoderm cells located in the distal region at midline migrate in concert with the adjacent paraxial mesoderm [29–33], and that the cells in the forward mesoderm migrate more rapidly than cells elsewhere in the mesoderm [34]. We therefore separately measured nuclear positions in the lateral (cyan in Figure 5B) and axial (magenta) areas at the first time-point. We also classified nuclear positions as ‘forward’ (orange) and ‘rear’ (green). Figure 5C and Movie S8 demonstrate that triangle shapes connected neighboring nuclei at the first time-point intersect each other after three hours. These results show that the mesodermal cells migrate not as a coordinated group, but rather individually, i.e., that mesodermal cells in the mouse embryo do not maintain cell–cell adhesion as they do in Xenopus or Zebrafish mesendoderm [35–38]. Furthermore, this property of the migration is not different significantly between the lateral and distal or the forward and the rear regions, contrary to previous assumptions [30,34]. The average speed was calculated to be $1.17 \pm 1.40$ μm/min ($n = 452$ nuclei), and the average ratio of actual migration length to linear displacement over 30 minutes was $2.98 \pm 1.13$ ($n = 220$ nuclei).
Discussion

Visualizing and understanding cell behavior in the whole mouse embryo during gastrulation has been a long-standing goal in developmental biology. In this study, we developed the techniques of digital scanned light-sheet microscopy (DSLM) combined with mouse embryo culture system and computational analyzing tools for 3D tracking. Using these techniques, we performed live imaging of whole mouse embryos during gastrulation and analyzed the migrations of epiblast and mesodermal cells. Tracking of nuclear movements of the epiblast and mesoderm revealed that the bidirectional movement of INM is likely to be caused by active cell-autonomous forces rather than passive pushing forces from neighboring nuclei, and that mesodermal cells migrate individually rather than as a sheet. We also first report INM-like migration in the E5.5 epiblast, which is not yet pseudostratified.

We visualized a living mouse embryo during gastrulation till a depth of approximately 140 μm from the distal end. The resulting image showed stripe patterns and decreasing resolution along the optical axis (Figure 2A), which are shadows cast by refraction and scatter from the front region of the sample to the illumination light. These shadows can be reduced using multidirectional illumination techniques [39,40]. Other light sheet techniques, such as structured illumination or multi-photon light sheet illumination, will also improve penetration when imaging mouse embryo during gastrulation.

The techniques we present here will be applicable to the study of other aspects of mouse development, for instance, migration of distal visceral endoderm, intercalation of definitive endoderm to visceral endoderm, and tracking of primordial germ cells. These topics are of significant interest, but have not yet been observed in live specimens because of the difficulty in live imaging of the mouse embryo. The phototoxicity problem still needs to be overcome to image the embryos for sufficient length of time: use of longer wavelength will be a solution in near future.

Experimental Procedures

Mice

Histone H2B-GFP and -mCherry transgenic mice were obtained from T. Fujimori (National Institute for Basic Biology) [41,42]. Heterozygous embryos from intercrosses between wild-type ICR and Histone H2B-GFP (Figure 2, 3, 4, S2) or H2B-mCherry (Figure 5) were used. All animal experiments were carried out along the guidelines and the approval of The Institutional Animal Care and Use Committee of National Institutes of Natural Sciences (Permit Number: 10A087, 10A90).

Embryo culture and imaging conditions

Mouse embryos were dissected into phenol red–free Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), preserving a portion of Reichert’s membrane at the eciplacental cone. Embryos were
moved to the pocket space of the specially designed embryo holder which has been filled with medium culture from window of the cylinder (Figure 1C). Embryos were set into the hole of the holder and then the holder was pushed until the end of the cylinder. The embryos stayed at the position without window in order to prevent exposure of the embryo to air. The cylinder and holder was set at the stage and inserted into the culture medium in the chamber. The cylinder is elongated due to the length of cylinder holder. Embryos were cultured at 37°C with 5% CO2, 5% O2, and 80% N2 in medium containing 40% phenol red-free DMEM, 10% FCS (Invitrogen), 50% rat serum, 100 μM Trolox (Cayman), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Gas was mixed by a GM-6000 from TOKAI Hit and blown into the air gap of the chamber. The sample holder was attached to the sample positioning system, which comprises three linear translation stages (M-111.2DG, Physik Instrumente) and one micro-sample positioning system, which comprises three linear translation stages (M-116DG, Physik Instrumente). Three-dimensional image stacks were acquired every 1.5 or 3 min with a 500 ms exposure time per image and a z-spacing of 2.58 μm.

Microscopy

We used a Digital Scanned Laser Light Sheet Fluorescence Microscope (DLSM) that was modified from our previously reported implementation [8]. An argon-ion laser (532 nm, Melles Griot) was used as the light source. The wavelength of the laser beam (488 or 568 nm) was selected using an acousto-optical tunable filter (AA.OPTF.nG-400–650 nm-PV-TN, AA Opto-Electronic) and scanned through the sample using a two-axis high-speed scan head (VM5000+, GSI Lumonics). The scanned light sheet was created with an f-theta lens (S4.LF.T061/065, Sill Optics) and a low-NA objective lens (Plan-Apochromat 5x/0.16, Carl Zeiss). Fluorescence emitted from probes was detected using a water-immersion lens (Achromplan 20×/0.5, Carl Zeiss) and recorded with a CCD camera (Orca AG, Hamamatsu) through a long pass filter (RazorEdge RU 488 or 568, Semrock). The image data were recorded using our custom DLSM control software, which was developed in the Microsoft .NET framework. Illumination intensity was measured at the focal point of the illumination objective lens.

Fixation and labeling

Dissected embryos were fixed and permeabilized overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS, Sigma) with 0.1% Triton X-100 (Sigma). After thorough washing, embryos were stained with Alexa Fluor 546 phalloidin (1:100, Invitrogen) and 1 μM DRAQ5 (1:1000, Biotatus) for one hour at room temperature.

Pre-processing of image data and analysis of tracked epiblast and mesodermal cells

The acquired data were cropped and aligned in time and space with custom macros in ImageJ (National Institutes of Health). For tracking nuclei in epiblast, we used a custom-made assistant program for manual tracking, which records positions indicated by the user. For mesodermal cell tracking, we used another custom-made program that automatically track nuclei after the user points the position at first time point. The tracking results were confirmed by human eye, and wrong ones were deleted. Both tools were written in C++ (code available upon request). The automated tracking is based on an image recognition algorithm pointing the center of the nuclei. To reconstruct nuclear positions as 3D, good depth penetration is achieved due to the low numerical aperture used in the illumination sub-system. Since the emitted fluorescence photons are collected in parallel for all pixels in the focal region of the detection system. Since the emitted fluorescence photons are collected in parallel for all pixels in the camera, LSFM acquire images at high speed and with low illumination intensity. Good depth penetration is achieved due to the low numerical aperture used in the illumination sub-system. We performed data analysis in Microsoft Excel. Nuclear density in Figure 4B was calculated as the number of nuclei per 100 μm curve along the apical or basal surface of epiblast. The curve was drawn 5 μm from the apical or basal tips of the nuclei. The meddle was drawn at the center of the epiblast along its apical–basal axis. A nucleus was counted when the curve crossed the apical-basal axis at a point more than 20% of the axis length from the nearest end. The number of the nearest-neighbor nuclei shown in Figure 4C was calculated as a distance between a nucleus and nearest nucleus in a cone having bottom of same spherical radius as the nucleus and height of twice radius at the nuclear center toward apical or basal side. Nuclear radius is set as 11 μm at apical side and 16.6 μm at basal side and it increases linearly along apical–basal axis. These values were measured with ImageJ (apical n = 30, basal n = 42).

Supporting Information

Figure S1 Comparison of conventional and light sheet-based fluorescence microscopy (A). (top) Optical paths of excitation (Ex) and detection (Det) in a conventional fluorescence microscope. Illumination and detection axes are parallel, and fluorophores outside the in-focus region are excited, resulting in low contrast. (bottom) The excitation and detection axes in light sheet-based fluorescence microscopy are perpendicular to one another. Fluorophores are excited in a region that overlaps with the local region of the detection system. Since the emitted fluorescence photons are collected in parallel for all pixels in the camera, LSFM acquire images at high speed and with low illumination intensity. Good depth penetration is achieved due to the low numerical aperture used in the illumination sub-system.

Figure S2 Effect of phototoxicity by illumination on the development of mouse embryos during gastrulation.

Top row shows section images of the embryos at first time point (E6.5) and bottom row at 10 hours later (E7). In control, the embryo normally developed in the microscope chamber without illumination. When the embryo was illuminated throughout culture for 10 hours, it exhibited abnormal growth. When illuminated for...
only first 3 hours, the growth was indistinguishable from the control. Estimated illumination power per hour is 4.6 mJ. (TIF)

Figure S3 Quantitation of division orientations in the epiblast. (A) Distribution of division orientation. Blue, pink, and red indicate divisions parallel, oblique, and perpendicular to the apical surface, respectively. A, anterior; P, posterior; L, left; R, right; D, distal; Pr, proximal. (B) Percentage of parallel, oblique, and perpendicular divisions relative to epithelial surface. Measuring procedure is as described for Fig. S4. (TIF)

Figure S4 Method for measuring the three-dimensional division orientation of epiblast nuclei. (A) The distribution of directions of nuclear divisions in the epiblast was calculated in planes both parallel and perpendicular to the proximal–distal axis. The angle was measured between the line along the epithelial surface and the division axis at anaphase. (B) The orientation $\theta$ was calculated from the following equation using $\theta_p$ and $\theta_i$. The measured division directions were pooled into three groups with identical curved surface areas: parallel ($0^\circ \leq \theta_i < 19.5^\circ$), oblique ($19.5^\circ \leq \theta_i < 41.8^\circ$), and perpendicular ($41.8^\circ \leq \theta_i < 90^\circ$).

\[
\theta = \arctan \left( \frac{\cos \theta_p \times \cos \theta_i}{h} \right)
\]

Movie S1 Live imaging of Histone H2B–GFP mouse embryo at E6.5. The optical section shown here is located about 78 μm from the distal end of the embryo. The time interval is 1.5 min. Gastrulation and mesodermal cell movement are clearly demonstrated; in the epiblast, interkinetic nuclear migration can be observed. (MOV)

Movie S2 Live imaging of mouse embryo at E6. The optical section is located 65 μm from the distal tip of the embryo. The time interval is 3 min. (MOV)

Movie S3 Live imaging of mouse embryo at E5.5. The optical section is located 40 μm from the distal tip of the embryo. The time interval is 3 min. Although the epiblast is not pseudostratified, INM-like movement occurs. (MOV)

Movie S4 Reconstructed epiblast nuclei from distal view. Tracked positions were reconstructed as 3D computer graphics using POVRay [http://www.povray.org]. Red, blue and green arrows indicate the anterior–posterior axis, right–left axis, and proximal–distal axis, respectively. (MOV)

Movie S5 Reconstructed epiblast nuclei from a lateral view. Tracked positions were reconstructed as 3D computer graphic. Left side is shown. Red, blue, and green arrows indicate the anterior–posterior axis, right–left axis, and proximal–distal axis, respectively. (MOV)

Movie S6 Reconstructed trajectories of mesodermal cells. Selected trajectories are shown on the embryo. The embryo consists of two surfaces: the outer visceral endoderm (blue) and a boundary surface between the visceral endoderm and mesoderm (green). Red semi-cylindrical shapes show the primitive streak, pointing in the posterior direction. Red, blue, and green arrows indicate the posterior-anterior axis, left–right axis, and proximal–distal axis, respectively. (MOV)

Movie S7 Time-lapse migrations of mesodermal cells. Each mesodermal cell migrates, leaving a trail. The embryo consists of two surfaces: the outer visceral endoderm (blue) and a boundary surface between the visceral endoderm and mesoderm (green). Red semi-cylindrical shapes show the primitive streak, pointing in the posterior direction. Red, blue, and green arrows indicate the posterior–anterior axis, left–right axis, and proximal–distal axis, respectively. (MOV)

Movie S8 Time-lapse changes of triangles connected neighboring nuclei at the first time point. Mesodermal cells are divided into two regions, lateral (cyan) and distal (magenta). The embryo consists of two surfaces: the outer visceral endoderm (blue) and a boundary surface between the visceral endoderm and mesoderm (green). Red semi-cylindrical shapes show the primitive streak, indicating posterior side. Red, blue, and green arrows indicate the posterior–anterior axis, left–right axis, and proximal–distal axis, respectively. As time goes by, the triangles become larger and overlap each other, indicating that each cell migrates individually. (MOV)

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Author Contributions

Conceived and designed the experiments: TI SN. Performed the experiments: TI HK. Analyzed the data: TI KN AM. Contributed reagents/materials/analysis tools: KN PJK HK EHKS AM. Wrote the paper: TI SN.

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