A microfluidic array for large-scale ordering and orientation of embryos

Kwanghun Chung1,3, Yoosik Kim2,3, Jitendra S Kanodia2, Emily Gong1, Stanislav Y Shvartsman2 & Hang Lu1

Quantitative studies of embryogenesis require the ability to monitor pattern formation and morphogenesis in large numbers of embryos, at multiple time points and in diverse genetic backgrounds. We describe a simple approach that greatly facilitates these tasks for *Drosophila melanogaster* embryos, one of the most advanced models of developmental genetics. Based on passive hydrodynamics, we developed a microfluidic embryo-trap array that can be used to rapidly order and vertically orient hundreds of embryos. We describe the physical principles of the design and used this platform to quantitatively analyze multiple morphogen gradients in the dorsoventral patterning system. Our approach can also be used for live imaging and, with slight modifications, could be adapted for studies of pattern formation and morphogenesis in other model organisms.

Cell differentiation in embryos can be spatially controlled by the graded distribution of morphogens, chemical signals that act as dose-dependent regulators of gene expression. Some of the first morphogen gradients had been identified in the *Drosophila* embryo, in which dorsoventral patterning is initiated by the nuclear localization gradient of Dorsal (Dl). Dl is an NF-κB transcription factor, which subdivides the embryo into three germ layers1–3; the dorsoventral pattern of the embryo defines the dorsoventral pattern of the adult (Fig. 1a,b). The regions exposed to high, medium and low levels of Dl contribute to the formation of the mesoderm, the nervous system and the skin of the embryo, respectively.

Quantitative analysis of developmental systems controlled by morphogens requires information about both the regulatory regions of genes comprising the network and the spatial distribution of patterning signals. The dorsoventral patterning system in *Drosophila* is arguably one of the best understood systems with regard to its sequence-specific transcriptional regulation. However, information about the distribution of patterning signals is currently lacking, mainly because of technical difficulties associated with imaging the spatial distribution of proteins and transcripts along the dorsoventral axis of the embryo4,5. When imaged on a regular microscope slide, embryos are oriented with their major axis parallel to the coverslip, and their dorsoventral orientation is essentially random. As only a small fraction of embryos can be used for quantitative imaging, previous analyses of signals in the dorsoventral system relied on data collected from about ten embryos6,7. To enable high-throughput analysis of the dorsoventral patterning signals, we developed a microfluidic embryo-trap array, a device in which hundreds of embryos are oriented vertically in a few minutes. Such ‘end-on’ orientation allows for dorsoventral-axis data to be easily collected for multiple embryos. Previously, end-on imaging has been possible only for very small numbers of embryos, which had to be individually and manually placed into an upright position5,6.

Here we describe the design and the physical principles of the embryo-trap array, and use it to quantify morphogen gradients in fixed embryos and to monitor nuclear divisions in live embryos. The device enables high-throughput analysis of the dorsoventral patterning system at the level of the inductive cues and their signaling and transcriptional targets in multiple genetic backgrounds. Using this device to image a large number of embryos, we resolved an outstanding issue regarding the spatial extent of the DI morphogen gradient.

**RESULTS**

**Design of the embryo-trap array**

The array is a one-layer microfluidic device fabricated from polydimethylsiloxane (PDMS), an optically transparent elastomer widely used in biological microfluidics8,9. To allow for imaging of a large number of embryos, the array needs to have traps that are densely packed, which is an engineering challenge. Conventional approaches using hydrodynamics for cell trapping typically do not achieve such high packing density10,11 mostly owing to the requirement to properly balance flow resistance, resulting in a relatively large space between neighboring traps. The mechanism used in our design, in contrast, does not rely on resistance change upon the occupation of traps and therefore allows for dense arraying of ~700 traps in the space of a microscope slide (Fig. 1c,d). Our design consists of a serpentine fluid-delivery manifold and an array of cross-flow channels (Fig. 1c,d). The 700-µm-wide serpentine channel is wider than the major axis of the embryo (~500 µm), allowing embryos of any orientation to move easily through it.

1School of Chemical and Biomolecular Engineering, and Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia, USA.
2Department of Chemical and Biological Engineering and Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, New Jersey, USA.
3These authors contributed equally to this work. Correspondence should be addressed to H.L. (hang.lu@gatech.edu).

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Figure 1 | Microfluidic embryo-trap array for high-throughput arraying of vertically oriented Drosophila embryos. (a) Image of an adult Drosophila (left) with dorsal, posterior, ventral and anterior directions indicated. Scale bar, 1 mm. (b) Micrograph of early embryo stained using antibody to DL. (Anterior is to the left, and dorsal is at the top.) Scale bar, 100 µm. (c) Photograph of the device (left) and a micrograph of the boxed region (right). Scale bar, 1 cm (left) and 500 µm (right). (d) Detail of the embryo-trap array design (top view). (e) Scanning electron micrograph of the trap structure. Scale bar, 100 µm. (f) Schematic showing the embryo trapping process: an embryo is guided into the trap (top); the flow around the embryo orients it vertically (middle); the trap contracts and secures the embryo (bottom). The yellow plane represents imaging focal plane. Blue arrows show the direction of bulk flow in the serpentine channel. (g) Schematic of the imaging setup. Inset, representative confocal image of an embryo stained with antibodies to DL, Twist and dppERK. (h) A section of the array with trapped embryos (dark circular object in each trap). Scale bar, 500 µm.

This feature is particularly important for robust handling of nonspherical objects such as Drosophila embryos. Each cross-flow channel includes a truncated cylindrical trap where the embryo is located for imaging; the trap is connected to a narrowing channel and a long and narrow resistance channel (Fig. 1d,e). When an embryo approaches an empty trap, flow through the trap guides it into the trap (Fig. 1f). The shape of the trap dictates that the embryo is in an upright position for imaging (Fig. 1g) such that each embryo on every device is oriented with its dorsoventral plane positioned horizontally. Oriented embryos, which appear round when viewed from the top, are thus arrayed on the device (Fig. 1h).

Using a computational fluid dynamics approach (Online Methods), we engineered the hydrodynamic resistances of the cross-flow channels. A simplified smaller array in a three-dimensional computational model (Supplementary Fig. 1) demonstrated that our design satisfied the following criteria. First, all the traps were exposed to similar flow rates (Fig. 2a). If the flow in the array has large variations in different rows or columns, the trap occupancy will be severely compromised; optimal design, however, yields highly repeatable near-perfect occupancy, as we found experimentally (Supplementary Fig. 2). Second, the bulk of the embryo suspension flows along the serpentine manifold (Fig. 2a,b). The bulk flow through the main channel efficiently sweeps out extra and improperly trapped embryos (Supplementary Video 1). Additionally, too low a cross-flow through the traps prevents embryos from being introduced into the traps, resulting in inefficient trapping, but too-high cross-flow causes embryos to accumulate near traps and clump together (Fig. 2b). Thus, optimal design of an array that works well with Drosophila embryos requires proper parameter choice, including geometry and operating pressure range.

Another important mechanism for orientation of embryos in our device is Dean flow (with a Dean number greater than 100 throughout the device), an effect in which curvature of the channel induces a secondary non-axial flow. This hydrodynamic effect was apparent in the stream-line trace (Fig. 2c) and in frames from an embryo-loading video (Fig. 2d). The Dean flow and the diverging and converging flow through the cross-flow channels focus the embryos toward the traps (as opposed to embryos distributing in random locations in the bulk flow) and increases the frequency with which embryos contact the traps and are loaded into them. The presence of the secondary Dean flow at the bends of the channel not only greatly improves trap occupancy but also maximizes loading efficiency because an embryo has many opportunities to be in contact with an empty trap. Essentially every single embryo entering the device was trapped, a feature that will be very useful for studies in which one has to work with small numbers of embryos in complex genetic backgrounds. When imaging, we filled about 90% of the traps with embryos.

During loading, the entire device is under a slight positive pressure. Because PDMS is an elastomer, the pressure can expand the trap opening to facilitate loading (Figs. 1f and 2e–j). Confocal microscopy characterization of trap behavior under different pressures (Fig. 2c,h) demonstrated that at ambient
Figure 2 | Operating principles of the embryo-trap array. (a, b) Volumetric flow rate in the serpentine main channel (a) and through the cross-flow channels (b) at each trap. Widths of the resistance channels in the optimal design (Fig. 1d), low-resistance design and high resistance design were 40 µm, 80 µm and 20 µm, respectively. Dummy columns are the first and last columns of the device. (c) Schematic of streamlines plotted from the numerical computational fluid dynamic model as the fluid turns the corner in the main channel. (d) Optical images at the indicated time points show an embryo (circled) migrating along the wall of the serpentine channel. Scale bar, 800 µm. (e–j) Three-dimensional characterization of the trap by confocal microscopy at 0 psi (e–g) and 6 psi (h–j). Single-frame top view from the middle of the device (f, i; dotted red circle represents dorsoventral plane of an embryo). Single frame cross-sectional view of the trap opening (g, j; dotted red ellipse represents vertically oriented embryo). Dotted white lines, locations where cross-sections of the trap opening (g, j) were acquired. Scale bars, 100 µm.

condition (0 pounds per square inch (psi)), the traps had smaller openings (not enough for an embryo to be loaded or released) as compared to their size under 6 psi of positive pressure. When operating the device, we first connected the device at the outlet to a pressure-drop tube to raise the average pressure of the device to ~6 psi to open the traps. Then we introduced the embryo suspension into the device using a syringe or a pressure source (for example, compressed air).

Figure 3 | Spatial extent of the Dl gradient. (a) Confocal images of vertically oriented embryos stained for Dl and stained with DAPI. A merge is also shown. (b–e) Average gradients of nuclear Dl from four representative experiments. Error bars are s.e.m. (number of gradients used is indicated in each plot). The arrow denotes the dorsoventral (DV) position beyond which the nuclear Dl gradient can be considered ‘flat’. DV distances are normalized: x = 0 for ventral and x = 1 for dorsal. (f, g) Early (f) and late (g) expression patterns of Dl and zen. (h) Schematic of regulatory models that can be used to account for the two phases of zen expression (top schematic depicts early expression). U is a uniform activator and pMAD is phosphorylated MAD. (i–k) Pairwise comparison of Dl gradients in wild-type and mutant backgrounds. Nuclear Dl gradients from the wild-type embryos (i), embryos from Δr1/4 females (j), and average gradients for both genetic backgrounds (k). Error bars, s.e.m. (n = 70 for wild type and n = 82 for mutant).
Supplementary Fig. 4  

Under flow conditions, embryos at the traps experience non-uniform pressure and shear by the surrounding fluid; the resulting force flips the embryo vertically, inserting it into the cylindrical trap (Fig. 1f and Supplementary Fig. 3). This is achieved entirely passively by hydrodynamics, without user intervention or control. Upon completion of loading, we reduced the injection pressure, and the trap opening contracted, securing the embryo inside in an upright position (with dorsoventral axis parallel to the coverslip; Fig. 1f and Supplementary Video 2). This lock-in feature allows the device to be disconnected from the rest of the hardware, to transport it for imaging or to store it with the embryos embedded. Operation of the device consists of two simple steps and does not require a computer, valves or other off-chip components except a pressure source, and thus non-experts can use it easily. Because the embryos have different sizes and shapes and because antibody staining can be highly variable, typically many embryos are needed. We quantified 4′,6-diamidino-2-phenylindole (DAPI) staining in many embryos in the trap array, which revealed that the variability of the supposedly uniform DAPI staining was negligible compared to morphogen gradients that we typically quantify. We conclude from these data that the device did not introduce illumination bias.

Quantitative imaging of pattern formation

We used the embryo-trap array to analyze the distribution of nuclear Dl. The ventral-to-dorsal distribution of nuclear Dl is induced by localized activation of the Toll receptor on the ventral side of the embryo. Before Toll activation, Dl is sequestered in the cytoplasm, in a complex with its binding partner Cactus. In response to Toll signaling, Cactus is degraded, and Dl moves into the nucleus, where it binds the regulatory regions of its target genes.

One of the outstanding questions regarding dorsoventral patterning is the spatial extent of the DI gradient. More specifically, it is not clear over what part of the dorsoventral axis the Dl gradient is ‘flat’, and where it therefore cannot act as a patterning signal. This has been a matter of intense debate in recent publications.

The disagreements in the literature may be traced to current methodological limitations in quantification of the DI gradient. Although end-on imaging provides information about the entire dorsoventral axis, it has only been possible to apply it to a few embryos until now. Lateral imaging, in contrast, can be applied to analyze more embryos and to image more gradients, but it is limited to only a fraction of the dorsoventral axis. Our platform substantially increases the statistical power of end-on imaging, allowing us to investigate the spatial extent of the DI gradient.

The lowest level of nuclear Dl is at the dorsal-most point of the embryo, which corresponds to the least Toll activation. If the amount of nuclear Dl at an arbitrary position x along the dorsoventral axis is statistically indistinguishable from the nuclear Dl level at the dorsal side of the embryo, then the DI gradient can be considered ‘flat’ between the position x and the dorsal-most position. We compared the distribution of nuclear Dl along the dorsoventral axis to the nuclear Dl levels at the dorsal-most point of the embryo. In each of over ten independent experiments, we collected at least 50 DI gradients 70 µm from the poles of embryos during the last nuclear division.
Indeed, previous studies revealed that the later phase of the Dl gradient, which suggests a more complex mode of regulation.

This system is dominated by feedforward loops, a network motif in which a gene is controlled both by the primary input, such as Dl, and by one of its more proximal targets (summarized in Fig. 4a). For instance, Snail (Sna), a transcription factor expressed in the future mesoderm, is activated both by Dl and by Twist (Twi), a transcription factor that is directly activated by Dl. Patterning of the neurogenic ectoderm requires a two-peaked pattern of signaling through the bone morphogenetic protein (BMP) pathway, which is spatially regulated by Dl and its multiple targets.

We analyzed the distribution of other regulators of dorsoventral patterning. As an example, we consider the regulation of zerknüllt (zen), a transcription factor expressed on the dorsal side of the embryo19, shown), supporting the notion that the ventrally activated MAPK pathway, which is spatially regulated by Dl and its multiple targets.

We used the embryo-trap array to characterize Twi expression gradients as well as gradients of MAPK and BMP signaling (Fig. 4b–g). Twi and BMP signaling gradients we observed were consistent with the ones reported previously6,7,17,23, but we quantified the gradient of phosphorylated MAPK (dpERK) here to our knowledge for the first time. We observed MAPK phosphorylation at the ventral-most region of the embryo (Fig. 4g). Furthermore, we found that Cic, a transcriptional repressor that is degraded as a consequence of its phosphorylation by MAPK24, is substantially downregulated at the ventral side of the embryo (data not shown), supporting the notion that the ventrally activated MAPK contributes to dorsoventral patterning.

**DISCUSSION**

Until now, end-on imaging was not ideally suited for quantitative and statistical studies of pattern formation5,6,18. Using our microfluidic embryo-trap array, hundreds of embryos can be oriented in an upright position in a matter of minutes. Data for dozens of embryos are sufficient for statistical analysis of spatial patterns in both wild-type and mutant backgrounds.

In the future, the temporal resolution of end-on imaging can be increased by grouping the images collected from fixed samples into distinct temporal classes. This could be based on cytological markers, such as the nuclear density in the syncytium.
or the extent of membrane invagination during cellularization. In preliminary experiments we established that live embryos can be loaded into and imaged in the device as well. We obtained videos of cell divisions in the early embryo as well as in an embryo undergoing gastrulation (Fig. 5 and Supplementary Videos 3 and 4). We observed that some of the embryos hatched in the device, but we did not confirm whether those that hatched were the ones that we imaged. The timing of nuclear divisions observed in our device was very close to that observed in the classical studies of the early Drosophila embryo.25 This suggests that our imaging platform can be used to study the real-time dynamics of embryonic development.

Unlike the anterioposterior patterning system, which has been a subject of extensive mathematical modeling and computational analysis,26,27 comprehensive quantitative models of the dorsoventral system have yet to be developed.28,29 This is now a feasible goal, enabled by the efficiency of end-on imaging in our platform. Furthermore, embryo-trap array–based imaging is not limited to the analysis of pattern formation in the early embryo. Other related developmental events, such as gastrulation, could be readily analyzed using this system. Devices for related fly species can be readily designed by modifying the trap size for embryos that are smaller or larger than those of Drosophila. Finally, because we demonstrated a general method for handling non-spherical objects, which is more difficult than handling cells, we expect that similar microfluidic designs can be used to image pattern formation and morphogenesis in other model organisms of developmental genetics.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS
K.C., E.G. and H.L. designed, fabricated and tested the device. J.S.K. wrote the image processing and statistical analysis programs for gradient quantification. K.C., Y.K., S.Y.S. and H.L. designed the experiments and wrote the paper.

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ONLINE METHODS

Microfluidic device fabrication. A mold was first fabricated by photolithographic processes. First, a negative photore sist (SU8-2100, MicroChem) was spin-coated twice at 400–600 r.p.m. onto a silicon wafer to form an ~500-µm-thick layer. Features on a transparency mask were transferred to the SU-8 coated wafer by standard UV-light photolithography. The mold was then treated with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane vapor (United Chemical Technologies) to prevent adhesion of PDMS during the molding process.

For fabricating the PDMS devices, a mixture of PDMS (parts A and B in 15:1 ratio) was poured onto the mold to give a ~1-mm-thick layer, which was partially cured at 70 °C for 20 min. A mixture of PDMS (part A and B in 10:1 ratio) was then poured on to form an ~4-mm-thick layer, which was then cured at 70 °C for 2 h. After peeling off the 5-mm PDMS layer, the individual devices were cut out, and access holes were punched in the PDMS. The devices were then treated with oxygen plasma and bonded to a coverslip.

Microfluidic device operation. Drosophila embryos were suspended in 100 ml of PBS buffer in a glass bottle, which was connected to the inlet of the device. The outlet of the device was connected to long PE90 tubing. The high resistance of the long PE90 tubing makes the pressure drop along the device less than 20% of the total pressure drop. This allows the traps to expand uniformly throughout the device. To load the embryo suspension into the device, a constant pressure source (~6 psi) was applied to drive the flow into the device. Precise pressure is not critical. After loading, the injection pressure was slowly decreased to 0 psi. All tubing was then disconnected from the device for imaging and storage.

Confocal microscopy to characterize trap-array performance was done on a Zeiss LSM 510 V1S confocal microscope. The device was filled with fluorescent dextran (molecular weight, 70,000 Da; Oregon Green; Invitrogen) solution. The pressure (0 psi to 6 psi) was controlled using a portable air compressor. Note that during normal operation of the device, a thumb-driven syringe was connected to the inlet of the device. The outlet of the device was connected to PE90 tubing. The high resistance of the long PE90 tubing makes the pressure drop along the device less than 20% of the total pressure drop. This allows the traps to expand uniformly throughout the device. To load the embryo suspension into the device, a constant pressure source (~6 psi) was applied to drive the flow into the device. Precise pressure is not critical. After loading, the injection pressure was slowly decreased to 0 psi. All tubing was then disconnected from the device for imaging and storage.

Fly strain and whole-mount immunostaining. OreR flies were used as a wild-type strain and dl(6) flies were used as dl heterozygous mutant strain in this study. Flies were raised, and embryos were collected at 25 °C. Antibody staining was performed as described previously21. The following primary antibodies were used: rabbit anti-dpERK (1:100, Cell Signaling), mouse anti-Dl (1:100, Developmental Hybridoma Bank), guinea pig anti-Twist (1:40, a gift from M. Levine) and rabbit anti-phospho-SMAD (1:3,500, a gift from D. Vasiliauskas, S. Morton, T. Jessell and E. Laufer, Columbia University). DAPI (1:10,000, Vector Laboratories) was used to stain nuclei, and Alexa Fluors (1:500, Invitrogen) were used as secondary antibodies.

To visualize the zen transcript, fluorescence in situ hybridization was used as described previously30. Embryos were hybridized with digoxigenin (DIG)-labeled antisense probe to zen mRNA overnight at 60 °C. Sheep anti-DIG (1:20; Roche) was used as primary antibody and Alexa Fluors (1:500) were used as secondary antibodies.

Microscopy and gradient quantification. Imaging was performed on a Zeiss LSM 510 confocal microscope with a Zeiss 20× (numerical aperture (NA), 0.6) A-plan objective. High-resolution images (1,024 × 1,024 pixels, 12 bits depth) were obtained from the focal plane ~70 μm from either the anterior or posterior pole. For live imaging, a Leica SP5 confocal microscope was used with 63× (NA 1.3) glycerin objective. Images were obtained every 7 s from the focal plane ~70 μm from the anterior pole. We could distinguish anterior and posterior poles by looking for the presence or absence of the pole cells, which are located at the posterior tip of the embryo. Fixed embryos were imaged in 90% glycerol solution and live embryos in PBS buffer.

Protein gradients were extracted from confocal images by using a Matlab (MathWorks) program described previously21. DAPI staining was used to determine the positions of nuclei, which were then used to quantify the ventral-to-dorsal nuclear concentration gradient of the protein of interest. To orient the extracted gradients, the embryos were also stained with Dl, whose gradient can be used to identify the dorsal-most and ventral-most points of the embryos. Briefly, the extracted nuclear DI gradient was fitted with a Gaussian curve, and the raw data were oriented such that the maximum of the Gaussian fit was set as the ventral-most point of the embryo, that is, x = 0 mm.

Characterization of flow profile in the microdevice by numerical simulation. Simulations were performed using a commercial finite element package, COMSOL (COMSOL, Inc.). The three-dimensional geometry of the section of the device is shown in Supplementary Figure 1a. The actual geometry was simplified to contain four actual trap columns to reduce the size of the model and the number of mesh elements. The number of traps in each column in the model (23 traps total) is the same as that in the actual device. Incompressible steady-state Navier-Stokes equations were solved to obtain the velocity and pressure profiles. The pressure at the outlet was fixed at atmospheric pressure, and the pressure at the inlet was set to obtain a volumetric flow rate equal to the measured value.

Characterization of hydrodynamic force on an embryo by numerical simulation. The simulation described above was used to calculate hydrodynamic force on an embryo located in the trap. The embryo was simplified as described in Supplementary Figure 2 at 60° to the trap inlet. The total force in the x direction was calculated using the post-processing feature of COMSOL, which results in a torque.