Published in final edited form as:

Phys Biol. 2011 August ; 8(4): 045006. doi:10.1088/1478-3975/8/4/045006.

Extracellular matrix fluctuations during early embryogenesis

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Abstract

Extracellular matrix (ECM) movements and rearrangements were studied in avian embryos during early stages of development. We show that the ECM moves as a composite material, whereby distinct molecular components as well as spatially separated layers exhibit similar displacements. Using scanning wide field and confocal microscopy we show that the velocity field of ECM displacement is smooth in space and that ECM movements are correlated even at locations separated by several hundred micrometers. Velocity vectors, however, strongly fluctuate in time. The autocorrelation time of the velocity fluctuations is less than a minute. Suppression of the fluctuations yields a persistent movement pattern that is shared among embryos at equivalent stages of development. The high resolution of the velocity fields allows a detailed spatio-temporal characterization of important morphogenetic processes, especially tissue dynamics surrounding the embryonic organizer (Hensen's node).

1. Introduction

The importance of the extracellular matrix (ECM) during morphogenesis and organogenesis is comparable to that of the cells [1]. In addition to providing structure to the interstitial spaces between cells, the ECM acts as a scaffold for cell adhesion. Cellular adhesion results in a physical coupling of the intracellular cytoskeleton and the extracellular environment, the ECM. This contact mediates cell migration (movement relative to the ECM) and also allows the cells to exert mechanical forces. In addition to serving mechanical roles, cell binding to the ECM can also elicit signal transduction events across the cell plasma membrane [2].

The dynamic relationship between cells and their local ECM environment influences not only their adhesion and migration, but also their polarity, survival, proliferation and differentiation [3]. The mechanisms by which the ECM contributes to the specification of cell fate are not understood, but both biochemical and mechanical signals may play important roles. As the ECM binds and sequesters soluble growth factors, it can modulate—either restrict or promote—access of ligands to specific cell surface receptors. Thus, the ECM is positioned to regulate the spatial and temporal localization of most growth factors after their secretion. Such ECM-bound growth factors may also stimulate directional cell migration through chemotaxis [4, 5]. Physical (mechanical) cues from the microenvironment are also likely transmitted through the ECM–integrin interaction and influence gene expression. For example, ECM stiffness contributes to cell fate determination of mesenchymal stem cells [6].

Embryonic tissues, i.e. cells and their ECM environment, undergo large deformations during organogenesis. Our earlier studies demonstrated that morphogenetic movements also involve substantial rearrangements of ECM constituents during bird embryogenesis, specifically during three pivotal processes referred to as gastrulation [7], ingression [8] and segmentation [9, 10] (see supplementary data for anatomical notations and diagrams available at stacks.iop.org/PhysBio/8/045006/mmedia). Similar ECM movements are also present in the ancient Hydra [11].

In this study, we investigate the movements of two ubiquitous ECM components that are present in vertebrate embryos within the first few hours of embryogenesis—fibronectin and fibrillin-2. We begin with a brief overview of ECM biology for the non-specialist readers. We then analyze the large-scale movement patterns in early, segmentation stage embryos. We show that the motion is inherently noisy and exhibits large fluctuations. By analyzing confocal recordings we show that despite their large spatial extent, the lifetime of the fluctuations is very short, less than a minute. We argue that the likely source of the fluctuations is individual cell contractile activity. Thus, not only spatial but temporal scales separate morphogenetic movements from individual cell action.

2. The ECM—an overview

The ECM is the non-cellular portion of tissues and organs [2, 12]. The hallmark of multicellularity is the presence of an ECM, as the existence of an organism with more than one cell depends upon inter-cellular adhesion. Indeed, the evolution and development of multicellular organisms is completely dependent upon the synthesis of a fibrous ECM scaffold upon which complex arrangements of epithelial sheets can be formed into tissues [1]. The ECM is present from the earliest stages of development in the embryo, and indeed the ECM of maternal origin invests some oocytes and eggs. Even the most primitive of Metazoans, such as the Hydra, have an ECM scaffold (mesoglea), required for the formation of a correct body plan [13, 14].

In adult tissues the ECM is usually categorized either as interstitial, or as forming a specialized structure termed a basement membrane. The basement membrane is a sheet-like structure that adheres to cell surfaces on one side and is often continuous with the interstitial ECM on the other side. Thus, it both isolates and connects cells to their environment as well as forms a biophysical support for overlying tissue [15].

The ECM throughout an organism is heterogeneous in both biochemical and biophysical properties. Each tissue has a unique composition of ECM components, which have a dynamic relationship with the cells of that tissue. Cells secrete and help assemble ECM, and the ECM in turn provides structural support and biochemical cues to the cells. In addition, the ECM is constantly being remodeled by enzymatic or non-enzymatic processes, which may alter its physical (material) properties or its biochemical signaling state via the release of bound growth factors. Aging and disease also alter the composition of the ECM in a given tissue, as the normal balance between ECM degradation and synthesis becomes disregulated [16].

2.1. Properties of ECM components

The ECM is comprised mainly of water, proteins and polysaccharides. Macromolecules of the ECM include glycosaminoglycans (GAGs) and proteoglycans, collagens, non-collagenous glycoproteins and elastin (reviewed in [17]).

GAGs are enormous polysaccharide chains with a net negative charge that attract small cations (Na^+ , Ca^{++}). The result is that GAGs bind water, thus leading to the swelling of

interstitial spaces. The presence of a hydrated ECM appears to promote the invasion and migration of cells, particularly in embryos [18]. With the exception of one GAG, hyaluronan, this class of polysaccharides are covalently linked to core proteins to form an even more complex class of macromolecules, the proteoglycans.

The predominant class of ECM components are the so-called glycoproteins. Glycoproteins are extended polypeptides modified with various amounts of covalently linked carbohydrates that can be found in fibrillar or nonfibrillar forms. Fibrillar proteins include some collagen family members, elastins, fibronectins, fibrillins and laminins. Collagens are the main structural and tensile elements in multicellular animals, and are the most abundant fibrous proteins in vertebrates. Collagens embedded in the interstitial ECM provide tensile strength, participate in cell adhesion, support cell migration [3] and limit the distensibility of tissues. Certain fibers provide elasticity to tissues that experience repeated stretch [19, 20]: elastic fibers are composite structures composed mainly of tropoelastin [21] and fibrillins, elaborately cross-linked to one another via extracellular enzymes [22]. Fibronectin is prominent at tissue boundaries, especially at sites of active cell movements and thus at sites of shear motion between tissues [23].

Throughout evolution, innovations, domain shuffling and co-evolutionary events expanded the repertoire of existing ECM molecules and ECM interacting molecules [24]. New kinds of ECM enable enormous possibilities for tissue/organ specificity, local microenvironmental complexity and ECM-dependent intracellular signaling, which in turn contribute to major tissue and body plan innovations which are characteristic of vertebrates and their immediate ancestors.

2.2. ECM can display multiple physical properties

The ECM contributes to maintaining the physical shape of tissues and organs [25]. The ECM also designates tissue and organ boundaries, which may be critical to maintain tissue function and structural integrity, for example, separating the skin from an underlying muscle or bone. There are a finite number of ECM macromolecules, but by mixing components with differing material properties the resulting biomaterials can manifest an extreme range of functional properties [26]. For example, type I collagen can be organized into optically transparent orthogonal lattices (cornea) [27], parallel bundles (tendon), concentric shells (bone) and two-dimensional interlocking fibrils (skin) as discussed in [1]. Dilute collagens in a highly hydrated mixture of GAGs can fill spaces with soft matter such as the vitreous humor of the eye or the lubrication chamber of a skeletal joint; conversely collagens embedded in calcium salts contribute to the material properties of hard tissue such as bones and teeth. Age-dependent alteration of a tissue's molecular components also influences the physical properties of the assembled and heterogeneous ECM. For example, as collagens and elastic tissue age the fibrils are chemically cross-linked, resulting in stiffer fibers with reduced elasticity [28].

2.3. Turnover of the ECM

In addition to ECM synthesis, assembly and organization, there is an important role for ECM turnover in development [29, 30]. Cells secrete whole families of enzymes that are tailored to degrade specific categories of the ECM. The main family is the metalloproteinases, which are regulated by the so-called tissue inhibitors of metalloproteinases [31]. Together these molecules modulate the turnover of the ECM via complex biochemical pathways.

Degradation of the ECM may serve to either remove the ECM as a barrier to cell motility [29], or result in the release of biologically active fragments of ECM molecules that in turn

regulate motility, proliferation and cell survival [32]. Further, ECM degradation can release explicit signaling molecules non-covalently bound to the ECM. Thus, the spatial-temporal regulation of protease expression and/or secretion can control when and where bioactive factors are bound to or released from the ECM. This dynamic and ongoing regulation of ECM accumulation and degradation modulates a host of developmental processes.

2.4. Role of cells in organization of the ECM

In addition to secretion of ECM components, cell activity is required for the organization of the ECM scaffold. The vast majority of research on ECM fiber assembly has been conducted in culture dishes. In densely populated cell cultures, the secreted ECM molecules are first assembled in distinct globules on the cell surface. Next, through the motion of several adjacent cells, the ECM foci are stretched [33] or otherwise organized into progressively larger filaments [34–36]. Thus, the process requires both specific cell surface receptors for fibronectin (called integrins) and cell motility/contractility (reviewed in [24, 37]).

While useful, the rigid physical environment of culture-based studies does not account for the tissue level deformations present in embryos. Nor are cell cultures truly three-dimensional environments in terms of active cellular motility. On the other hand, studying ECM fiber assembly in whole embryos is technically challenging. As a consequence, the role of individual cellular activity (1–100 μm), versus tissue level motion (100–1000 μm), during formation of tissue (cell-ECM) composites is poorly understood.

2.5. Localization of fibronectin and fibrillin-2 in bird embryos

Fibrillin-2 is an evolutionary ancient protein, with slender filaments detectable in bird embryos at 6–8 h of incubation (see supplementary figure S2 available at stacks.iop.org/PhysBio/8/045006/mmedia). A central fibrillin monofilament marks the future cranial–caudal axis and disperses into a fringe of radial fibers surrounding Hensen's node, the embryonic organizer tissue [38]. Fibronectin is present in vertebrates during formation of the middle embryonic layer (mesoderm) [23, 39]. Fibronectin coats the basal (under) surface of both the future top (ectoderm) and bottom (endoderm) layers (see supplementary figure S3 available at stacks.iop.org/PhysBio/8/045006/mmedia). The general organization of fibronectin and fibrillin-2 is quite similar in early bird embryos [10]. Both molecules exhibit a punctate pattern at Hensen's node and in the caudal (tail) part of the embryo. Both accumulate at the dorsal and ventral surfaces of the mesodermal layer, and co-exist in filaments that traverse the mesoderm (figure 1, and movie 1 (available at stacks.iop.org/PhysBio/8/045006/mmedia)).

3. Methods

3.1. Embryo preparation

Fertile quail eggs (*Coturnix coturnix japonica*) are incubated in a humidified chamber at 37 °C until the appropriate stages as determined by Hamburger and Hamilton (HH) [40]. Embryos are manipulated *ex ovo* as described in [41] and [42]. Briefly, the incubated eggs are carefully opened to keep the vitelline membranes on the dorsal aspect of the embryos intact (supplementary figure S1 available at stacks.iop.org/PhysBio/8/045006/mmedia). The yolk and albumen are gently poured into a sterile Petri dish. The vitelline membrane is exposed by removing the viscous albumen with a transfer pipette, and a paper ring (Whatman 52 filter paper), the inner diameter of which is slightly larger than the size of the embryo, is placed around the embryo. The ring is cut along its perimeter beginning at the cranial or caudal end using angled iridectomy scissors (Fine Science Tools, Foster City, CA). The ring-mounted embryo is gently pulled free by grasping the paper ring at the cranial or caudal end and pulling at a small angle. The embryo is gently submerged (ventral side up)

into ePBS to remove yolk. A transfer pipette may be used to gently pulse ePBS across the surface of the embryo to remove any remaining yolk. Once cleaned, the embryos are placed ventral side up onto embryonic culture dishes [41] for experimental manipulation.

3.2. Cell and ECM labeling

Fluorochrome-conjugated antibodies are introduced into live embryos mounted on paper rings. To label the ECM components, we use an Alexa-488 or Alexa-555 (Invitrogen, CA) conjugated nonperturbing primary fibrillin-2 antibody (JB3) or a similarly conjugated fibronectin antibody (B3D6, both from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). In live embryos, antibodies are delivered by the microinjection of 5–15 nl volumes 1–16 times per embryo at 1 ng/nl concentrations. A micropipette (18 μ m bore) and pneumatically driven Pico-Injector (Harvard Apparatus, Holliston, MA) mounted to a hydraulic micromanipulator assembly (Narshigie Scientific Instrument Laboratory, Tokyo) is used to deliver the antibodies to the interstitial space.

3.3. Microscopy

For long-term, wide field (10 $\times$), time-lapse imaging, immunolabeled quail embryos were recorded using an automated microscopy system as described in detail elsewhere ([42, 43]). Briefly, this imaging system is based on a computer-controlled epifluorescent microscope (Leica DMR series), equipped with a motorized stage and connected to a cooled digital CCD camera (QImaging Retiga-SRV). Most experiments utilized a 10 $\times$ objective (0.25 NA), which determines the limit of resolution to $\sim$ 1 μ m. Images are acquired using both epifluorescence and differential interference contrast (DIC) optics. One time point (frame) of the time lapse sequence typically consists of 4–8 images taken at slightly overlapping xy locations of the embryo (tiles), with 7–9 focal (z) planes in each tile. The length of the time interval between consecutive frames depends on the number of focal planes, fields and optical modes and is usually within the range of 2–20 min. Up to 12 embryos can be imaged per recording. During image acquisition the embryos are kept at 37 $^{\circ}$ C in a thermostatically controlled chamber.

For short-term, high-resolution (40 $\times$), time-lapse imaging, labeled whole quail embryos were placed ventral side down in glass bottom microwell dishes (P35G-0-14-C, MatTek Corporation, Ashland, MA). Once positioned, a Teflon membrane (97L0038, YSI Inc., Yellow Springs, OH) ring assembly was placed above the embryo to allow oxygen transfer while maintaining a humid environment; four drops ($\sim$ 20 μ l each) of ePBS were placed around the inner edge of the MatTek dish, but outside of the membrane ring (see [44]). Embryo chambers were placed in a heated environmental chamber at 37 $^{\circ}$ C on a Nikon Eclipse TE2000-E inverted microscope equipped with a Nikon A1R Confocal Laser Microscope System (Nikon, NY). Images were collected with a Nikon Plan Fluor 40 $\times$ /1.30 oil objective. The number of Z-stacks, the distance between optical sections and the time interval between consecutive images varied per embryo.

Static confocal images of fixed labeled embryos were collected on a Zeiss LSM510 upright microscope using a Zeiss Plan-Neofluar 25 $\times$ /0.8 oil immersion objective. Embryos were fixed post-labeling with 3% paraformaldehyde for 30 min at 4 $^{\circ}$ C. Embryos were mounted on glass slides in ProLong Gold antifade reagent with DAPI (Invitrogen, CA). Twenty-five z planes were collected at 2.4 μ m spacing.

3.4. Velocity fields

ECM displacements are estimated for a pair of images by particle image velocimetry (PIV), using the algorithm of [45] implemented in MatLab (Mathworks, Inc.). Briefly, the displacement field is first estimated using large image segments. These estimates are refined

in the second step by using smaller image segments which are less specific but allow better spatial resolution. The presented velocity fields were obtained without the application of a final smoothing (spline fitting) step, instead we kept the results of the second cross-correlation analysis. The statistical properties, however, remain the same irrespective of whether a final smoothing step was conducted. As the ECM label is highly non-uniform, for the analysis of confocal data we filtered the PIV-derived displacement vectors based on the local standard deviation of fluorescence intensity. Thus, only those displacement vectors that characterize a detail-rich region were kept for analysis. Velocity fields were then obtained as

$$\mathbf{v}(\mathbf{x}, t_i) = \frac{d\mathbf{x}(\mathbf{x}, t_i)}{\Delta t}, \quad (1)$$

where $\mathbf{v}(\mathbf{x}, t_i)$ is the velocity vector and $d\mathbf{x}(\mathbf{x}, t_i)$ is the displacement vector at position $\mathbf{x}$ and time t_i . Δt is the time difference between consecutive time frames.

To graphically represent velocity fields, vector directions are color coded using the scheme shown in the inset of figure 2(C). This procedure assigns ‘pure’ colors (blue, green, red and yellow) to the four directions along the axes of symmetry (left, anterior, right and posterior). An arbitrary direction is then represented as a blend (linear combination) of the two colors corresponding to the axes of the quadrant the direction is pointing to. To represent areas where the velocities are small (and the direction is ambiguous), we modified the color saturation (brightness) according to

$$h(\mathbf{x}) = h_{\max} \sqrt{v(\mathbf{x})/v_{\max}}, \quad (2)$$

where $v(\mathbf{x})$ is the magnitude of the velocity at $\mathbf{x}$, $h_{\max}$ is the maximal saturation and $v_{\max}$ is a suitably chosen upper limit, usually $30 \mu\text{m h}^{-1}$. The magnitudes of the velocity vectors are also presented on a linear scale in the supplementary figures available at stacks.iop.org/PhysBio/8/045006/mmedia.

For an arbitrary field $\phi(\mathbf{x}, t)$, temporal autocorrelations were calculated using the formula

$$C_t(\phi, \tau) = \frac{\langle \phi(\mathbf{x}, t) \phi(\mathbf{x}, t + \tau) \rangle_{\mathbf{x}, t}}{\langle \phi^2(\mathbf{x}, t) \rangle_{\mathbf{x}, t}}, \quad (3)$$

where $\langle \dots \rangle_{\mathbf{x}, t}$ denotes averaging over all possible locations $\mathbf{x}$ and times t . Similarly for spatial, in-plane autocorrelations:

$$C_r(\phi, r) = \frac{\langle \phi(\mathbf{x}, t) \phi(\mathbf{x}', t) \rangle_{|\mathbf{x} - \mathbf{x}'| \in B(r), t}}{\langle \phi^2(\mathbf{x}, t) \rangle_{\mathbf{x}, t}}, \quad (4)$$

where $\mathbf{x}$ and $\mathbf{x}'$ are in the same optical plane and $B(r)$ denotes the interval $[r - b; r + b]$. Our choice of the bin width b is $33 \mu\text{m}$ for the wide field and $1.5 \mu\text{m}$ for the confocal data. Spatial autocorrelations along the dorsal–ventral (z) axis were calculated for the confocal data as

$$C_z(\phi, z) = \frac{\langle \phi(\mathbf{x}, t) \phi(\mathbf{x}', t) \rangle_{\mathbf{x} - \mathbf{x}' = \pm z \mathbf{e}_z, t}}{\langle \phi^2(\mathbf{x}, t) \rangle_{\mathbf{x}, t}}, \quad (5)$$

where $\mathbf{e}_z$ denotes a unit vector along the dorsal–ventral axis.

Two-dimensional divergence of a velocity field $\mathbf{v}(\mathbf{r})$ has been calculated for each point $\mathbf{r} = (x, y)$ as

$$\text{div } \mathbf{v}(x, y) = \frac{\mathbf{v}(x+\Delta x, y) - \mathbf{v}(x-\Delta x, y)}{2\Delta x} + \frac{\mathbf{v}(x, y+\Delta y) - \mathbf{v}(x, y-\Delta y)}{2\Delta y} \quad (6)$$

where Δx and Δy are the horizontal and vertical grid spacings used in the refined PIV calculation, respectively.

4. Results

4.1. ECM movements

To study ECM movements during early embryonic development, quail embryos, between HH stages 4 and 6 (see supplementary figure S2 and movie 2 available at stacks.iop.org/PhysBio/8/045006/mmedia), were microinjected with fluorophore-conjugated antibodies against fibronectin or fibrillin-2. The immunolabeled specimens were recorded for 2–3 h using a wide-field automated microscope system (movie 3). ECM displacements were extracted from the obtained image sequences by cross-correlation (PIV) analysis (figure 2). The velocity field $\mathbf{v}(\mathbf{x}, t)$, at position $\mathbf{x}$ and time t , is calculated in a frame of reference co-moving with the somites; for example, the spatial average of $\mathbf{v}$ within the boxed area shown in figure 2(A) is zero (movie 4).

The obtained speeds of ECM movements can exceed $100 \mu\text{m h}^{-1}$, fast enough to substantially reorganize ECM distribution within a few hours (figure 3(A) and supplementary figure S5 available at stacks.iop.org/PhysBio/8/045006/mmedia). These movements are slightly faster in the earlier stages of development. The color variations in figure 2 occur over large distances; thus, the $\mathbf{v}(\mathbf{x}, t)$ velocity field describing ECM movement is smooth. In accord, the spatial autocorrelation functions (4) reveal that velocity vectors, separated by up to a half millimeter, are correlated (figure 3(B)). Furthermore, the observed movements are shared by both fibronectin and fibrillin. Embryos immunolabeled with both antibodies yielded almost identical vector fields for each ECM component: the cross-correlation coefficient is 0.91 ± 0.1 ($n = 4$).

Surprisingly, successive velocity field snapshots obtained in 15 min intervals show considerable variability (figure 2(D), movie 4). Still, the temporal autocorrelation function of the velocity data (3) is positive for several hours (figure 3(C)), which clearly indicates a movement pattern that is persistent in time. This persistent movement pattern, $\mathbf{V}(\mathbf{x}, t)$, gradually changes the structure of the ECM and is expected to be similar in embryos at the same developmental stage. We estimate $\mathbf{V}$ from a series of velocity field snapshots ($\mathbf{v}$) by suppressing the fluctuations. In figures 2(C) and (E) we present estimates obtained by local temporal averaging

$$\mathbf{V}_0(\mathbf{x}) = \langle \mathbf{v}(\mathbf{x}, t) \rangle_t, \quad (7)$$

or by a linear fit

$$\mathbf{V}_1(\mathbf{x}, t) = \mathbf{A}(\mathbf{x}) + \mathbf{B}(\mathbf{x})t, \quad (8)$$

where parameters $A(\mathbf{x})$ and $B(\mathbf{x})$ are chosen to minimize $\langle [V_1(\mathbf{x}, t) - \mathbf{v}(\mathbf{x}, t)]^2 \rangle_t$ for each location $\mathbf{x}$ (movie 5). Since the duration of the analyzed observations is 3–4 h, fitting higher order polynomials does not seem necessary. In the following we will use local linear fits (8) to estimate V .

4.2. Persistent motion pattern

Representative V_0 fields, calculated from embryos at various stages of development, are shown in figure 4. The complex movements can be best described by decomposing the pattern into parallel processes. Convergent-extension-like movements (see the supplementary data available at stacks.iop.org/PhysBio/8/045006/mmedia) take place lateral to the notochord: Hensen's node and the streak move caudally, the head region moves cranially, while the ECM surrounding the lateral plate mesoderm (LPM) moves medially. In the caudal embryo, along the streak, the LPM-associated ECM moves laterally. The combination of these movements results in a vortex-like pattern on both sides of the embryo. The region where the ECM converges medially elongates with progressively later stages of development. The moving boundary separating medial convergence from the more caudal domain, characterized by lateral expansion, is often asymmetric: on the right side of the image (left side of the embryo) the domain of medial-ward movement is larger, and lateral expansion takes place only at more caudal positions (see the dashed lines in figure 4). The formation of the future foregut is also noticeable as early as HH stage 5 as a distinct, cranio-laterally moving region projecting from the second pair of somites.

The divergence of the velocity field is a straightforward measure to characterize the capacity of these movements to accumulate or disperse the ECM. The local mass-balance equation for the 2D projected ECM density ρ is

$$(\partial_t + \mathbf{V} \cdot \text{grad})\rho = -\rho \text{div} \mathbf{V}. \quad (9)$$

Thus, the local accumulation or dilution of the ECM during a short time frame Δt can be expressed as

$$\frac{\Delta \ln \rho}{\Delta t} \approx -\text{div} \mathbf{V}. \quad (10)$$

Divergence fields obtained from the embryo in figure 2 are shown in movies 7 and 8, for $\text{div} \mathbf{v}$ and $\text{div} \mathbf{V}_1$, respectively. As shown in figure 4(E), in earlier stages the divergence is negative (ECM accumulates) in the cranial embryo, while it is positive (ECM is dispersed) around the primitive streak. The most obvious structures are two stripes parallel to the embryonic axis where the ECM accumulates. The location of these stripes is coincident with the somitic and presomitic mesoderm, and the accumulation continues even after the formation of the first few somites. The rate of the motion-related increase in ECM density can be substantial, as high as 50 %/h.

4.3. Fluctuations

We attribute the difference $\mathbf{u}$ between a velocity snapshot $\mathbf{v}$ and the persistent motion pattern $\mathbf{V}$ to intrinsic fluctuations within the tissue. Representative velocity fluctuations are shown in figure 2(F), obtained from three consecutive images of movie 6. The magnitude (figure 5(A)) and the spatial extent (figure 5(B)) of the fluctuations are somewhat smaller, but comparable to those of $\mathbf{V}$ (figure 3).

The fluctuations $\mathbf{u}$, however, are changing rapidly: consecutive snapshots at 15 min interval are already uncorrelated (figure 5(C)). The integral of the autocorrelation function is expected to be zero for a stationary signal with zero mean. Therefore, any short-range positive correlations are expected to be offset by longer range (negative) anticorrelations. These anticorrelations are, however, finite size effects in the sense that their magnitude is expected to decrease by increasing the length of observations. In figures 5(C) and (D), the horizontal gray lines estimate the anticorrelations expected in a stationary finite sample with the corresponding positive autocorrelation structure and zero mean.

The fluctuations result in local contractions and expansions of the ECM in apparently random patterns (figure 6, movie 9) which are similarly short lived (figure 5(D)) and which lack an obvious correlation with anatomical structures. In summary, data presented in figures 2–6 demonstrate that ECM movement during morphogenesis is very *noisy*. While one can extract a smooth and persistent pattern which is shared across embryos at a given developmental stage, a velocity snapshot taken at an arbitrary time point is so distinct that their cross-correlation value is only 0.5.

4.4. High-resolution confocal data

To gain additional independent information on ECM fluctuations, we obtained higher spatial and temporal resolution confocal recordings of embryos immunolabeled for fibronectin (movie 10) and fibrillin-2 (movie 11). These recordings cover a smaller, $150\ \mu\text{m} \times 150\ \mu\text{m}$ area, and thus do not yield information regarding the persistent large-scale motion pattern $\mathbf{V}$. Instead, the ~ 10 s long sampling cycles (frame rates) allow a higher resolution characterization of the dynamics of the fluctuations. Furthermore, the ability to restrict imaging to $0.7\ \mu\text{m}$ thin optical sections allows us to compare ECM movements along the dorso-ventral (DV) axis.

To estimate shifts of the specimen and movements of the surrounding tissue, we fitted a space-independent $\mathbf{V}_1(t)$ linear function, analogous to (8), over the spatial average of all observed velocity vectors, i.e. we minimized

$$\langle [\mathbf{V}_1(t) - \langle \mathbf{v}(\mathbf{x}, t) \rangle_{\mathbf{x}}]^2 \rangle_t. \quad (11)$$

Then, the local velocity vectors characterizing ECM fluctuations were obtained as

$$\mathbf{u}(\mathbf{x}, t) = \mathbf{v}(\mathbf{x}, t) - \mathbf{V}_1(t). \quad (12)$$

Figure 7(A) shows a representative example for in-plane ECM movements $\mathbf{u}$ within a single optical section. To compare movements across the DV axis, 4–7 planes, each separated by $3\text{--}9\ \mu\text{m}$, were imaged to ensure that the collection of the planes is completed within 15 s. The velocity autocorrelation plot for the DV axis reveals that while the ECM movement is not identical across the embryo, it is clearly positively correlated (figure 7(B)). The autocorrelation magnitudes as well as the spatial decay along the DV axis are comparable to those obtained for the in-plane (horizontal section) spatial correlations (figure 7(C)), which in turn are consistent with those obtained from wide-field recordings (figure 5(B)). The correlation time of the fluctuations is, despite their large extent, less than a minute (figure 7(D)).

5. Discussion

The persistent flow field $\mathbf{V}$ and various related derived quantities such as divergence are convenient ways to describe the typical ECM movements that occur during morphogenesis, and provide much higher spatial resolution than our previous attempts ([9, 10]). In particular, the accumulation of the ECM (negative $\text{div}\mathbf{V}$) observed in the somitic and presomitic mesoderm is consistent with the fact that the ECM density around the somites is high, and some of its constituent filaments are obtained from lateral areas [9]. As we only traced a 2D projection of ECM movements, an increase in the 2D projected density can reflect both an increase in the physical (3D) density and a thickening of an ECM-rich volume.

ECM filaments are especially suitable to map tissue movements since the ECM meshwork (unlike a cell layer) is an inert object incapable of active movements. In addition, the ECM is the microenvironment for some embryonic cell types such as mesenchymal cells or early vascular precursor cells. Thus, movement of the whole ECM meshwork is expected to carry any passive, adhesive cell. Furthermore, considering the tissue as a composite material of cells and the ECM, we expect that even passive objects not adherent to the ECM would move in a similar fashion. In fact, the co-movement of the ECM and injected microbeads was observed in recent experiments exploring heart development [46] and Hydra development [11]. These studies also demonstrated the co-movement of endogenous and microinjected exogenous ECM components.

The observed left–right asymmetry in ECM movements around Hensen’s node complements recent results indicating the rotation of the node in avians [47, 48]. While previous investigations focused on individual cell movements at somewhat earlier stages of development, our data suggest the sustained presence of a counterclockwise rotating tissue movement around the node (in a ventral view).

The fact that the ECM of early embryos moves substantially also affects modeling efforts [49]. Theoretical models operating with morphogen gradients should take into account that the concentration fields are likely to deform through these movements. Recent experimental studies revealed that active cell movements (relative to the surrounding ECM) are more random than previously suggested [7, 50]. A substantial portion of the apparent non-random ‘drift’ motion is a result of passive cell movements.

The identification of the motors driving the observed ECM (and tissue) movements remains a frustrating problem of developmental biology and tissue mechanics. Knowledge about residual stresses within the tissue is essential to conclusively map the driving forces of this motion [51–53]. One force driving the observed movements is likely to be the contractile activity of cells. Here, we speculate that the observed divergence pattern is consistent with an anisotropic, lateral contraction by the cells of the presomitic and somitic mesoderm, which are clearly functionally distinct from the mesodermal cells of the lateral plate [50].

The biological relevance of the fluctuating movement of ECM filaments is unknown. *In vitro* studies of ECM assembly revealed similar behavior, with spatial correlations well exceeding 100 μm (see figure 6(b) of [34]). Such continuous stretching and releasing of the filaments may reveal cryptic binding sites, which have been demonstrated by *in vitro* analysis of fibronectin [54, 55]. The long correlation length is consistent with the idea that the tissue is in mechanical equilibrium; therefore, a local change in cell traction is expected to immediately alter tissue deformations elsewhere.

Cells can actively remodel, pull and drag the ECM. Thus, our view according to which cells move relative to a conveyor belt such as the ECM (i.e. active cell motion is the difference

between local cell and ECM movements) is obviously an approximation. As an example, one can imagine a scenario in which individual ECM filaments are pulled within a completely non-motile array of cells. Sufficiently large displacements between two interwoven three-dimensional structures (the ECM and cells), however, are difficult to envision. Furthermore, recent experiments revealed that in the case of an actively moving cell population, vascular sprout cells, the local ECM rearrangement accompanying cell invasion is negligible compared to the magnitude of the observed fluctuating ECM motion [56]. Thus, for tissue-scale movements, the ECM likely acts as an inert conveyor belt. On much smaller scales individual cells can slowly alter its structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH R01 grants HL087136 (AC), HL085694 (BJR), HL068855 (CDL), GM76692 (AC and CDL, PI is James A Glazier, Indiana University); the Hungarian Research Fund OTKA K72664 (AC), and the G Harold & Leila Y Mathers Charitable Foundation (AC, CDL, BJR).

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Figure 1.

Fibronectin (red) and fibrillin-2 (green) filaments, visualized by confocal microscopy. *A*: maximum intensity projection of a $300\ \mu\text{m} \times 300\ \mu\text{m}$ large and $60\ \mu\text{m}$ deep volume onto a frontal (xy) plane (see supplementary data for anatomical terms available at stacks.iop.org/PhysBio/8/045006/mmedia). *B*: a $300\ \mu\text{m}$ long, $30\ \mu\text{m}$ wide and $60\ \mu\text{m}$ deep sub-volume, projected onto a transverse (xz) plane (similar to supplementary figure S4, section y, available at stacks.iop.org/PhysBio/8/045006/mmedia). While the two ECM components are not completely colocalized, they form a composite meshwork. The ECM accumulates at the dorsal (d) and ventral (v) sides of the mesoderm, but filaments are also seen traversing the mesoderm. The approximate location of Hensen's node is marked by an

asterisk. See movie 1 (available at stacks.iop.org/PhysBio/8/045006/mmedia) for the corresponding three-dimensional visualization.

Figure 2.

Calculation of ECM movements. A three somite (HH stage 7) embryo is shown, using both DIC (panel A) and anti-fibrillin-2 immunofluorescence (panel B) optics. Major annotated anatomical landmarks are the area opaca (ao), notochord (nc), Hensen's node ($\odot$) and the first (S1) and third (S3) somite pairs (see supplementary figure S2 available at stacks.iop.org/PhysBio/8/045006/mmedia). The boundary demarcating the extraembryonic area opaca is marked with dotted lines. C: average ECM movements V_0 characterizing the entire 3h long recorded time period. As described in section 3.4, the velocity directions are color coded according to the scheme shown in the inset: left, right, anterior and posterior directions are indicated with blue, red, green and yellow colors, respectively. The color saturation (brightness) increases with the speed of movement, which is presented in the supplementary figure S5 available at stacks.iop.org/PhysBio/8/045006/mmedia. The velocity field is presented in a frame of reference co-moving with the region containing the somites (boxed area). D: ECM movements between consecutive frames, as obtained by PIV analysis. The three snapshots (Da, Db and Dc) are separated by 15 min. E: fluctuations are reduced by fitting the velocity field V_1 at each grid point of analysis. The V_1 vector fields shown in panels Ea, Eb and Ec correspond to the velocity snapshots shown in panels D. F: fluctuations, the difference between the velocity snapshots and the fitted V_1 field. The asterisks mark a region where fluctuations reversed direction during the time period shown. Movies 2–6 (available at stacks.iop.org/PhysBio/8/045006/mmedia) correspond to panels A, B, D, E and F, respectively.

Figure 3.

Statistical characterization of ECM movements at three stages of development. *A*: speed distribution (density), binned into $10 \mu\text{m h}^{-1}$ wide categories. *B*: spatial autocorrelation of the velocity field. *C*: temporal autocorrelation of the velocity field. *D*: temporal autocorrelation of the divergence field. Data are averaged from four (HH5, red), five (HH6, green) and ten (HH7, blue) embryos. The error bars represent standard deviations.

Figure 4.

Persistent ECM movements during progressively later stages of development. *A*: a ventral view of embryo morphology, visualized by DIC optics. Anatomical features such as somites, the notochord and Hensen's node are marked with boxes, a vertical line and a circle, respectively. *B*: ECM immunofluorescence. *C*: magnitude and directionality of the persistent ECM movement pattern, represented by intensity and color as in figure 2(C). Arrows indicate motion direction at selected positions as a guide to the eye. *D*: speed magnitudes are color coded according to the scale in the inset. Speed values are given in $\mu\text{m h}^{-1}$. *E*: divergence of ECM movements (V_0), color coded according to the scale in the inset (blue: dilution, red: accumulation). Rows *a–d* contain data obtained from the same embryo. The dashed lines mark the boundary between the medially and laterally moving regions of the lateral plate mesoderm. Artifacts of the image-tiling process are marked by asterisks.

Figure 5.

Statistical characterization of ECM fluctuations at three stages of development. *A*: speed distribution (density), binned into $10 \mu\text{m h}^{-1}$ wide categories. *B*: spatial autocorrelation of the velocity fluctuations. *C*: temporal autocorrelation of the velocity fluctuations. *D*: temporal autocorrelation of the divergence of fluctuations. Horizontal gray lines indicate the anticorrelations expected in a stationary finite sample with zero mean. Data are averaged from four (HH5, red), five (HH6, green) and ten (HH7, blue) embryos. The error bars represent standard deviations.

Figure 6.

Fluctuating divergence of ECM movements. *A, B*: divergence was calculated from consecutive velocity fields, separated by 15 min. *C*: divergence of the persistent flow field $\mathbf{V}$. *D, E*: divergence of the velocity fluctuations, corresponding to the same frames shown in panels *A* and *B*, respectively. Movies 7–9 (available at stacks.iop.org/PhysBio/8/045006/mmedia) correspond to panels *A, C* and *D*, respectively.

Figure 7.

Fluctuating ECM movements, assessed by confocal microscopy. *A*: a single, 0.7 μm thick optical plane of fibrillin-2 immunofluorescence. The PIV-estimated velocity vectors are superimposed: The green lines indicate the calculated displacements of the red dots. As the ECM layer separating the mesoderm from the endoderm is not perfectly flat, only an irregular area within the optical section contains high ECM density. *B*: velocity autocorrelations in the dorso-ventral (*z*) direction. *C*: velocity autocorrelations within the frontal (*xy*) planes. *D*: temporal autocorrelation of ECM movements.