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Revealing epithelial morphogenetic mechanisms through live imaging

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Epithelial morphogenesis is guided by mechanical forces and biochemical signals that vary spatiotemporally. As many morphogenetic events are driven by rapid cellular processes, understanding morphogenesis requires monitoring development in real time. Here, we discuss how live-imaging approaches can help identify morphogenetic mechanisms otherwise missed in static snapshots of development. We begin with a summary of live-imaging strategies, including recent advances that push the limits of spatiotemporal resolution and specimen size. We then describe recent efforts that employ live imaging to uncover morphogenetic mechanisms. We conclude by discussing how information collected from live imaging can be enhanced by genetically encoded biosensors and spatiotemporal perturbation techniques to determine the dynamics of patterning of developmental signals and their importance for guiding morphogenesis.

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Introduction

During morphogenesis, epithelia respond to physical and biochemical signals with spatiotemporal precision to establish the final tissue forms required for functional organs. For decades, genetic manipulations combined with images of fixed specimens have helped uncover the molecular machinery that instructs epithelial morphogenesis. However, many dynamic cellular processes that drive morphogenesis (e.g. cell division, migration, rearrangements, signaling activity, gene expression) are missed when a sample is analyzed at a single point in time (Figure 1). For instance, the convergent extension of epithelial tissues results from cell rearrangements, but the dynamics of such movements cannot be observed without monitoring individual cells over developmental time [1]. Similarly, traveling waves of signaling activity propagate across tissues to guide morphogenesis [2,3•,4], but identifying such phenomena requires the ability to visualize signaling within the same specimen at multiple time points.

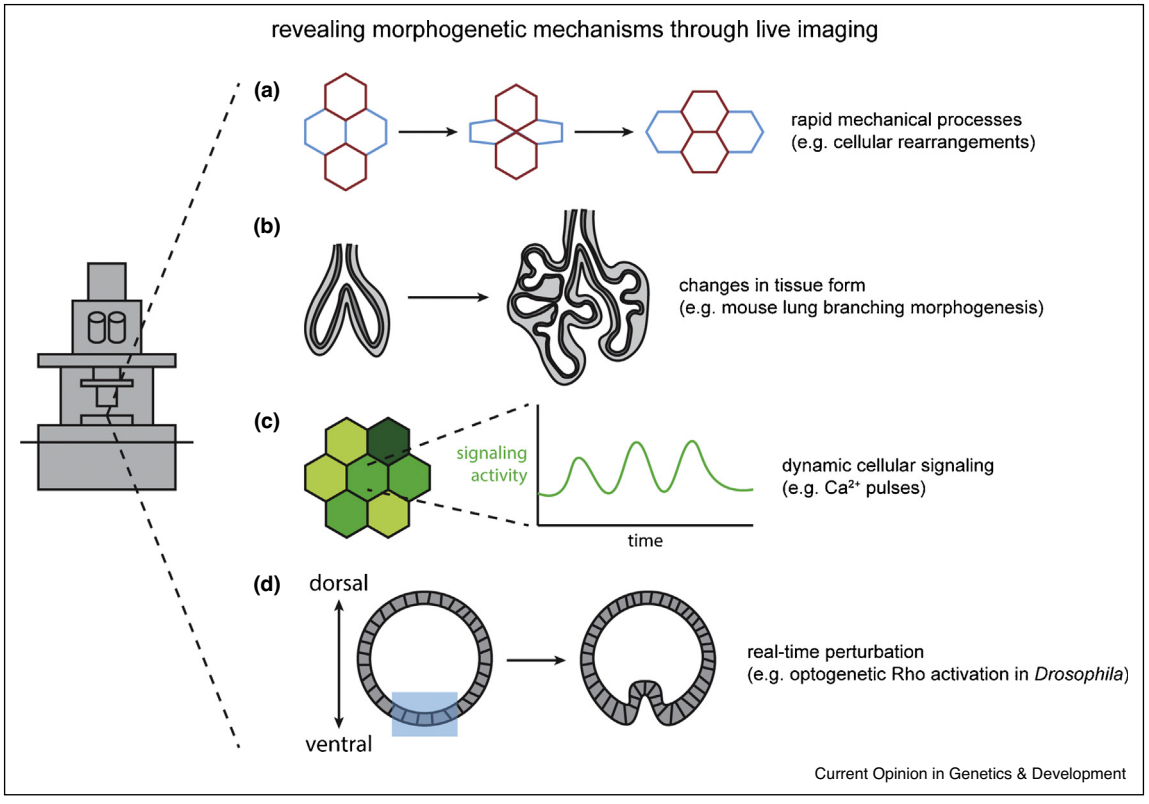
Advances in live imaging, fluorescent biosensors, and spatiotemporal perturbation approaches are enabling investigators to monitor and manipulate morphogenetic processes as they occur. In this review, we focus on recent insights into epithelial morphogenesis that were made possible by live imaging of developing systems. We begin with an overview of imaging technologies that have enabled real-time visualization of developing tissues with high spatiotemporal resolution and low phototoxicity. Next, we discuss recent studies in which live imaging was employed to reveal cellular shape changes, rearrangements, and movements that build tissue structures. We then discuss how live imaging has begun to reveal morphogenetic mechanisms in developing organs, such as the lung, which historically have been challenging to monitor with high spatiotemporal resolution. Finally, we suggest ways in which live imaging can be combined with genetically encoded biosensors and spatiotemporal perturbation approaches to answer outstanding questions about the processes of epithelial morphogenesis.

Strategies for live fluorescence imaging

To monitor developing systems by fluorescence microscopy, images at sequential time points must be constructed from photons emitted by the specimen, which contains a limited number of fluorophores. Consequently, spatial resolution, temporal resolution, signal-to-noise ratio, and the duration of imaging are parameters that must be optimized while accounting for photobleaching and phototoxicity [5]. These parameters are constrained by both the developing system of interest, as well as the method of imaging.

There are several imaging modes that can be used for real-time analysis of developing tissues. The simplest is widefield fluorescence microscopy. Here, the specimen is illuminated with light at specific wavelengths and a camera is used to capture emitted fluorescence. Because fluorescence is captured from the entire z-axis (thickness) of the sample, three-dimensional (3D) resolution is not

Figure 1



By monitoring developing systems in real-time, **(a)** rapid mechanical processes, **(b)** large-scale changes in tissue form, and **(c)** dynamic cellular signaling can be analyzed and combined with **(d)** real-time perturbations to understand the mechanisms driving morphogenesis.

possible without image deconvolution. Able to circumvent this challenge, confocal fluorescence microscopy is a relatively accessible method for 3D time-lapse imaging of developing systems [6]. Lasers illuminate selected regions of the sample in the *xy*-plane and a pinhole prevents the transmission of fluorescence from out-of-plane regions, thereby providing 3D resolution. However, out-of-plane illumination remains destructive to the sample due to phototoxicity. Destructive illumination is particularly challenging when using laser-scanning confocal microscopy. In this approach, a point laser illuminates a small region of the sample in the *xy*-plane and scans across the sample to produce a rasterized image. For real-time imaging, scanning across each imaging plane of a specimen is often too slow to capture relatively fast morphogenetic processes, and can result in phototoxicity to the sample. Spinning disk confocal microscopy increases the speed of imaging by passing the light simultaneously through multiple pinholes located on an opaque spinning disk. This approach provides faster imaging speed and lower phototoxicity at the expense of less resolution in the *z*-dimension, as compared to laser-scanning confocal.

More recently, developing tissues have been imaged using two-photon microscopy [7]. Rather than illuminate samples with a wavelength of light sufficient to excite fluorophores with a single photon, higher-wavelength light is used to excite fluorophores with two, lower-energy photons. This excitation is limited to the focal point, preventing photobleaching and photoxicity from out-of-plane excitation and allowing larger depths of imaging due to less absorbance of the exciting photons. As with laser-scanning confocal, the speed of image acquisition remains an issue.

Often the ideal approach for imaging large samples with high speed and low phototoxicity, light-sheet fluorescence microscopy (LSFM) has gained popularity for monitoring developing systems in real-time. In LSFM, a thin sheet of light is illuminated across the sample using one objective, and the emitted fluorescence is collected using another perpendicularly oriented objective. The sheet of light is confined to the plane of interest and photons from the imaged plane are simultaneously detected by a camera, enabling large regions of a sample to be imaged with high speed and low phototoxicity.

Despite these advantages, LSFM is still limited by light scattering and aberrations that are inherent to thick specimens. Recently developed LSFM strategies, such as dual selective-plane illumination (diSPIM) [8,9], multiview selective-plane illumination (MuVi-SPIM) [10], and isotropic multiview (IsoView) microscopy [11], have improved spatiotemporal resolution by collecting and deconvolving images collected from multiple sheets of light.

Some developmental events involve growth over several orders of magnitude, which limits the duration over which specimens can be monitored. Imaging multiple, adjacent regions of a specimen to generate a mosaic image can help capture larger portions of a sample, but changes in optical properties and the geometry of the sample may require one to adapt imaging strategies throughout the experiment. McDole *et al.* introduced an adaptive LSFM technique that continuously optimizes spatial resolution of rapidly growing specimens by tracking their size and movement, and correspondingly adjusting the imaging volume and focus at reference regions [12•]. This adaptive LSFM approach enabled *in toto* imaging of mouse embryogenesis over a two-day period, which was used to build a dynamic atlas of post-implantation mouse development [12•]. Excellent summaries of the uses of light-sheet imaging in studies of development have been written elsewhere, which we recommend for those interested [5,13].

Tracking cellular flows

During morphogenesis, cells dynamically change their shapes and neighbors. These changes occur over minutes to hours, and understanding the relative role of cellular flows in morphogenesis requires imaging at high spatiotemporal resolution. In the early *Drosophila* embryo, tissues fold on the scale of minutes (Figure 2a) and this folding is driven by neighbor exchanges (Figure 2b) and pulses of actomyosin contraction (Figure 2c). By imaging cells within the ventral furrow of the *Drosophila* embryo every 5–6 s, Martin *et al.* revealed pulses of actomyosin contraction that promote a change in cell shape known as apical constriction, which is required for gastrulation [14]. Subsequent work using live imaging revealed that this apical constriction requires F-actin turnover, which links the actomyosin network to adherens junctions to balance the transmission of intercellular forces [15]. Similar pulses of actomyosin contraction drive the shrinkage of dorsal-ventral-oriented junctions to facilitate germband extension in the *Drosophila* embryo [16,17] (Figure 2c), and are observed during gastrulation [18] and neurulation [19] in the *Xenopus* embryo, suggesting pulses of actomyosin contraction are a conserved driver of morphogenetic processes.

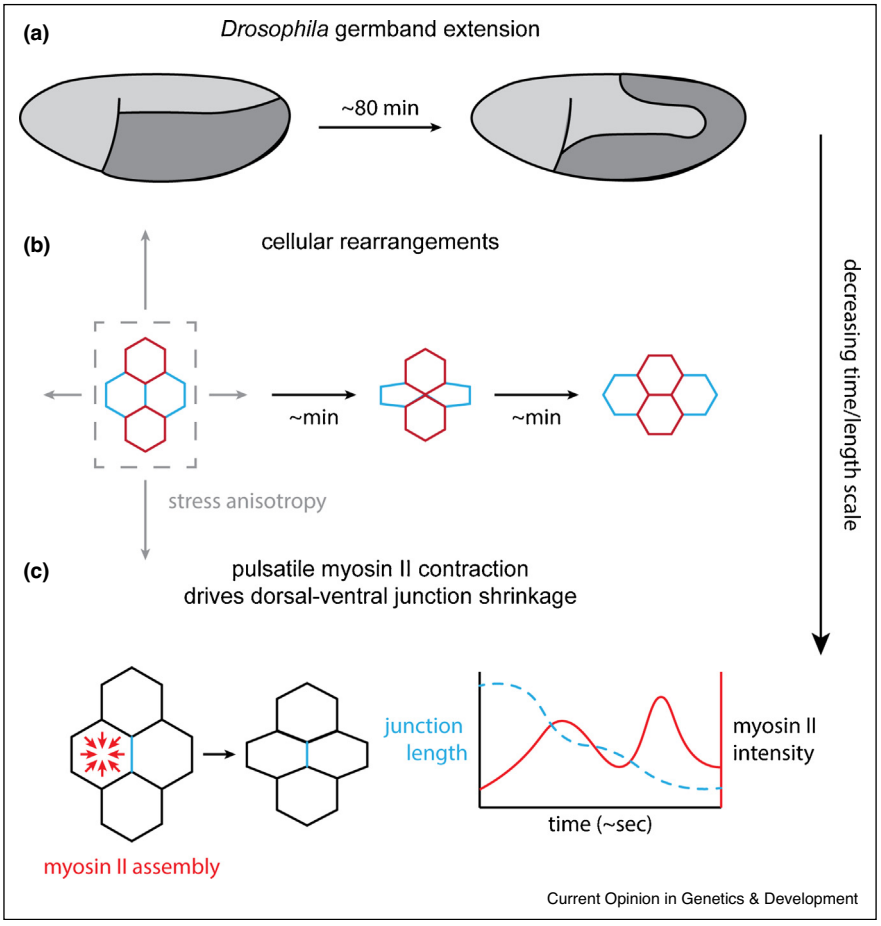
Live imaging of epithelial tissues has revealed dynamic collective motions that range from fluid-like (rapid rearrangements) to solid-like (relatively static). The transition between these two states is known as a jamming transition [20,21]. Such flows are not limited to cells in culture; unjamming of cells in the *Drosophila* embryo permits germband extension, where an increase in neighbor arrangements promotes convergent extension of the tissue [1] (Figure 2b). Whereas unjamming during *Drosophila* germband extension is driven by changes in cell shape and alignment, a density-dependent jamming transition is thought to underlie anterior-posterior axis elongation in the developing zebrafish embryo [22]. Fluid-like tissue behavior is also critical to events such as gastrulation in the quail embryo, where a ring of contractile actomyosin at the boundary between the extraembryonic tissue and embryo proper drives vortical flows, giving rise to the primitive streak [23•]. In this case, fluid-like movements in the embryo require cellular rearrangements, which are downstream of cell division [24].

Vortical flows have also been observed during other stages of development; while these flows occur on the millimeter-scale in the quail embryo [23•], similar flows occur on the scale of hundreds of micrometers in other systems [25,26]. Embryo-wide cellular flows during *Drosophila* germband extension include vortical flow patterns at the anterior and posterior ends of the embryo [26]. To try to understand the origins of these cellular flows, Streichan *et al.* were able to predict morphogenetic movements during *Drosophila* gastrulation by combining *in toto* live imaging with a continuum mechanics model, in which local mechanical stresses were estimated from the distributions of myosin [26]. During morphogenesis of mammalian skin, polarized hair follicles arise from symmetric, multicellular placodes. Monitoring hair follicle formation in explanted embryonic mouse skin revealed that anterior–posterior polarization and asymmetric specification of cell fate arise from counter-rotational cell movements that are promoted by actomyosin contractility downstream of planar cell polarity signaling [25]. Taken together, the studies described above underscore how tissue morphogenesis results from rapidly occurring cellular processes, making live imaging a necessary tool for understanding organismal development.

Tracking changes in tissue form: branching morphogenesis

Challenges inherent to imaging thick, opaque specimens have confined much of our understanding of morphogenesis to early developmental stages and transparent model systems. For instance, time-lapse analysis of the development of branched organs such as the lung has been restricted to cultured explants, where light scattering still limits spatial resolution. Nevertheless, recent efforts to image lung branching morphogenesis have uncovered the physical forces that drive shape changes of the airway epithelium. Live imaging has revealed a high degree of tissue fluidity in the mesenchyme of the embryonic chicken lung, which is thought to facilitate matrix

Figure 2



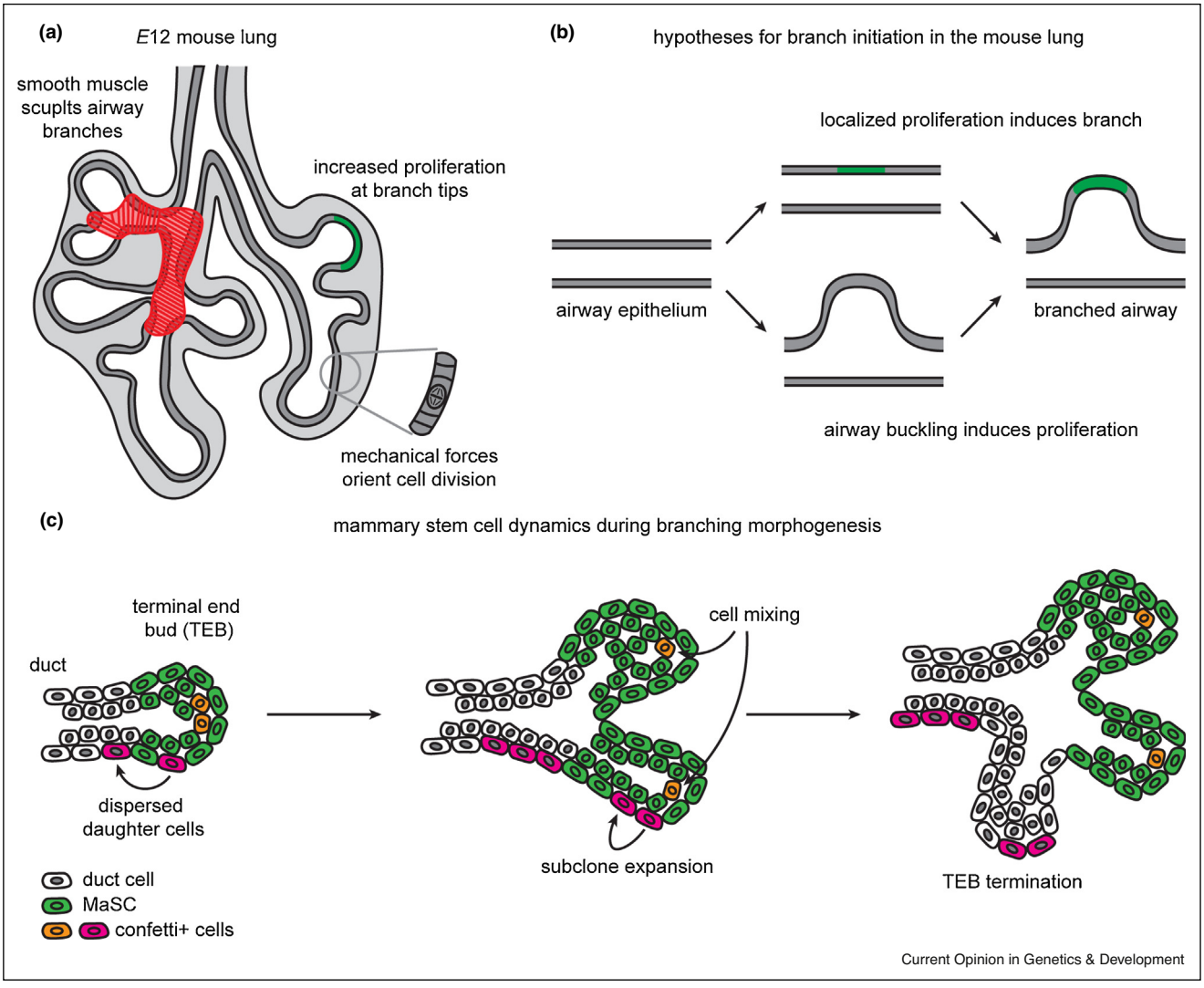
Time scales of epithelial morphogenesis. **(a)** Morphogenesis of the *Drosophila* germband epithelium occurs through convergent extension of the tissue, which is promoted by **(b)** cell intercalation caused by anisotropic stresses [1]. **(c)** To facilitate convergent extension of the germband epithelium, dorsal-ventral-oriented junctions are shortened by pulses of actomyosin contractility [16,17].

remodeling during branching morphogenesis [27]. Imaging the dynamics of airway smooth muscle differentiation in embryonic mouse lung explants revealed that this process provides mechanical forces that sculpt both terminal bifurcations [28] and domain branches [29] (Figure 3a). Branch formation also requires oriented cell divisions that are regulated by mechanical forces [30,31]. Real-time tracking of cell-division events in the airway epithelium revealed two different dynamic behaviors of the mitotic spindle before division, which result in different distributions of cell division angles that affect the overall shape of the airway [31] (Figure 3a).

While airway epithelial cells remain in the epithelium during cell division, cells at the tips of branches in the early ureteric bud tend to delaminate from their neighbors and divide in the lumen. One daughter cell maintains a thin protrusion that contacts the basal surface of the tissue, while the other daughter cell reintegrates into the epithelium at a site up to three cell diameters away [32].

Similar phenomena are observed during branching of both the pancreas [33] and salivary gland [34]; in these organs, live imaging revealed that cells at the tips of buds delaminate from the extracellular matrix (ECM), enter the body region of the epithelium where they divide, and reintegrate into the cap region as dispersed daughter cells. While the importance of cell mixing for tissue form and cell fate across branched organs remains unclear, lineage tracing combined with intravital imaging has helped reveal a critical role for cell mixing in branching morphogenesis of the mammary gland. Mammary stem cells that disperse into distal portions of the epithelial tree lose their self-renewing capability and exit the cell cycle after a handful of divisions. Thus, the presence of mammary stem cells at terminal end buds determines whether branches will bifurcate or terminate [35] (Figure 3c). As live imaging approaches continue to be applied to large-scale morphogenetic events whose analyses have historically been limited to single time points, we will expectedly discover novel morphogenetic mechanisms,

Figure 3



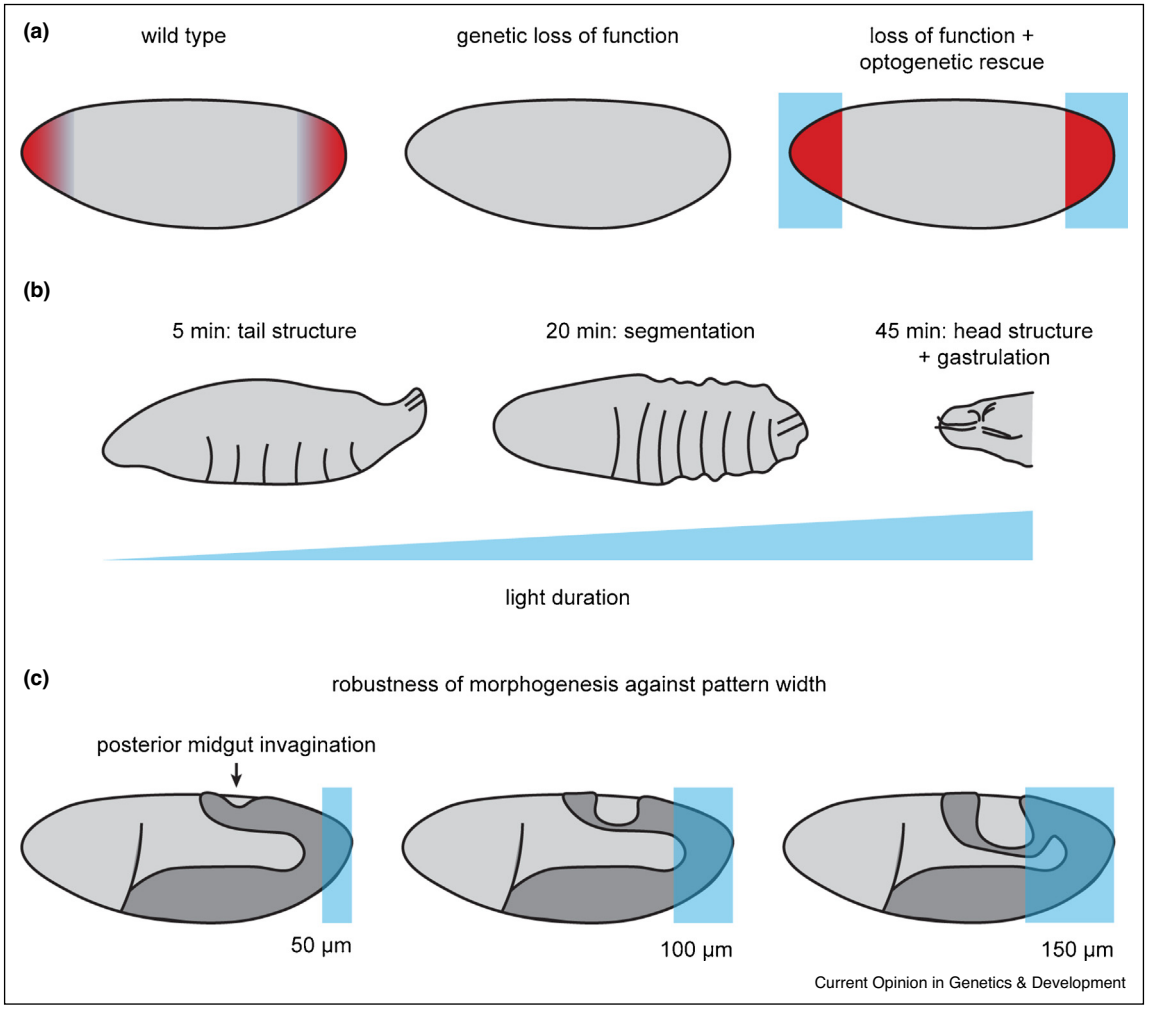
Mechanisms of branching morphogenesis revealed by live imaging. **(a)** Airway smooth muscle wraps around the epithelium to sculpt terminal bifurcations and domain branching. Within the epithelium, mechanical forces orient cell division along the long axis of the airway. **(b)** Whereas increased proliferation is observed in cells located at the tips of branches in the developing lung, it remains unclear whether branches are initiated by localized proliferation, or whether branches are initiated by buckling of the epithelium. **(c)** Proposed model for mammary branching morphogenesis: mammary stem cells (MaSCs) at terminal end buds (TEBs) expand and mix, but differentiate and exit the cell cycle after several rounds of division upon leaving the TEB. After TEB bifurcation, a fraction of TEBs terminate, while others continue to branch [35].

as well as similarities in morphogenesis across organisms and/or tissues.

Enriching information gained from live imaging

To date, many insights into the dynamics of epithelial morphogenesis were made possible in part by relatively simple nuclear and membrane markers as a means of tracking cellular motion. How can we better understand the source of tissue-wide movements and their influence on other aspects of development? Several biochemical patterning events correlate with mechanically driven morphogenetic processes. For instance, Erk signaling is enriched at branch tips in the developing mouse lung [36]. In many cases, however, it is unclear whether biochemical patterning is a consequence of tissue mechanics, or vice versa. Fluorescent reporters of gene expression [37,38], kinase activity [2,39,40], cytoskeletal signaling [14–19], and mechanical stress [41,42] have all garnered use in studies of developing tissues and can be combined with markers used for simple cellular tracking. For example, Ishii *et al.* recently used a FRET-based reporter to monitor Erk signaling in the developing murine cochlear duct, which revealed traveling waves of Erk activity that guide collective cell migration towards the apex tip of the duct, facilitating its elongation [3•].

Figure 4



Optogenetic rescue of a patterning mutant. **(a)** Genetic loss of function combined with optogenetic control of signaling can be used to recapitulate biochemical signaling patterns observed during embryogenesis. **(b)** Temporal control over signaling makes it possible to determine durations of signaling necessary to specify tissue structures during development. **(c)** Spatial control over signaling can be used to test the robustness of morphogenetic events against aberrant signaling patterns [45•].

Similar traveling waves of Erk activity influence other developmental processes, such as scale regeneration in zebrafish [2] and invagination of the tracheal placode in the *Drosophila* embryo [43], further highlighting the importance of spatiotemporal signaling dynamics for shaping tissues. In the *Xenopus* embryo, neural tube closure is driven by actin-based apical constrictions that are preceded by cell-autonomous, asynchronous pulses of Ca^{2+} signaling, a finding that was enabled by combining biosensors for Ca^{2+} and cytoskeletal signaling [19]. A growing toolbox of biosensors for genes and signaling pathways will reveal whether other biochemical signals cooperate with physical forces to shape tissues within model organisms.

Towards more precise tests of necessity and sufficiency

Genetic knockouts can help determine whether a signal is necessary for morphogenesis, but the signals being manipulated may only be important for a short window of time, or in a small population of cells. Thus, disrupting gene expression in a permanent, tissue-wide manner cannot permit one to uncover the times and locations at which signals are employed during morphogenesis. Optogenetic tools for modulating protein activity using light have been used to reveal the biochemical and physical signals that are necessary and/or sufficient for morphogenesis [44]. By combining a genetic loss of Erk signaling at termini of the *Drosophila* embryo with

optogenetic control of the Ras/Erk cascade, Johnson *et al.* revealed that 90 min of Erk signaling at embryo termini is sufficient to rescue normal development [45•] (Figure 4a, b). Moreover, posterior midgut invagination and germband extension are robust to a 3-fold variation in the width of the terminal Erk signaling region, demonstrating the robustness of these morphogenetic mechanisms against a broad range of biochemical signaling patterns (Figure 4c). Both optogenetic inhibition [46] and activation [47] of cytoskeletal signaling in the *Drosophila* embryo have been used to elucidate contractile events driving gastrulation. Notably, optogenetic control of RhoGEF2 was used to reveal that Rho activation is sufficient to drive apical constriction and tissue folding reminiscent of gastrulation [48]. A multitude of other optogenetic tools have been employed in developing systems to perturb signaling, including the Notch/Delta pathway [49], the morphogen Bicoid [50], and the transcription factor Twist through a generalizable tool for nuclear export [51]. Thus, spatiotemporally perturbing and monitoring development in real time will likely be an increasingly accessible tool for studying morphogenesis.

In closing, advances in live imaging, fluorescent biosensors, and spatiotemporal perturbation techniques have enabled elucidation of the cellular processes that are responsible for driving epithelial morphogenesis, largely in specimens of small size and high optical transparency. Even within imaging-amenable systems such as *Drosophila*, however, much remains to be understood about how biochemical signaling, gene expression, and physical forces cooperate to shape epithelial tissues. Within larger systems such as the developing mouse lung or mammary gland, even less is known about the minutes-scale dynamics of cellular processes and the patterning instructions that are required to sculpt these organs. Efforts to expand these live-imaging techniques to more complex developing systems will shed light on morphogenetic mechanisms that share similarities with other organisms and/or tissues, while also revealing novel mechanisms that have thus far remained undiscovered.

Conflict of interest statement

Nothing declared.

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