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Roadmap on multiscale coupling of biochemical and mechanical signals during development

Pierre François Lenne, Edwin Munro, Idse Heemskerk, Aryeh Warmflash, Laura Bocanegra-Moreno, Kasumi Kishi, Anna Kicheva, Yuchen Long, Antoine Fruleux, Arezki Boudaoud, et al.

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Roadmap on multiscale coupling of biochemical and mechanical signals during development

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Abstract

How interactions between mechanics and biochemistry lead to the emergence of complex cell and tissue organization is an old question that has recently attracted renewed interest from biologists, physicists, mathematicians and computer scientists. Rapid advances in optical physics, microscopy and computational image analysis have greatly enhanced our ability to observe and quantify spatiotemporal patterns of signalling, force generation, deformation and flow in living cells and tissues. Powerful new tools for genetic, biophysical and optogenetic manipulation are allowing us to perturb the underlying machinery that generates these patterns in increasingly sophisticated ways. Rapid advances in theory and computing have made it possible to construct predictive models for how cell and tissue organization and dynamics emerge from the local coupling of biochemistry and mechanics. Together, these advances have opened up a wealth of new opportunities to explore how mechanochemical patterning shapes organismal development.

In this Roadmap, we present a series of forward-looking case studies on mechanochemical patterning in development, written by scientists working at the interface of the physical and biological sciences, and covering a wide range of spatial and temporal scales, organisms and modes of development. Together, these contributions highlight the many ways in which dynamic coupling of mechanics and biochemistry shape biological dynamics: from mechanoenzymes that sense force to tune their activity and motor output, to collectives of cells in tissues that flow and redistribute biochemical signals during development.

Introduction

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Dynamic coupling of biochemistry and mechanics governs the organization of cells into tissues and organs during organismal development. Biochemical signals control force production and transmission within and between cells. These forces are resolved into subcellular deformation and flow, cell shape change and movement, and ultimately into collective movements and deformations of cell populations that drive the emergence of complex tissues and organs.

At the same time, force, deformation and geometry feedback at multiple spatial and temporal scales to shape biochemical signals. Molecular motors and other mechanoenzymes sense force to tune enzymatic activity and motor output. Local deformations remodel cytoskeletal networks and adhesive contacts. Flows redistribute biochemical signals. Local changes in cell geometry promote the recruitment of curvature sensing proteins. At the tissue level, dynamic changes in cell shape and position, local remodeling of cell-cell contacts, feedback to control signalling between cells and across tissues. The dynamic coupling of local biochemistry, force generation and deformation governs the emergence of order at all scales: from adhesion complexes to self-organized structures such as the mitotic spindle and contractile actomyosin arrays, to the characteristic shapes of motile cells, to the emergence of complex functional structures such as muscles, branched vascular networks or the convoluted brain.

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3 How mechanochemical interactions lead to the emergence of complex cell and tissue organization is
4 an old question. However, in recent years, this question has attracted a resurgence of interest from
5 biologists, physicists, mathematicians and computer scientists. Rapid advances in optical physics,
6 microscopy and computational image analysis have greatly enhanced our ability to observe, quantify
7 and perturb spatiotemporal patterns of signalling, force generation, deformation and flow in living
8 cells and tissues. Rapid advances in theory and computing have made it possible to construct
9 predictive models of how cell and tissue organization and dynamics emerge from local coupling of
10 biochemistry and mechanics. Physics provides a rich perspective on biological cells and tissues as
11 active self-organized living matter, while biology provides a seemingly endless supply of examples in
12 which different properties of living matter are encoded in molecular hardware.
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16 In this Roadmap, we bring together a series of forward-looking perspectives from scientists working
17 at the interface of the physical and biological sciences. We highlight both recent successes and
18 ongoing efforts to understand how the complex organization of cells and tissues emerge through
19 mechanochemical coupling. We emphasize the need to understand mechanisms that govern the
20 production, propagation, degradation and dissipation of biochemical and mechanical signals, and
21 how these mechanisms determine length and time scales on which signals are coupled and one type
22 of signal can relay the other. By presenting case studies drawn from different systems and
23 approached from different perspectives, we aim to highlight both the unique insights that can be
24 obtained from studying particular systems and general themes that transcend the details of
25 individual systems.
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29 **Dynamics of signalling in self-organized stem cell assemblies**

30
31 Idse Heemskerk¹ and Aryeh Warmflash²

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40 **Status**

41
42 Studying mammalian embryogenesis presents particular challenges as in utero development
43 precludes observation and manipulation of live embryos. Recently, recapitulation of embryonic
44 processes in vitro using pluripotent stem cells (PSCs) has emerged as powerful alternative (reviewed
45 in [1,2]). Both two- and three-dimensional systems have been developed, and while the three-
46 dimensional systems show a remarkable degree of patterning and morphogenesis, to date, a lack of
47 reproducibility in size, shape, and pattern has largely hampered quantitative studies. In contrast, two
48 dimensional systems, while largely incapable of undergoing morphogenesis, are quantitatively
49 reproducible, and have enabled investigators to begin to understand how self-organized paracrine
50 signalling produces patterns in mammalian development.
51
52
53

54 The first two-dimensional patterning system mimicked gastrulation: the organization of germ layers
55 in the mammalian embryo, starting from human PSCs (hPSCs) [1]. The patterning in this system is
56 controlled by the same BMP, Wnt, FGF, and Nodal signals that pattern the embryo in vivo, and the
57 germ layers are arranged in the same order, although they are placed adjacent to each other within
58 the same plane rather than in a layered structure as in vivo. Here, we focus on our expectations for
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3 continued discovery with these two-dimensional systems as well as the road towards quantitatively
4 reproducible three-dimensional systems.
5

6 **Current and Future Challenges**

7 *Interpretation of dynamic signals*

8
9
10 Recently, studies using micropatterned hPSCs showed that patterning does not result from the
11 formation of static signalling gradients, but rather from dynamic waves of signals which propagate
12 from the edge inwards specifying cell fates in their wake [1,3]. Despite this progress, the precise
13 relationship between signalling activity and fate remains unclear. How the site of gastrulation,
14 known as the primitive streak, is specified by Wnt and Nodal signalling remains an important
15 outstanding question. It is clear that both signals are required, and that the cell fate is not a simple
16 function of the concentration or duration of signalling, but how cells integrate these signals into a
17 fate decision is not understood. Similar questions pertain to how signals create patterns at other
18 developmental stages. For example, a number of groups have recently developed two-dimensional
19 hPSC models for ectodermal patterning into neural, neural crest, and future epidermis[4–6],
20 however, the precise mapping between signalling dynamics and cell fates remains obscure here as
21 well. In the future, quantitative measurements of signalling and fate simultaneously in live cells
22 should provide insight into this question.
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25

26 *Formation of signalling patterns*

27
28 Complementary to the question how extracellular signals are interpreted is the question of how
29 these signals are generated and patterns of signalling activity that drive cell fate patterning are
30 formed. To date, most studies have avoided this question by studying reporters of signalling activity
31 directly without monitoring the ligands and inhibitors that modulate this activity. However, most
32 models of patterning refer to diffusible extracellular ligands and their inhibitors (reviewed in [7]),
33 and therefore directly observing these molecules is necessary for validating or disproving these. This
34 is a particularly challenging problem as these molecules are secreted into the extracellular space and
35 are often effective at very low concentrations. However, it is essential to observe the low
36 endogenous levels since overexpression of these molecules may severely perturb their behavior.
37
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39

40 *Integration of chemical and physical signals*

41
42 An emerging area is the intersection between mechanics and patterning. Mechanics may play an
43 important role in specifying cell fates as several of the signalling pathways involved in early
44 development are known to be mechanosensitive, including Hippo and Wnt signalling. For example,
45 recent work in the mouse blastocyst directly links mechanical forces generated by myosin
46 contractility to Hippo pathway activity and trophectoderm differentiation [8]. This suggests a similar
47 mechanism may control extraembryonic differentiation of hPSCs. Little is known about the role of
48 tissue mechanics in mammalian gastrulation or about its impact on early cell fate decisions in hPSCs.
49 This question can be separated into the role of substrate mechanics (including extracellular matrix),
50 and the role of intercellular forces. While soft substrates were found to enhance mesoderm
51 differentiation (and in some settings extraembryonic differentiation) a broader understanding of
52 how substrate properties affect self-organized patterning is missing[9]. Moreover, essentially
53 nothing is known about the role of intercellular forces, which have not been measured or
54 manipulated in embryoid systems.
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Advances in Science and Technology to Meet Challenges

Measuring endogenous ligand levels in live cells

Two-dimensional patterning systems may be ideal for studying the formation of signalling patterns as they provide optimal imaging conditions. There is evidence that many ligand-receptor interactions occur basal to the cells [1], that is between the cells and the coverslip, so it is possible that TIRF microscopy could be used to image these with single molecule resolution. This would be an exciting opportunity as it would represent the first live imaging of an endogenous extracellular morphogen gradient outside of limited studies that have been performed in *C elegans* and *Drosophila*. Even if this fails, some insight may be gained indirectly. For example, recent studies using an inhibitor of Wnt secretion and live cell imaging of signalling activity have suggested that in the 2D gastrulation model, Wnt ligands are capable of long-range diffusion while Nodal activity propagates primarily by autoactivation of Nodal production [3]. Other studies of Wnt have suggested that its range may be context dependent: it appears to be short range in the murine intestinal crypt but long range in *C elegans* embryos so a system where the mechanisms of ligand dispersal could be dissected is of great interest.

Reproducible stem cell assemblies in 3D

Because of their high degree of reproducibility and ease of observation, two-dimensional stem cell systems have enabled highly quantitative studies that revealed surprising new features of pattern formation. However, no morphogenesis occurs in these systems, restricting their use to studying isolated features of pattern formation. Moreover, 2D models are only available for a small number of developmental processes. 3D stem cell systems display more realistic morphogenesis, and, a wide range of 3D organoid systems are available [10]. However, detailed quantitative studies to shed light on the mechanisms of self-organization in such systems are made very difficult by a lack of reproducibility: shape, size, and pattern show qualitative trends, but cannot be quantitatively compared between individual organoids/embryoids. For 3D models of the early embryo this situation has significantly improved in the recent past [2]. Mouse stem cells have been made to form assemblies that closely resemble the early mouse embryo in shape and size. With microfluidic devices, hPSCs have also been made to form highly reproducible 3D structures resembling part of the early human embryo. If this technological development continues it may soon be possible to gain deeper insight into how signalling orchestrates the interplay of patterning and morphogenesis. This will require not only controlling 3D development precisely, but also enabling high resolution live cell microscopy, which remains a challenge for these systems.

Concluding Remarks

This is an exciting time for studying development with self-organizing stem cell systems. Already a number of models have been created that have led to significant insights into signalling dynamics and patterning. Developing technologies to measure extracellular ligands in space and time, to probe the mechanics of these structures, and to grow reproducible 3D cultures promises to expand this field to analysis of morphogenesis and its interplay with cell fates and tissue patterning.

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36 **Interplay between tissue growth, morphogen signalling, and pattern** 37 **formation in development**

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40 *equal contribution

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42 **Status**

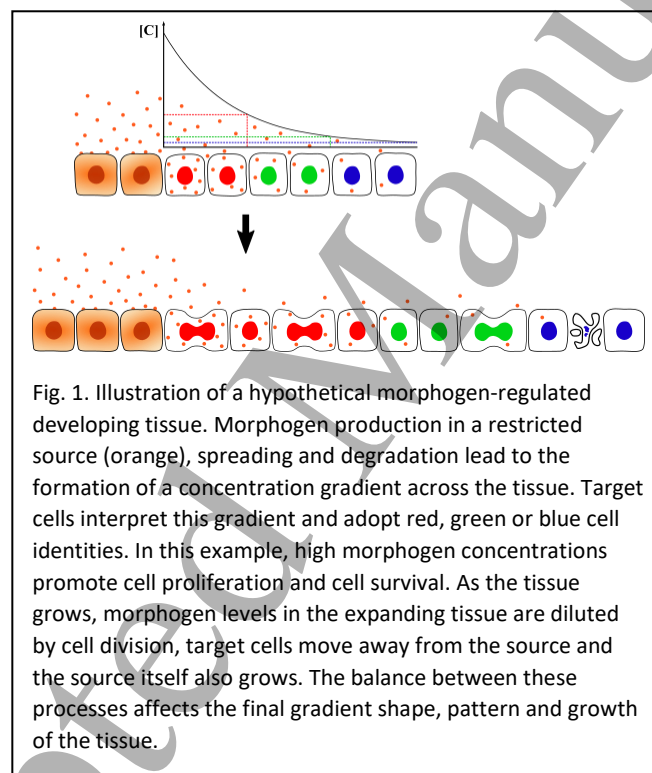
43 Tissue growth is essential for the transformation of a fertilized cell into a mature organism. To
44 generate organs with reproducible shape and size, growth must be tightly coordinated with the
45 specification of diverse cell fates and the signals that direct tissue patterning and morphogenesis.
46 Yet, it is remarkable how little is known about this coordination.

47 During the past few decades, much progress has been made in unraveling how cell fates are
48 specified. With the help of genetics, in vitro models of ES cells differentiation into diverse cell fates
49 and single cell sequencing technologies, a highly detailed picture of how cell fates are molecularly
50 defined has emerged. Furthermore, our understanding of how morphogen signalling is interpreted
51 to specify cell identities has advanced considerably since the French flag problem was first posed by
52 Wolpert. It is currently well established that patterns of cell fate specification emerge from the
53 dynamics of gene regulatory networks driven by signals that spread across tissues [1].
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The greater understanding of the temporal dynamics of pattern formation has emphasized the fact that this process happens at the same time as tissues grow. However, tissue growth itself is poorly understood. While the cell-intrinsic regulation of the cell cycle has been extensively studied, it is unclear how global tissue-level control of tissue size is achieved.

Research in the *Drosophila* wing imaginal disc has contributed much to our knowledge on this question. This organ possesses an intrinsic size control mechanism, which allows it to measure its absolute dimensions. Signalling by the morphogens Dpp and Wg is required for the correct growth of the wing disc, although the precise mechanism of how size regulation is achieved is still a matter of intense investigation [2]. While the existence of intrinsic size control mechanisms is not well established in other systems, it is clear that organs have reproducible dimensions and shapes characteristic for their species. Furthermore, similar to imaginal discs, the signalling molecules that specify cell identities also regulate cell cycle progression and cell survival in many different organs and systems.

Our current knowledge paints a picture in which multiple feedbacks exist between tissue growth, morphogen signalling and pattern formation (Fig. 1). On one hand, morphogens control both cell



fates and tissue growth. On the other, signalling profiles and cell positions are modulated by tissue growth. Furthermore, pattern can locally alter the parameters of tissue growth. Further investigation of these feedbacks is necessary to get an insight into how they work together to help achieve reproducible organ size and shape during development.

Current and Future Challenges

A major obstacle to advancing our understanding of the interplay between growth, signalling and pattern formation is the poor understanding of tissue growth itself. This is perhaps not surprising – growth control is multifaceted and occurs at multiple scales. Systemic and nutritional inputs act alongside with tissue-level regulators, such as morphogens and survival factors, to regulate the dynamics of cell-intrinsic machineries. Although it is poorly understood how individual cells interpret

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3 tissue-level signalling to regulate their cell cycle progression, in many cases these signals have
4 quantitative, rather than all-or-none, effects on tissue size. For example, overexpression of Dpp
5 results in enlarged wing discs, whereas hypomorphic mutants produce smaller wings [2].
6 Nevertheless, it is still debated whether morphogens act in an instructive or permissive manner to
7 control growth. It is also unclear how size is sensed, how perturbations in size are corrected and how
8 morphogens contribute to these processes.
9

10 Tissue-level regulators of growth converge downstream on the core molecular machineries driving
11 cell cycle progression, cell death and cell growth. This can occur indirectly via the effects of
12 morphogens on pattern formation. For instance, in the neural tube progenitor identities are initially
13 specified by morphogen signalling, subsequently different progenitor types exit the cell cycle at
14 distinct rates, thereby changing the pattern of progenitor domains [3]. Signalling by extrinsic factors
15 can also directly affect core cell cycle and cell survival regulators. What key components and
16 interactions are regulated to alter the cell cycle speed, the probability to progress versus exit the cell
17 cycle or undergo apoptosis and how cell type specificity of this regulation is achieved are open
18 questions.
19

20
21 While we need a better understanding of how tissue growth is controlled, it is also necessary to
22 consider that growth may affect its own tissue-level regulators in several ways. Signalling molecules
23 can be diluted due to growth and advected away from their source of production, thereby altering
24 morphogen gradient shape. Moreover, tissue growth changes the position of target cells with
25 respect to the tissue boundaries and sources of morphogen production (Fig. 1). At the same time,
26 growth within the sources of morphogen production alters the overall morphogen production rate
27 over time. This contributes to temporal changes in signalling in individual cells. For instance, the
28 posterior elongation of the vertebrate body axis over time leads to the displacement of cells away
29 from Fgf produced in the tailbud. The restricted time interval of Fgf activity is key for correct pattern
30 specification [4].
31

32 A central challenge that lies ahead is not only to advance our knowledge of the interactions between
33 signalling, pattern and tissue growth, but also to understand the emergent properties of these
34 interactions. Tissue growth could be viewed as a process that modifies itself via its relationship to
35 signalling and pattern. This is likely to be fundamental for the self-organisation properties of
36 developmental systems [5]. Furthermore, in analogy to principles from control theory, these
37 feedbacks could underlie the reproducibility of organ size determination and patterning.
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39

40 **Advances in Science and Technology to Meet Challenges**

41 Tissue growth and pattern formation are dynamic processes that elicit quantitative changes in tissue
42 morphology and depend on multi-scale regulation. In order to capture these defining features it has
43 been of crucial importance to obtain highly resolved spatiotemporal quantitative data and use
44 theoretical models to interpret and understand the underlying complexity.
45

46 In recent years, the advances in microscopy and imaging assays have allowed in vivo observation and
47 quantitative measurements of the dynamic cell and molecular behaviors that underlie tissue growth
48 and pattern formation. For instance, light sheet microscopy has allowed capturing the cellular
49 morphodynamics in *Drosophila*, zebrafish and mouse embryos, as well as organoids, over extended
50 periods. Combined with real-time data processing to accommodate for significant embryo growth,
51 this has recently allowed imaging of large and complex specimens such as the postimplantation
52 mouse embryo up to organogenesis stages [6]. At the molecular level, assays based on fluorescence
53 recovery after photobleaching, fluorescence correlation spectroscopy and photoactivation allow
54 monitoring of the off-steady state kinetics of morphogens as they spread through tissues.
55

56 Photoactivatable proteins fused to receptors in opto-genetic chimeras, as well as fluorophores with
57 defined half-lives that constitute fluorescent timers have provided novel methodology to study the
58 dynamics of morphogen signalling and gradient formation [7,8].
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3 New developments in genetics and genome engineering have also been instrumental. These have
4 permitted tagging of endogenous ligands, as well as precisely manipulating components of signalling
5 pathways. In addition, advanced mosaic cell labelling techniques have allowed dynamic tracking of
6 cell lineages as well as properties of tissue growth in different species. For instance, analysis of the
7 size and shape of clones of lineage-related cells labelled at defined developmental stages has
8 provided information on the growth rate and anisotropy of tissue growth in the mouse neural tube
9 [9] and heart [10]. This provides useful information for understanding the links between tissue
10 growth and morphogenesis. Furthermore, the ability to track growth parameters simultaneously
11 with cell lineage makes clonal analysis a promising approach for understanding how pattern
12 formation and growth are coordinated during development.
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14

15 The advances in the type, quality and resolution of experimental data demand new theoretical
16 models to interpret these data. Dynamical systems and diffusion models that capture key
17 biophysical properties and interactions have been useful for understanding specific processes, such
18 as morphogen gradient formation, gradient scaling with tissue size or interpretation of morphogen
19 signalling by downstream patterning regulatory networks. The development of models that
20 integrate the knowledge of how these different processes interact, however, is still a challenge.
21 Computational models in which cells are represented as discrete units interacting via mechanical
22 forces are increasingly used for obtaining a more realistic picture of how growth, signalling and
23 morphogenesis dynamically interact during development. Such models are likely to become more
24 elaborate as they develop as indispensable platforms for synthesis and analysis of experimental
25 data.
26
27
28

29 **Concluding Remarks**

30 Recent technological advances are leading to substantial progress in understanding the mechanisms
31 of morphogen signalling, pattern formation and tissue growth. Establishing experimental and
32 theoretical methods that would allow unraveling the complex interactions between these processes
33 and understanding the emergent properties of morphogen-driven systems is a key future challenge.
34 The open questions discussed in this review are indicative of an exciting time to be working in the
35 field of developmental biology, where there is still much to be discovered and understood.
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38

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42
43

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26 The biophysical basis of robust plant morphogenesis

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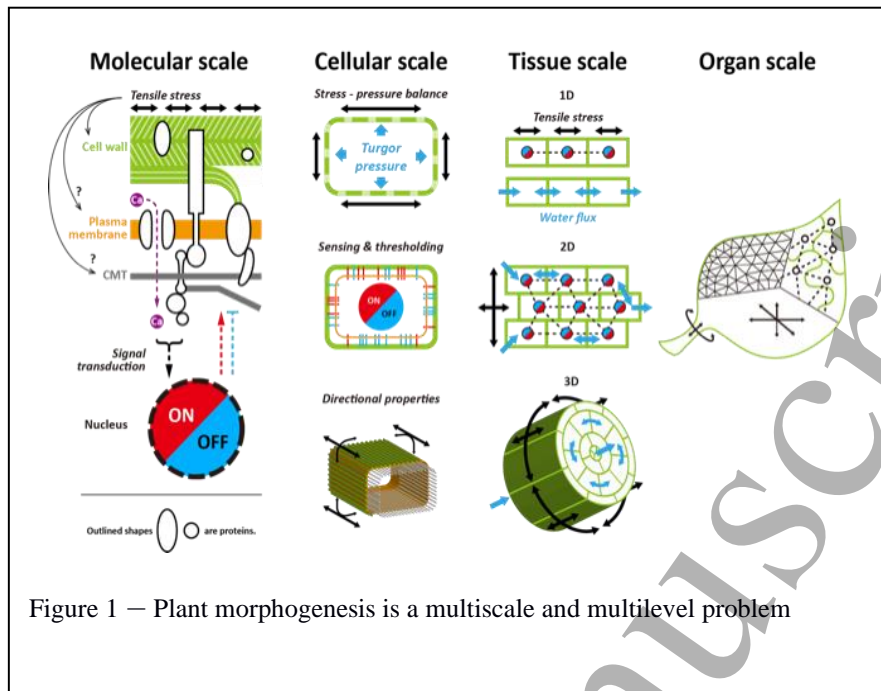
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33 Status

34 Morphogenesis, or the transformation of a developing organism to achieve a well-defined shape and
35 size, has historically been envisioned through genetic programming. As such, the reproducibility of
36 organ morphology observed in nature (e.g. symmetry of the two hands of a human, invariance of
37 flowers within a plant) find its explanation in that every organ follows approximately the same
38 programmed transformation. Nevertheless, a growing organism encounters multiple internal and
39 external perturbations (mechanical, nutritional, pathological, etc.), therefore a certain variability in
40 final organ shape and size would be expected. The relatively low variability at organ level suggests
41 that, besides following a preprogrammed transformation, organogenesis responds to perturbations
42 and can limit the fluctuations in final organ morphology. Tissue mechanics has emerged as a key
43 component in morphogenesis, both in making the link between gene expression and shape changes
44 and as a buffering mechanism to achieve developmental robustness. We are, however, far from fully
45 understanding the role of tissue mechanics in morphogenesis.
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Since their growth involves relatively simple physics and is slow enough to be easily observed, plants are ideal systems to investigate these issues. Plant cell growth involves the balance between the turgor pressure (hydrostatic pressure caused by an actively maintained osmotic flow) and the tension in the encasing cell wall. On developmental timescales, part of the elastic tension in the cell wall is released by its expansion via remodeling and new material delivery. Mechanical concepts in plant development date back to the XIXth century, with, for instance, experiments demonstrating that the plant epidermis is generally under tension. Such concepts were somehow overlooked during the XXth with the notable exception of scientists such as Paul Green, until a revival prompted by recent studies (reviewed in [1]). Positioning of flower primordia (early floral buds) at the shoot tip was associated with changes in cell wall composition and stiffness, hinting to a link between gene expression and tissue mechanics. The cytoskeleton of cortical microtubules (CMT) was found to respond to mechanical forces, contributing to robustness of morphogenesis at the shoot apex. Similar processes were identified in the root, while new approaches have been developed or renewed to quantify plant mechanics at cell or tissue scales, including pressure probe, nano-indentation (notably atomic force microscopy), and extensometry (reviewed in [2]).

Many theoretical approaches were developed in parallel. While a class of models, kinematic models [3], try to find the minimal local rules relating gene expression to growth patterns that reproduce the complex shapes observed in nature, other models try to relate these shapes to tissue mechanical properties [1,4]. From this biomechanical prospect, different questions arise depending on the considered scale. At subcellular scale, models relate tissue elasto-plastic properties to the cell wall composition or assess the cell wall response to mechanical stress [1]. At cellular scale, models try to relate cellular parameters, like cell size, wall tension and turgor pressure, which can in return describe the upper organ scale. Cellular models like vertex models reduce the complexity of the cellular functioning to the consideration of few features and deduce the tissue behaviors at a macroscopic scale. Other models adopt a coarser viewpoint and describe tissues as continuous media. While coarse-grained models allow depiction of more general tissue properties, cellular models can consider more subtle effects like the coupling between hydraulics and cell mechanics.

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3 Altogether, these mechanical models investigate the plant tissues mechanical response and its
4 impact on the morphological robustness.
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6 Here we discuss new challenges in the mechanics of plant morphogenesis, that stem from the
7 parallel rise in quantitative experiments and theoretical models.
8
9

10 **Current and Future Challenges**

11 Biological complexity underlies many aspects of the challenges in understanding plant
12 morphogenesis.
13

14 *Plants as complex biomechanical systems.* The cell wall is a thin layer composed of many
15 polysaccharides (cellulose, pectins, hemicellulose) and structural proteins with variable chemistry
16 and composition, all of which are ultimately regulated by genes. The mechanical properties of the
17 cell wall differ according to direction (in-plane, across thickness) and may vary in space and in time,
18 according to cell type and/or to cell status [5]. These relevance to growth of these properties is
19 unclear as they are measured at times scales much shorter than those of growth. Turgor pressure
20 depends on the regulation of all cellular osmolytes (involving movement, active transport, and
21 chemical reactions) and water movement in/out of the cell [6]. It is still unclear how cell growth rate
22 is exactly determined, while there is a strong need to globally bridge the genetic, biochemical and
23 biomechanical statuses of cell wall, which are mostly considered separately.
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26 *Specificity of mechanosensing and mechanotransduction.* Whereas the molecular basis of
27 mechanosensing (force-induced protein conformational changes) is generally recognized, their
28 collective behaviors are less understood: how much signal, detected by different proteins, is
29 required to initiate active response? How does the microtubule cytoskeleton respond directionally
30 to forces? Given the biological complexity, how is specificity ensured in mechanical signal sensing
31 and transduction; for example, is stress, strain or secondary signal sensed, and where [7]; and how
32 mechanically-stimulated Ca^{2+} signature, a common secondary messenger, is perceived differentially
33 than biotic stress and other signals [8]? Capturing the key determinants is crucial to understand
34 specific responses to mechanical signals.
35
36

37 *Mechanical variability at multiple scales.* Numerous studies revealed that most, if not all, plant
38 biomechanical properties are spatially variable [9]. If and how variability contributes positively to
39 robustness is still an open question. What is the temporal variability of mechanical properties? What
40 are the corresponding characteristic (correlation) times and lengths? How are mechanical properties
41 averaged over space and time and how does such averaging relate to morphological robustness?
42 Finally, as heterogeneity in mechanical properties and/or in growth rate induce mechanical stress in
43 the tissue, do mechanosensing and mechanotransduction contribute to robustness, and if so, how?
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47 **Advances in Science and Technology to Meet Challenges**

48 Conceptual and technical advances in data acquisition, data analysis, physical modeling and
49 simulations are required to meet the challenges mentioned above.
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51 *Multidimensional data acquisition.* Current biomechanical studies are largely limited to the surface
52 of plant tissue (epidermis and outer cell wall). So far, researchers have coped with this limitation
53 resorting to the theory that plant growth is controlled by the epidermis. Yet it is increasingly realized
54 that inner tissues also contribute significantly to plant biomechanics. Increased detection distance
55 and spatial resolution will promote optical imaging of deeper tissues. The use of biosensors (e.g.
56 sensor of cytoskeletal tension or of pressure), validated in the epidermis with direct biophysical
57 measurements, will give mechanical information about deeper tissues. Meanwhile, higher temporal
58 resolution demands automation with minimized invasiveness (e.g. reduced phototoxicity and effects
59 of mechanical probing). All these will benefit from new methods and optimizations for
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3 optical/mechanical measurements, like super-resolution optical microscopy, elastography (optical
4 inference of mechanical properties), or high-speed atomic force microscopy [2, 10].
5

6 *Data analysis and pattern recognition.* Compared to physical systems, the number of replicates in
7 biophysical experiments is often technically limited, while biological variability complicates
8 conclusions. Assessing 3D tissues, to multiply the quantity of data produced, will require the
9 development of fully automated methods of data extraction. To establish correlations between
10 biological data of increasing dimension (geometry, fluorescent reporters, mechanics... at multiple
11 scales), it is now common to use linear correlation analyses, mutual information, and dimensional
12 reduction such as principal component analysis. Methods from information theory and machine
13 learning could become even more powerful when they include constraints provided by physical
14 laws.
15

16 *Models and quantitative comparison with experiments.* Whereas models are increasingly compared
17 qualitatively with experiments, the lack of quantitative comparisons leaves it unclear whether
18 current theoretical frameworks are sufficient to describe morphogenesis in plants. This raises the
19 issue of inferring model parameters from experiments, as performed for traditional physical
20 systems. Inference could appear as unfeasible, given the complexity of some biophysical models and
21 their large number of parameters. Nevertheless, classical approaches of dimensional reduction in
22 continuum mechanics, such as homogenization (from discrete to continuous models) or
23 approximations of thin sheets (for leaves) or rods (for stems), may simplify models and reduce the
24 number of their parameters, facilitating model exploration and parametrization.
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29 **Concluding Remarks**

30 Time is ripe to further integrate experiments and theory, and bring plant biophysics closer to the
31 quantitative standard set by traditional physical systems. This will involve closing the cultural gap
32 between biologists and physicists, enabling research to combine molecular genetics, mechanical
33 measurements, and modelling. We will thus make progress in a multiscale and multilevel
34 understanding of the biophysics of plant morphogenesis.
35

36 **Acknowledgments** – Apologies to those whose work could not be mentioned due to limited space.
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Dynamics of morphogen gradient formation and signalling during development

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Status

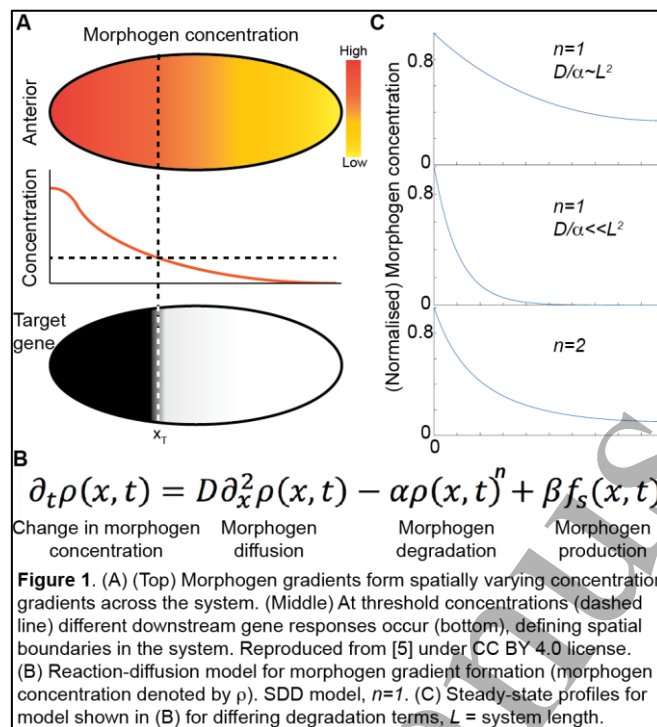
Morphogens provide crucial spatial and temporal information to developing systems. They form concentration gradients across the system (which can be a single tissue or the whole embryo), with the gene response dependent on the local morphogen concentration (Figure 1A). The morphogen concept was proposed by Alan Turing in 1952 [1], yet the first experimentally identified morphogen, Bicoid in the early *Drosophila* embryo [2], was not found until the 1980's. Subsequently, a range of morphogens have been identified – e.g. Fibroblast Growth Factor (FGF), Wingless (member of Wnt family) and Bone Morphogenetic Proteins (BMP) - that play essential roles in many developmental processes, from gene boundary specification to regulating growth. Defects in the morphogenetic program result in a plethora of birth defects, which are often lethal. Hence, understanding how gradients form and how they are interpreted is essential in deciphering how complex form emerges during development.

A classic model of morphogen gradient formation is the source, diffusion, degradation model [3] (Figure 1B). A source of morphogen is localized to a specific spatial region of the tissue. The morphogen then randomly diffuses through the tissue. Finally, protein degradation helps shape the gradient (Figure 1C). The SDD model results in an exponentially-decaying morphogen concentration in steady-state; as commonly observed *in vivo* [3,4]. Different forms for the degradation can result in power-law-like gradients (Figure 1C). The mechanism of morphogen degradation (trapping) shapes the gradient and also determines the time scale on which the morphogen reaches its steady-state [5].

Development is highly coordinated temporally; multiple events must occur in specific order to ensure that develop proceeds correctly. For example, during specification of the vertebrate neural tube, the temporal sequence of cell fate determination is highly dynamic, with temporally varying gene expression in cells at particular spatial positions [6]. However, in most developing systems there is still much to learn about how processes are coordinated both temporally and spatially to ensure robust morphogenesis.

Current and Future Challenges

Dynamics of morphogen gradient formation



What are the dynamic processes that form morphogen gradients, and on what timescales do these processes act? Answering these question remains hotly debated for most morphogens. Bicoid is likely the most under-stood morphogen in terms of its dynamics [2], and it is well-described qualitatively by the SDD model [3,5]. Despite being accessible for live imaging and quantitative approaches – such as fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) - there remain challenges in understanding how the Bicoid gradient forms. In particular, what processes are determining the Bicoid diffusion, what is the role of the cytoskeleton in modulating Bicoid transport, how do the dynamics depend on the increasing density of nuclei as the embryo develops, and how is production of Bicoid temporally regulated?

Quantitative techniques such as FCS and FRAP provide important information about the morphogen dynamics, but at different spatial and temporal scales. FCS provides a very local readout of the molecular motion. FRAP, which is typically done on large spatial scales than FCS, gives a readout of the effective time scales for cellular-level dynamics, which incorporates multiple processes, such as diffusion, binding, internalization, and degradation. Typically, FCS estimates larger diffusion constants for morphogens than FRAP, as shown with Bicoid in *Drosophila* [3] and Nodal in zebrafish [7]. There remains a major challenge to formulate a robust, biologically-relevant framework that incorporates different time and spatial scales of dynamics into models of gradient formation.

In the *Drosophila* wing disc, the Dpp and Wg gradients may also form in a SDD-model-like manner [4]. Yet, alternative transport mechanisms, such as cytoneme-mediated transport via filopodia-like protrusions [8], have been proposed. In this scenario, morphogen transport is more directed than random diffusion. It remains a hurdle to experimentally dissect the modes of morphogen transport in the wing disc, with improved quantitative measurements using new tools necessary to distinguish different mechanisms. From a theoretical perspective, the transport of morphogens by cytonemes has received only limited attention. Further modeling may help reveal when different transport mechanisms (*e.g.* random diffusion or directed transport) are favorable. Such modeling needs to

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3 account for the cost of generating the cytoneme network, not just the dynamics of morphogen
4 transport through the network.
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6 Models of morphogen gradient formation typically ignore details of the extracellular space and
7 growth of the tissue. Morphogens do not diffuse freely, but move through a dense
8 microenvironment that restricts their spread [7]. Tissues typically grow in 3D, yet most models of
9 morphogen gradient formation neglect such details. To build-up a more precise understanding of
10 gradient formation requires incorporating the microenvironment structure and dynamic changes in
11 the tissue architecture into improved models of gradient formation.
12

13 It is unclear for nearly all morphogens *when* information encoded within the gradient is interpreted
14 and over what time frame such interpretation occurs. Few, if any, morphogens reach a steady-state.
15 Therefore, *when* cells read out the concentration is important. How do systems regulate the
16 beginning and end of morphogen readout? Further, how do cells integrate the signal reliably?
17 Temporal and/or spatial averaging are required to reliably interpret the signal [3]. The mechanisms
18 for such averaging are generally poorly understood, particularly in vertebrate systems.
19

20 Detailed models have been formulated for how downstream genes respond to morphogenetic
21 inputs [9]. There are also many models for how morphogen gradients form [3-5]. Yet, there are
22 currently a paucity of models that incorporate both the *dynamics* of the morphogenetic input and
23 the *dynamics* of the signal interpretation. In particular, a challenge is to develop more realistic
24 models of how downstream genes respond to temporally varying morphogen concentrations.
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28 **Advances in Science and Technology to Meet Challenges**

29 There have been significant advances in our ability to gain quantitative data on morphogen
30 formation from live embryos. Light-sheet microscopy enables 4D imaging at sufficient spatial and
31 temporal resolution and sensitivity to quantify the formation of morphogen gradients [5]. Light-
32 sheet imaging can also be adapted for FCS, providing spatial maps of morphogen dynamics. Such
33 information can be used to generate more accurate models of morphogen dynamics, which
34 incorporate the microenvironment in which the morphogens are transported.
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37 Biological tools, such as endogenous knock-in of fluorescent markers into morphogens, are enabling
38 more accurate measurement of the biologically-relevant morphogen concentration. Tandem
39 reporters, which incorporate two fluorophores with different folding rates, are a powerful tool for
40 measuring morphogen dynamics, as they provide information about the morphogen *age*, and not
41 just concentration, at a particular spatial position [5].
42

43 Optogenetic tools allow the exploration of the dynamics of morphogen gradient interpretation.
44 Bicoid combined with the optogenetic molecule Cry2, enables the activity of Bicoid to be switched
45 on/off at specific times and locations [10]. Such temporal control enables dissection of when and
46 where Bicoid is required for robust interpretation. Relatedly, new tools for measuring the live
47 readout of transcription and translation facilitate quantification of the cellular response to
48 morphogen inputs. Such readouts can be used to quantitatively test models of morphogen
49 interpretation.
50

51 From a modeling perspective, there is an increasing realization of the importance of incorporating
52 dynamics within models of cell fate specification via morphogens [6]. There have been substantial
53 advances in statistical thermodynamic models and reaction-diffusion models that more realistically
54 incorporate system geometry. With increasing computing power, and higher quality quantitative
55 data for testing and validating model predictions, hopefully theorists will explore the questions of
56 how morphogen gradients form within complex developing systems and how morphogenetic
57 information is temporally interpreted.
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Concluding Remarks

Morphogens are ubiquitous throughout much of development. The precise formation and readout of concentration gradients is essential. These processes must be tightly controlled in both *space* and *time*. Recent technological advances are giving access to the dynamics in unprecedented detail. With such information, more accurate models of gradient formation and readout can be formulated, that are essential in understanding how tissues reliably and reproducibly generate complex form.

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Coupling morphogenesis and patterning during amniote gastrulation

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Status

During vertebrate development, gastrulation is the first major morphogenetic event that lays down the three embryonic germ layers (endoderm, mesoderm and ectoderm) and the primary axis of the embryo. In amniotes (birds, reptiles and mammals), this process spans several days, during which the transmission of cell-generated forces remodels the early epithelial embryo into a multilayered organism (Figure 1A), while signaling and gene regulatory networks concomitantly specify cell fate

(Figure 1B). Whereas the execution of these two tasks must be continuously coupled to ensure the proper regional allocation of cell fate (Figure 1C), they have been studied mostly independently, arguably because of lack of a suitable experimental model. As a result, little is known about how morphogenetic and patterning programs interplay and coordinate.

Although avian embryos are largely regarded as classical embryological models, the recent resurgence of interest in a physical view of development repositions them as modern mechanobiological systems. Avian embryos are ideally suited for mechanical perturbations and live microscopy, owing to their flat geometry (two-dimensional epithelial disk), large dimensions (~ 4mm diameter) and external development. A striking feature of avian -and more generally amniote- embryos is their highly regulative development, in which cell fates, influenced by inducers and cell-cell interactions, can be rerouted upon external perturbations to produce the correct spatiotemporal arrangement of patterns. This is remarkably illustrated by the bisection of a gastrulating chicken embryo, which eventually produces two perfectly patterned and shaped embryos [1]. However, to date, the mechanisms underlying such fate redirection remain unclear.

To decipher how mechanical forces and molecular signals might interact to specify or maintain cell fates during gastrulation, it is crucial to obtain a clear view of both its mechanical and molecular control. Decades of genetic, molecular and developmental biology studies have characterized the signaling and gene regulatory networks governing germ layers specification, highlighting their high level of conservation amongst vertebrates. For instance, the Wnt and TGF β /Nodal signalling pathways have been shown to be critical for mesendoderm specification whereas the Yap/Tead pathway has been involved in embryonic vs. extraembryonic specification. More recently, a clear mechanical picture of avian gastrulation has emerged, showing that the seemingly complex tissue motions that accompany gastrulation can be explained simply by the generation of active forces at the margin between embryonic/extra-embryonic territories. The propagation of such forces throughout the embryonic epithelial disk, shown to behave as fluid-like material, then accounts for the observed large-scale, counter-rotational tissue flows [2].

In summary, with only three major cell types, well-defined molecular and genetic regulation, a clear mechanical description, and high amenability to mechanobiological approaches, the gastrulating avian embryo provides a powerful experimental platform to decipher how molecular and mechanical cues combine *in vivo*. Its study is therefore likely to uncover fundamental principles underlying embryonic development in general, with direct relevance to human development.

Current and Future Challenges

Whereas it has become clear that mechanical forces influence cell fate specification *in vitro* [3], pinpointing an unequivocal role *in vivo* has remained a challenge for several reasons:

- The precise material properties of the gastrulating embryo (e.g. viscosity, elasticity) remain to be characterized and mapped.

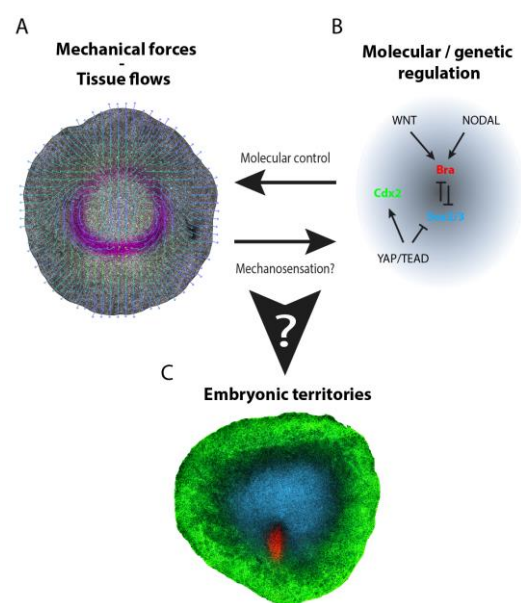


Figure 1 – Possible interplay between tissue mechanics (A) and regulatory networks (B) to specify embryonic territories during quail gastrulation (C). Active forces are in magenta in (A); embryonic, mesendodermal and extraembryonic territories are respectively in blue, red and green, in (C).

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3 This is a prerequisite not only to obtain an accurate physical description of gastrulation, but also to
4 perform controlled mechanical perturbations. Whereas *in vitro* cell cultures, owing to their great
5 accessibility and simple conformation are well suited for physical measurements using standard
6 apparatus (AFM, cantilevers, pipets, etc.), similar apparatus must be adapted to accommodate the
7 multilayered organization, culturing conditions and limited accessibility of live embryos [4]. This
8 remains a complex experimental challenge.
9

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11 - There remains a need for robust methods to modulate forces in living embryos through direct
12 physical interventions, rather than by modulating molecular motors and cytoskeletal components,
13 which may act pleiotropically on signalling pathways.
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15
16 - Because forces can propagate throughout tissues, much like molecular signals, disentangling the
17 effect of mechanical force vs molecular cues on gene expression is not a trivial task. In particular,
18 changing tissue geometry can affect tissue constraints but also the diffusion of signaling molecules
19 known to be critical during gastrulation.
20

21
22 - Dynamic changes in gene expression, following *bona fide* mechanical perturbations, must be
23 assessed in a quantitative manner to reveal relevant changes.
24

25
26 - Unbiased omics approaches must be designed to identify and characterize mechanotransductive
27 pathways beyond the already well-characterized candidates (e.g. YAP, beta-catenin, etc.) [5,6].
28

29
30 - To date, a physical description of tissue mechanics has been largely absent from models of
31 gastrulation [7,8]. As a result, important features such as cell and tissue movements have relied on
32 *ad hoc* hypotheses and conditions. A real challenge is the elaboration of theoretical models
33 grounded in quantitative descriptions of gene expression and tissue mechanics to obtain testable
34 predictions rather than simple plausibility studies.
35

36 **Advances in Science and Technology to Meet Challenges**

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38 The advances required to meet these challenges are diverse, spanning different areas of research.
39 For instance, the field needs input from experimental biophysicists and engineers to design devices
40 that can access the early embryo and subsequently probe and manipulate their mechanical states.
41

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43 Recent improvements in fluorescent *in situ* hybridization allowing quantitative, multiplexed
44 detection of single RNA molecules, make it possible to measure the expression levels of selected
45 candidate genes (see Figure 1C). However, whole transcriptome analysis would open the way to
46 unbiased detection of novel target genes and signatures of previously unidentified mechanosensitive
47 pathways. Single cell RNAseq analyses have proven to be successful in characterizing cell fates
48 trajectories in entire embryos, but such approaches disrupt tissue integrity and impose potentially
49 confounding mechanical perturbations. Spatial transcriptomics is emerging as a very promising
50 alternative tool because it offers unbiased sampling, while preserving tissue integrity and enabling
51 the recovery of spatial information [9].
52

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54 The unambiguous demonstration of the role of forces in controlling gene expression will require the
55 characterization of the underlying mechanotransduction pathways. This will require the
56 development of novel transgenic lines expressing fluorescent reporters and biosensors to readout
57 the dynamics of these pathways. This is necessary to gain actual mechanistic insights and not just
58 information about an ON/OFF state. Surprisingly, despite the well-demonstrated roles of numerous
59 signalling pathways during vertebrate gastrulation and development in general, only a handful of
60 tools exist to report the dynamics of their activities. Using CrispR-Cas9 genome editing to tag

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3 endogenous proteins, combined with dynamic approaches and mechanical perturbations, should
4 provide invaluable insights.
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6
7 Finally, to formulate and constrain quantitative theoretical models capturing the reciprocal
8 feedbacks between tissue mechanics (e.g. regions under compression or tension), dynamics of
9 signalling pathways and cell fate decisions, advances in image acquisition and analysis will be
10 needed. Whereas tremendous progress has been achieved regarding in toto imaging and analysis of
11 fly and fish embryos, the application of similar method to amniotes remains in an early but
12 promising stage [2,10].
13

14 **Concluding Remarks**

15
16 Addressing the role of forces in the coupling of morphogenesis and patterning during gastrulation
17 will require skills spanning different area of physics and biology from experimental (physical
18 measures), theoretical physics (conceptual frameworks) to morphogenesis (dynamic tissue-scale
19 analyses), transcriptomics and cellular biology (mechanotransduction characterization) approaches.
20 This will therefore necessitate joined efforts from experts of various backgrounds and
21 interdisciplinary-trained students.
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23

24
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Supra-cellular actin fiber arrays and their role in animal morphogenesis

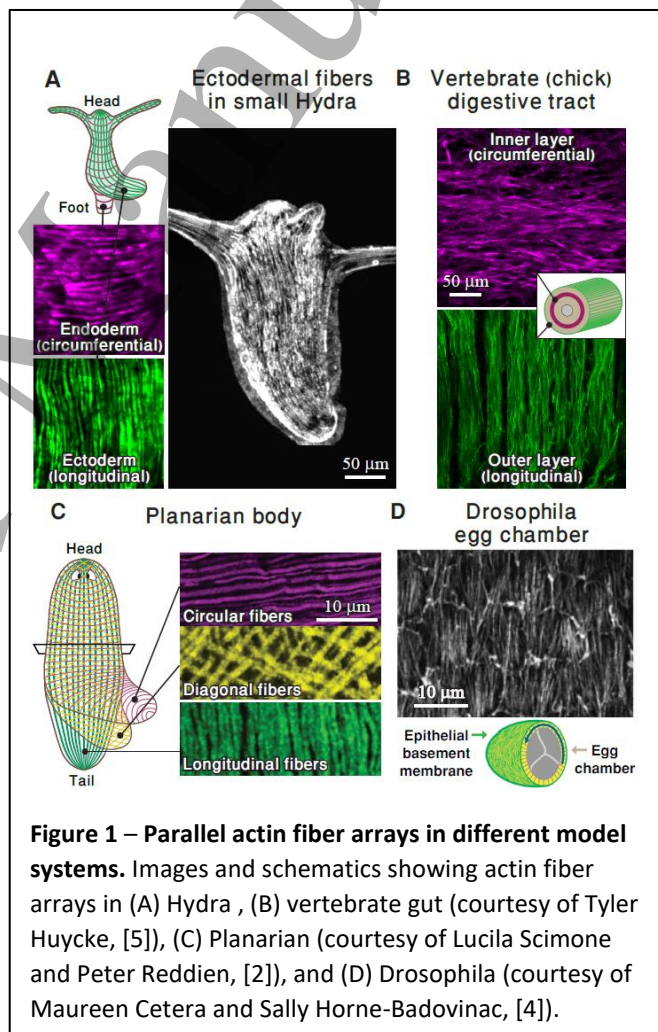
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Status

Parallel arrays of contractile actin fibers are a common organization theme seen across a variety of organisms, from the simple metazoan *Hydra*, through worms, insects and vertebrates [1–5] (Fig. 1). The contractile fibers are formed from bundles of actin filaments, bound together by cross-linking proteins, and decorated with myosin motors that generate forces by binding and moving along the filaments. Multiple fibers arrange into larger-scale structures ranging from cellular arrays of stress fibers, to supra-cellular arrays of fibers formed by mechanically linking actin filaments in neighboring cells through cell-cell junctions. The coordinated motor activity within these structures enables large-scale force-generation and synchronized contraction.

Actin fiber arrays fulfil important functional roles in patterning during morphogenesis as well as in normal tissue physiology. At the cellular level, aligned stress fiber arrays play a crucial role in controlling cell mechanics and mediating responses to environmental factors such as substrate stiffness, tissue geometry, and external stresses [6]. At the supra-cellular level, actin fiber arrays enable global, coordinated contraction, and provide long lasting structures that generate and mediate stable, directional mechanical cues. Interestingly, in systems ranging from *Hydra* and planarian to vertebrate smooth muscle and heart, parallel arrays of fibers are often found



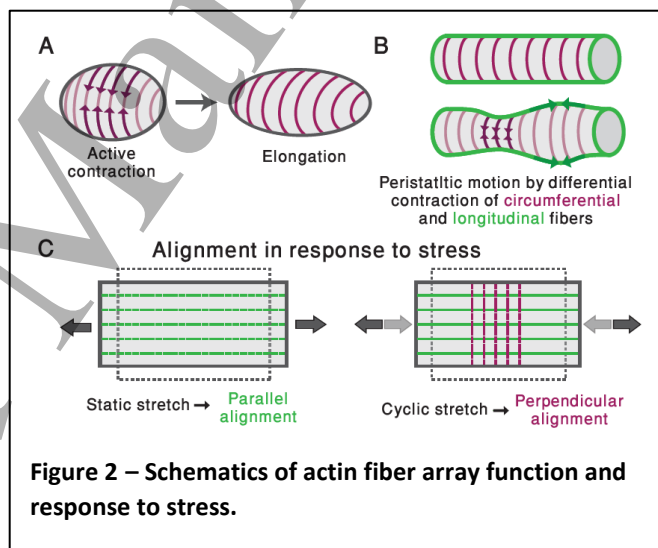
in two or more adjacent sheets of differing orientation (Fig. 1), facilitating the production of varying stress patterns which are important for tissue organization and function (Fig. 2A,B) [1,2,5,7].

The prevalence of parallel actin fiber arrays points towards some universal principles of organization governing the alignment and stabilization of these structures. *In vitro* experiments on single cells and cell monolayers have shown that fiber assembly and alignment is significantly affected by the mechanical environment. In particular, fiber arrays tend to orient parallel to the direction of externally applied static stretching, and at a uniform angle (often perpendicular) in response to cyclic stretching (Fig. 2C) [6]. Theoretically, these responses have been modelled assuming a minimization of an elastic energy, incorporating contributions from the fibers as force dipoles, as well as adhesions and/or coupling to a substrate. Interestingly, recent studies on the development of the smooth muscle layers lining the vertebrate gut (Fig. 1B) found a similar response of fiber orientation to internal stress patterns [5]. Once established, these ordered fiber arrays are stably maintained even in the absence of the stresses required for their initial formation and alignment [5]. In other cases, such as in the alignment of actin fibers at the basal epithelial layer surrounding the *Drosophila* egg chamber (Fig. 1D), the parallel alignment is reinforced through coordinated motion in which the entire egg chamber rotates along the direction of alignment [4]. Whilst the susceptibility to mechanical stress appears to be a common characteristic of actin fiber arrays, little is known about other aspects of their organization. For example, what determines the width of individual fibers or the spacing between them?

The contribution of actin fiber arrays towards patterning and directing tissue organization is just beginning to be appreciated. Several examples demonstrate that these arrays can act as a scaffold both mechanically, by providing structural stiffness in a particular direction, and biochemically, with muscle cells providing biochemical signals that correlate with their global orientation [2]. Furthermore, contraction of actin fiber arrays can actively drive tissue elongation, or even provide mechanical cues for the formation and alignment of a second, orthogonal array of fibers (Fig. 2). In *Hydra*, the alignment of actin fibers directs the formation of the body axis in regeneration from excised tissue segments [1,8], and defects (singularities) in this alignment have recently been shown to act as organization centers for the formation of morphological features [9].

Current and Future Challenges

Achieving systematic understanding of the mechanisms underlying the formation of actin fiber arrays during development and their role in patterning morphogenesis is an outstanding challenge. As a major force-generating system, structure and mechanics are intimately coupled in these arrays with substantial mutual feedback [5,6]. The complexity of this coupling is apparent even at the molecular level, where interactions between cytoskeletal and adhesion components include the formation of catch bonds and other force-dependent processes, which collaboratively, via mechanisms that are still largely unknown, regulate fiber assembly, binding, and turnover. Similarly, at the cellular and supra-



cellular level, assembly and patterning of actin fiber arrays often requires coordinated regulation of cell differentiation, intercellular junctions and cell-substrate adhesions, but the mechanisms involved are still unclear.

The functional roles of actin fiber arrays in morphogenesis are remarkably diverse, making them difficult to fully discern. Apart from the ability to directly drive changes in tissue shape by coordinated contraction (Fig. 2), actin fiber organization and contraction can provide mechanical cues, conveyed through either temporal and/or spatial alteration of the stress field, to regulate and coordinate important biological processes across the tissue [3,5,9]. Similarly, regular arrays of fibers can provide important positional information for biochemical cues that initiate and stabilize patterns of gene expression [2]. Although both are likely essential for morphogenesis, little is known about the mechanisms involved and how the different mechanical and biochemical processes are integrated to lead to robust morphological outcomes.

One of the most profound, yet poorly understood characteristics of actin fiber arrays is the remarkable duality of their regularity and stability on the one hand, and their flexibility and adaptivity on the other [8]. This duality seems essential for their function, providing structural memory and stability to the tissue, but at the same time facilitating their ability to rapidly adapt and drive the significant changes in shape and geometry that are widespread during morphogenesis [1,8]. How can the same structure be stable over extended timescales yet rapidly remodel when needed? While the mechanisms involved are still unknown, they clearly require complex inter-cellular coordination and feedback, potentially involving additional tissue-scale mechanical processes, such as directed cell migration or modifications of the extra-cellular matrix.

Advances in Science and Technology to Meet Challenges

Complementary advances in experimental and analytical tools, as well as the introduction of new conceptual approaches, can greatly enhance our understanding of the assembly, function, and role of actin fiber arrays in patterning morphogenesis. This requires the integration of ideas from physics, such as dynamics of active matter, elasticity and nematics, with the more prevalent biochemical approaches, as well as making use of the latest computational and mathematical tools.

Techniques for imaging actin dynamics and measuring mechanical properties within developing tissues, without compromising viability or interfering with the developmental process, have advanced considerably over the last decade [10]. Nevertheless, our ability to monitor the mechanical environment *in vivo* and apply mechanical perturbations in a spatially and temporally controlled manner is still limited and hinders our ability to study actin fiber organization and its influence on pattern formation. This is especially important given the ubiquity of the cytoskeleton in a multitude of cellular processes, making biochemical or genetic alterations of actin fiber arrays extremely disruptive and difficult to isolate, and highlighting the need for tools that enable controlled, quantifiable and versatile application of forces and constraints on live tissues.

Concluding Remarks

As a common organizational structure found across spatial and evolutionary scales, parallel arrays of actin fibers play an important role in directing and maintaining biological structure and patterning. Understanding the universal principles of organization underlying their assembly and function, which

involve multiple interacting biochemical and physical processes, will be an important step towards achieving an integrated understanding of morphogenesis.

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Mechano-chemical models for morphogenesis and collective cell migration

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Status

Tissue patterning and morphogenesis, as well as the coordination between these processes, in developing organisms rely on the feedback between mechanical and biochemical cues [1]. Similarly, phenomena like wound healing or tissue regeneration require information to be sensed and transmitted over long length and time scales. But how these properties are encoded molecularly, and able to emerge robustly across scales, in the presence of biological noise, tissue heterogeneity and extrinsic variability, remains poorly understood.

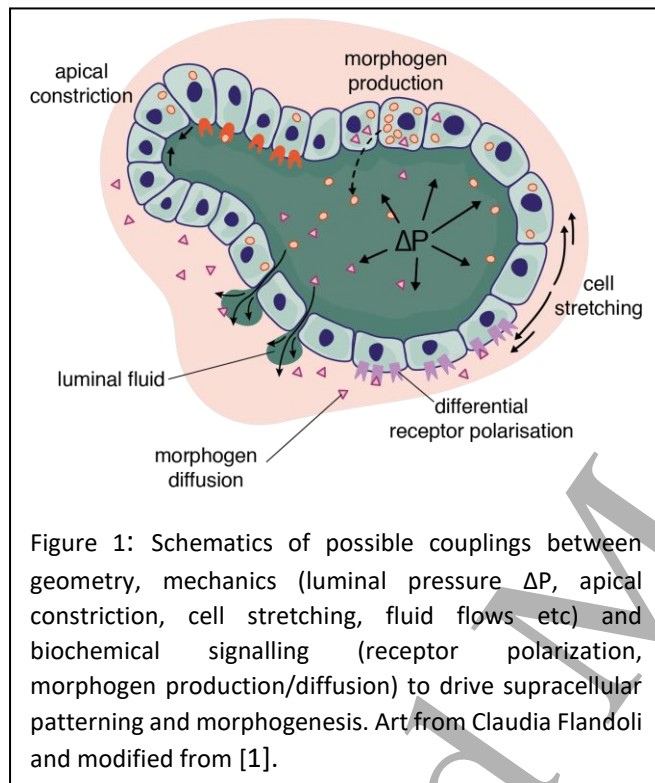
Biophysical and mathematical modelling has long-been used to help bridge this gap. In the early 20th century, D’Arcy Thompson used analogies from material science and soft matter physics to explain the typical hexagonal tiling of epithelia or the shape of spiral horns, thereby providing a mechanical framework to think about morphogenesis. In 1952, Alan Turing proposed a complementary, and highly influential, “chemical” theory of morphogenesis, where he neglected mechanical properties of tissues to concentrate on how simple biochemical reaction schemes of diffusing species (termed “morphogens”) can give rise to *de novo* patterns, such as tentacle patterns of *Hydra*. These ideas remained largely theoretical for decades, until advances in genetic manipulation, imaging and mechanical perturbations allowed them to start being tested [2]. However, shape acquisition and cellular patterning are often tightly correlated temporally, rendering an assessment of causation and the underlying mechanism difficult. Indeed, although theories of mechanochemical couplings date at least back to seminal works by Murray and Oster (among others) in the 80s, how to generically couple mechanical models of tissues with the complex biochemical signalling networks operating in each cell remains an open question [1].

Current and Future Challenges

Biochemical reaction-diffusion models have proved highly successful in predicting a number of non-trivial features in pattern emergence during development, namely in mouse hair follicle or digit patterning or early symmetry breaking in zebrafish embryos [2]. These models are highly and elegantly simplified, which is a key strength as it helps to distill universal principles of patterning, but also raises the key question of whether it is always possible to relate quantitatively the few parameters of the theory (diffusion coefficients, activation/inhibition rates) to biophysical observables [2]. At least three avenues of research are currently being undertaken to help answer these questions.

Firstly, how to extend Turing’s intuition from two-component systems to the complex biochemical regulatory networks present in cells (typically involving thousands of proteins/genes each) remains unclear, and graph-theoretical methods have recently been proposed to be a useful tool to bridge this gap [3], providing insights, for instance, into the importance of diffusible vs non-diffusible species in specific points of the interaction network. This allows for generalizations of classical work seeking to understand complex gene regulatory networks as “attractors”, or single-cell states, a perspective made even more topical by the recent advances of single-cell “omics” approaches.

Secondly, the interplay between morphogen reaction-diffusion and tissue geometry has also started to be explored. In cases in which epithelial sheets display curvature and signals with a stromal compartment, then a generic coupling exists that tends to modulate morphogenetic fields based on curvature. This has been proposed to occur for instance during intestinal morphogenesis [4]. The embryonic intestinal wall consists of an initially flat epithelium of developmental precursors, which evolves in a few days into its characteristic three-dimensional corrugated shape composed of both villi and crypts. In this system, cell fate specification is required to be tightly coupled to morphogenesis, as stem cells reside in the bottom of crypts, while differentiated cells move towards the top of villi [5]. It has thus been proposed that epithelial cell-derived Shh accumulates in concave stromal regions (i.e. towards the top of villi), due to the geometry of the gut, with the stroma then providing a feedback to drive local epithelial differentiation [4]. This allows intestinal cells to break



symmetry and adopt their characteristic fate and positional pattern during early development. During subsequent intestinal morphogenesis however, new villi and crypts must be created, which provides a model system to understand how cell fate and tissue shape adapt to growth. Interestingly, this second step involves a surprising degree of cellular plasticity: villi remodeling and fission allow differentiated cells to reposition from villi to nascent crypts, upon which they re-express stem cell markers, and can contribute long-term to the adult organ, as shown via lineage-tracing [5]. In the future, it would be interesting to better understand the mechanisms involved in villi remodeling and fate plasticity therein, and in particular whether these epithelial cells are specified by the same principles as those employed during earlier symmetry-breaking [4], as well as understanding what drives their eventual loss of plasticity [5]. Similarly, it

has been proposed that the early branching morphogenesis of mouse kidney arises from a coupling of receptor/ligand dynamics between epithelial and mesenchymal tissues and local tissue geometry [6]. Such coupling is essential for developmental and patterning robustness [6]. In both intestinal and branching morphogenesis, a next step would be to explicitly include tissue mechanics, rather than implicitly via an imposed geometry, as we discuss in the next paragraph.

Thirdly, the explicit interplay between tissue mechanics and reaction-diffusion has started to be explored in the past few years. Understanding this interplay remains highly challenging, due to the number of possible couplings to consider. Interestingly, however, coupling the two has been found to facilitate patterning, by lifting restrictive conditions on the network structure required for Turing patterns, but also by producing qualitatively different instabilities. Recent theoretical efforts include combining three-dimensional vertex models of epithelial mechanics with morphogen diffusion, assuming certain mutual feedbacks between cytoskeletal contractility, cell shape and morphogen concentration [7], or incorporating the biphasic, porous nature of three-dimensional tissues, assuming feedbacks between morphogen concentration, fluid flows and cellular growth [8]. Strikingly, a few experimental examples of *bona fide* mechano-chemical patterning have started to emerge in the past few years. This includes collective epithelial cell migration towards a wound *in vivo* or a free edge *in vitro*, where complex oscillatory patterns of stress, traction forces and cell

density have been observed, and shown to be accompanied by similar patterns of biochemical signalling, such as ERK/MAPK activity or YAP/TAZ nuclear localization, two key mechano-sensitive pathways. In line with a mechano-chemical loop, disrupting ERK/MAPK patterns by over-activation or inhibition of ERK also disrupted density patterns, long-ranged cell polarization and efficient wound-healing. ERK/MAPK signalling, traction forces and cell density thus feedback on each other to give rise to mechano-chemical waves, which allow epithelial cells to break symmetry and display long-range order in their collective migration towards wound edges [9]. This raises the possibility that mechano-chemical waves could help to transmit information across long distances, an intriguing concept to explore in other settings.

Advances in Science and Technology to Meet Challenges

Besides further theoretical research in systems biology, biophysics, and active matter, further advances will require the combination and development of a number of experimental tools. Firstly, spatio-temporally accurate measurements of mechanical forces *in vivo* remain a challenge. This has nonetheless started to be alleviated by improvements in two-dimensional and three-dimensional force inference methods (allowing force fields to be inferred from high-resolution microscopy images), as well as by direct measurement tools, such as micropipettes for cellular tensions, micro-pressure probes for hydraulic pressure or droplets for mapping local stresses in three-dimensional tissues. Secondly, improved live-measurements of intracellular protein activity (such as FRET or KTR systems), and reporters of cellular fate with high temporal precision will be needed to correlate biochemical activity with local geometrical/mechanical state. Thirdly, progresses in spatial transcriptomics and inferring temporal information from single-cell sequencing datasets will also be critical to achieve a more global “systems view” of these processes. Precise perturbation tools, such as optogenetics and microfluidics will also be needed to progress from correlations to causation. Lastly, advances in organoid/explant research (see contribution from Heemskirk et al) will also offer an exciting new platform to address many of these questions in more experimentally-tractable settings compatible with high-resolution live-imaging. It is striking for instance that although some systems, such as intestinal organoids, display a similar correlation between fate and shape as *in vivo*, other models, such as gastruloids, show precise fate specification despite divergent morphogenetic events [10].

Concluding Remarks

Understanding this will require truly multidisciplinary collaborations, involving biologists, engineers, and physicists, to develop new theoretical frameworks for mechano-chemical self-organization, as well as testing them via combinations of live-imaging, optogenetics, force manipulation, organoid culture, and microfluidics.

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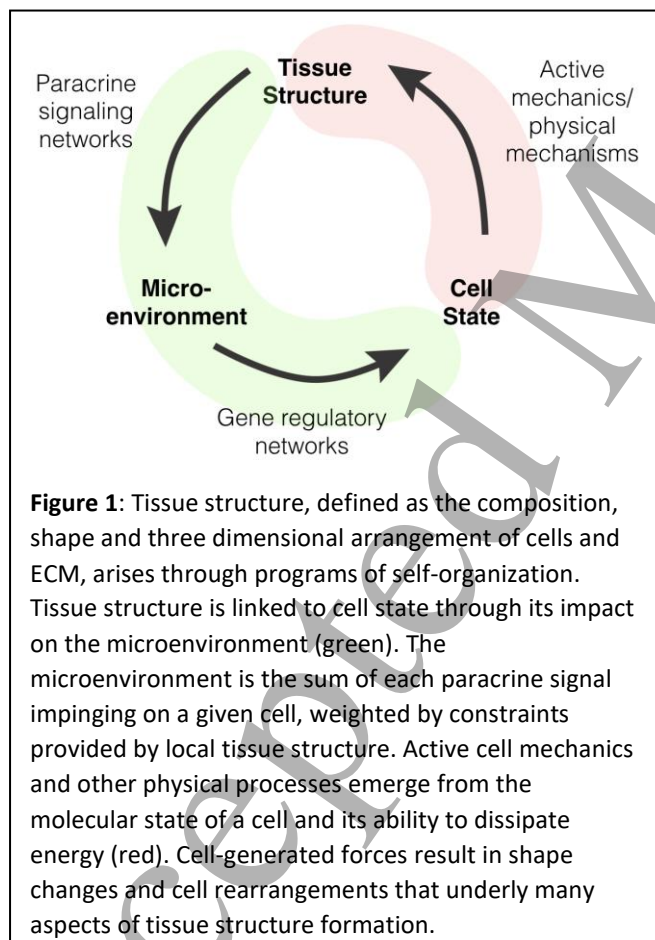
Guiding the formation of tissue structure through self-organization

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Status

Tissue engineers strive to build tissues with complex structures from simple cellular building blocks. This goal requires an understanding of how tissue self-organization emerges from the molecular and physical properties of different cell types. An important step toward this goal was Yoshiki Sasai's first report of stem-cell derived organoids, including an optic cup and a layered neural cortex, that self-organize in vitro[1]. Through analysis of the molecular and physical processes contributing to organoid formation, Sasai articulated several general principles for guiding the formation of tissues through self-organization[2]. Among them are working with the molecules and forces that drive tissue formation during development (rather than imposing constraints on the cells from the top-down); directing tissue formation beginning with cells in the correct molecular and physical state; and, arranging cells in a manner conducive to their self-organization by using the appropriate cell



numbers, cell-type composition, relative cell positions, and boundary conditions. Sasai noted that deviating from these principles would lead to undesirable or heterogeneous outcomes. His efforts anticipated many of the challenges tissue engineers currently face as they work to more efficiently guide the formation of tissue structure through self-organization.

Current and Future Challenges

Tissues assemble from living cellular building blocks whose physical properties can be altered by self-organization itself—a phenomenon unfamiliar to scientists and engineers who work with non-living building blocks such as atoms and molecules. Imagine the surprise of an organic chemist upon finding that carbon spontaneously turned into nitrogen in a specific chemical microenvironment. Unlike atoms, the physical properties of cells are plastic, and cell identity can change in different microenvironments. In adult tissues, structure, microenvironment and cell identity mutually reinforce each other,

resulting in a robust and steady state tissue structure (Figure 1). In developing tissues, however, structure, microenvironment and cell identity do not reinforce one another, resulting in continuing changes to tissue structure. In the context of development, changes to cell state lead to corresponding changes in cell mechanical properties that lead to unbalanced forces. These forces change tissue structure, thereby altering lines of communication between cells and modifying each

1
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3 cell's microenvironment. New microenvironmental signals are interpreted by gene regulatory
4 networks to determine whether to further change cell state. This cycle of structural changes,
5 microenvironmental changes, and cell state changes continue until the tissue arrives at the adult
6 steady state.
7

8
9 Since cell state is closely linked to the outcome of self-organization, a challenge for guiding tissue
10 formation is initiating the process with cells of the correct physical and molecular properties.
11 Isolating populations of single cells from developing tissues at the proper stage is one approach to
12 this challenge, but is problematic because the appropriate cellular characteristics may exist only
13 transiently outside the correct microenvironment. An alternative is to derive these cells in vitro by
14 exposing stem or progenitor cells to sequences of molecular, physical, and electrical cues. However,
15 this approach is hampered because the relevant cues are often unknown. Without better mapping
16 between microenvironment and cell state it will be difficult to systematically isolate cells capable of
17 robust self-organization.
18

19
20 Incomplete understanding of how molecular state maps to mechanical state poses an additional
21 challenge. The same mechanical property, such as the quantitative value of cell cortex tension, can
22 arise in different cell types within different gene expression programs. Thus, even if it were possible
23 to specify the molecular state of a cell by engineering its microenvironment or genome, the
24 particular state to program in order to dial in a desired physical property would still be unknown.
25

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27 Controlling the initial positions of cells in 3D represents yet another challenge. Cells respond actively
28 to their immediate surroundings when determining their next move. Thus, self-organization can
29 progress along different paths depending on the cues that cells receive from their immediate
30 surroundings, a phenomenon referred to as stigmery[3]. Therefore, the initial composition and
31 arrangement of cells, ECM molecules, and other components of a tissue can have a major influence
32 on the outcome of self-organization, leading the same cells to occasionally organize into different
33 three-dimensional structures when initially arranged in different positions.
34

35
36 Similarly, the boundary between a population of self-organizing cells and the surrounding
37 environment profoundly affects the outcome of self-organization. In particular, spurious mechanical
38 cues delivered to self-organizing tissues from their boundaries can lead to undesired outcomes, such
39 as the scattering of cells toward a stiff interface or the inversion of tissue structure due to a missing
40 mechanical interface[4]. In vivo interfaces change their shapes and physical properties along with
41 the tissues themselves. Control over the dynamics of interfaces in vitro has largely eluded tissue
42 engineers and represents yet another challenge.
43

44
45 Beyond these technical challenges, tissue engineers lack generalizable principles to predict how
46 living, non-equilibrium structures change in time to arrive at a steady state. Chemists and material
47 scientists rely on quantitative theories and laws, including statistical mechanics and kinetics, to
48 organize their observations and predict how non-living matter self-assembles. However, it is not
49 clear whether these theories break down when abstracted to non-equilibrium, or "active," materials
50 at the cell-to-tissue scale[5]. How these models must be modified to account for cell-cell
51 interactions and state changes adds yet another wrinkle[6]. Without foundational principles, our
52 ability to guide self-organization will be slow to progress.
53

54 **Advances in Science and Technology to Meet Challenges**

55
56 Numerous opportunities exist for scientists and engineers to further our understanding and control
57 of self-organization. In order to program cells efficiently methods are needed to identify the
58 physical and molecular cues in the microenvironment that direct cell state. In situ methods such as
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3 spatially resolved transcriptomics (e.g. Seq-FISH[7]) and proteomics (e.g. Multiplexed Ion Beam
4 Imaging[8]) represent the state of the art. However, these methods may be better at inferring cell
5 state as a function of cellular neighborhood than identifying the specific molecules or forces
6 responsible for directing cell state. Efforts to recreate specific microenvironments and then measure
7 their impact on transcription or translation may offer a complementary approach.
8
9

10 Along similar lines, progress in our ability to perform simultaneous molecular and physical
11 measurements on single cells will advance our ability to link the molecular state of a cell with its
12 active material properties. Achieving these measurements will likely require new devices for
13 performing these distinct modalities of measurement. Additional work is necessary to make these
14 measurements in situ, and to infer the physical properties and forces generated by groups of cells
15 within living tissues.
16
17

18 Development is a multistep process, and at every point the structure of tissues and their boundaries
19 are arranged so as to set the stage for the next step. Therefore, methods are needed to specify the
20 initial structure of a tissue and its boundaries in 3D culture so as to prepare for its subsequent self-
21 organization. Printing and other top-down technologies have been the focus for specifying initial
22 conditions, but they typically lack the spatial resolution to arrange single cells appropriately and do
23 not scale when many copies of a tissue are required. Synthetic biology methods may provide an
24 alternate path forward. For example, orthogonal genetic programs that direct self-organization
25 could be used to program the initial conditions of cells in culture, after which the synthetic programs
26 would be terminated allowing the cells to continue self-organizing autonomously[10].
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29 Dynamic tissue boundaries are fundamental to living materials, but do not have obvious parallels in
30 the non-living world. Therefore, recreating boundaries with the necessary properties in vitro poses a
31 major challenge. Material scientists are working to build boundaries from dynamic materials that
32 undergo modest changes to shape or stiffness when exposed to different stimuli, but these materials
33 cannot mimic the feedback between a tissue and its boundary that occurs in living organisms. The
34 best solution to this challenge will begin with studying the properties of living boundaries in
35 vivo. Areas for immediate focus include how the interface between parenchyma and mesenchyme
36 is assembled and maintained, as well as how adventitia and serosa support the back-side of the
37 mesenchyme[9]. Understanding the properties of these tissue interfaces, and recreating them in
38 vitro, will advance our capacity to guide tissue formation more efficiently and across larger length
39 scales.
40
41

42 Finally, the theoretical underpinnings of tissue self-organization are ripe for exploration. In
43 particular, basic research into the properties of active matter at the cell and tissue scale is called for.
44 Coupling these studies with dynamical systems theory will be necessary to incorporate information
45 exchange and cell state transitions. Whether unifying principles exist will only become clear as these
46 efforts mature.
47
48

49 **Concluding Remarks**

50
51 An improved ability to guide tissue self-organization will unleash new applications and spawn new
52 industries. However, realizing this potential will require important advances in science, technology,
53 and theory. These challenges represent exciting opportunities for young scientists and will require
54 an interdisciplinary effort.
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60

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Phase transitions, spatial control of mRNA localization and gene expression in syncytial fungi

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Status

Compartmentalizing the cytoplasm in both time and space is critical for cell function. In the last ten years membraneless organelles, also known as biological condensates and droplets, have been shown to compartmentalize the cytoplasm. These compartments exhibit liquid like properties and are thought to form through a process known as liquid-liquid phase separation (LLPS) in which a mixed solution separates into concentrated droplets and a dilute bulk phase. LLPS is driven by weak, multivalent interactions between proteins, often with disordered domains, and nucleic acids, such as RNA. Because these condensates are enriched for certain proteins and nucleic acids, they are

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3 predicted to increase reaction rates, sequester molecules, and serve as signalling or scaffolding hubs
4 (Hyman et al., 2014). However, there are still limited examples of specific biological functions that
5 require condensation. In syncytial fungal cells, which contain hundreds of nuclei and multiple sites of
6 polar growth within a single cytoplasm, compartmentalizing the cytoplasm is particularly important.
7 Biological condensates perform a role in localizing and regulating RNA to spatially and temporally
8 organize syncytial cells. In fungi, RNA containing condensates are important for polar growth,
9 symmetry breaking, nuclear cycling, and mating type switching (Langdon & Gladfelter, 2018).
10 There remain a number of unresolved questions about how biological condensates function in cells,
11 but thanks to the incredibly rapid advance of this field in the last decade, some of these problems
12 can now be addressed.
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16 **Current and Future Challenges**

17 One challenge for the field is to determine how ubiquitous phase separation is in cells. Whether
18 phase separation is a common phenomenon across the proteome, as seems likely, or is limited to a
19 small number of proteins, will likely be answered in the next decade. Another major challenge is
20 understanding the mechanisms by which condensates actually influence specific biochemistry
21 (McSwiggen et al., 2019). There is evidence of changes in reaction rates within condensates but the
22 examples for molecular function are still quite limited (McSwiggen et al., 2019). For proteins and
23 RNAs found to form biological condensates it will be important to answer several additional
24 questions.
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28 *How are condensates regulated in time and space?* Biological condensates must form at or be
29 transported to a specific location in the cell, and dissolve when necessary. The role of regulators that
30 can deliver specific post-translational modifications that may alter valency and interaction strengths
31 amongst key scaffolds in a condensate are likely critical and most await discovery. In addition, how
32 cells regulate the material properties of condensates including size, surface tension, viscosity,
33 elasticity, pore size, ect. is not understood. Study of free-living cells that cannot regulate their
34 internal temperature or control their environment, will be especially useful for understanding how
35 the physical chemistry and biochemical control that underlies the formation of condensates are
36 sensitive to or exploit these physical parameters to cope with extremes in the environment.
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40 *How is specificity achieved and maintained?* The formation of biological condensates is predicted to
41 occur through a generalizable thermodynamically-driven process involving many low-affinity
42 interactions. Many phase separating proteins share features such as being low sequence complexity
43 and predicted intrinsically disordered structure. How are specific combination of proteins and RNAs
44 brought together while excluding others, despite seemingly similar features? There are still very
45 limited models for how protein affinity may be encoded at the scale of individual amino acid
46 composition in low complexity sequences. Understanding how different droplets establish and
47 maintain their individual identities, rather than inter-mixing, is a crucial question in the field.
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50 *How do biological condensates affect RNA dynamics in cells?* Many biological condensates are
51 known to contain RNAs, but with few exceptions their function is not well understood. Learning
52 whether specific condensates promote or prevent translation, degradation and transport and under
53 what conditions will help us understand both basic cell physiology and dysregulation in disease.
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56 **Advances in Science and Technology to Meet Challenges**

57 Only in the last ten years has the concept of liquid-liquid phase separation, a well-described
58 phenomenon in chemistry and physics, been applied to biological systems as a way to understand
59 the spatial patterning of the cytosol. Since then the number of proteins believed to undergo some
60 form of phase separation either *in vivo* or *in vitro* has grown from zero to hundreds. As the number

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3 of proteins that undergo LLPS has increased a variety of computational models, from particle-based
4 Molecular Dynamics simulations to continuum models, have been developed to predict the
5 properties of phase separated systems and the behavior of individual molecules within the system
6 (Choi et al., 2020). A major challenge in all of the current modeling approaches is to capture the
7 complexity of molecular composition and interactions in an experimentally-accurate and
8 computationally-feasible manner.
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11 To ground these models and better understand how condensates function in cells it will be
12 necessary to measure the physical properties of the droplets and of the cellular milieu in which they
13 form. Unlike traditional membrane-bound organelles, droplets are dependent on the physical
14 environment around them and are sensitive to changes in pH, temperature and physical crowding
15 (Wei et al., 2017). The recent creation of *in vivo* probes to measure the mesoscale viscosity of the
16 cytoplasm, complemented by new approaches to manipulate elasticity and compression *in vitro*, will
17 be critical new tools (Delarue et al., 2018). In addition, advances in microscopy such as Fluorescence
18 Correlation Spectroscopy (FCS) allow the measurement of concentration and diffusion rates within
19 droplets with a high degree of accuracy (Choi et al., 2020).
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23 One key question is whether the physical properties of droplets, for example their viscosity, are
24 important for their function. One way to experimentally alter the properties of condensates is by
25 changing the temperatures of the system. Because fungi are able to tolerate temperature changes
26 they are an excellent model to test these questions. By using these new tools to carefully measure
27 the properties of condensates and measuring the functional consequences, it will likely be possible
28 to link material properties of condensates to their functions in cells.
29

30
31 Recent research combined with new tools has shed light on how identity is maintained in some
32 condensates. One critical factor in droplets that contain RNAs is the RNA itself. In the case of Whi3
33 droplets in *Ashbya gossypii*, distinct condensates with functions in the cell cycle and cell polarity are
34 composed of the same protein but contain different RNAs (Langdon et al., 2018). In this system, the
35 functionally distinct droplets seem to arise from specific RNA-RNA interactions that join or segregate
36 different RNAs which may then recruit distinct protein components in addition to Whi3. This
37 depends in part on the sequence of the RNA, but also on RNA structure which appears to scaffold
38 the two droplet types such that they do not mix (Langdon et al., 2018). New tools developed to
39 probe the structure of long RNAs through structure-dependent mutagenesis will allow researchers
40 to test the universality of this phenomenon (Smola et al., 2015). In addition to the role of RNA in
41 conferring identity, structural modelling and mutagenesis has suggested ways in which “hidden
42 order” within unstructured domains in proteins may convey specificity to these droplets (Choi et al.,
43 2020). These advances in studying the structures of RNA and protein within droplets will allow
44 researchers to probe how droplets maintain their identities, and to better understand ways that
45 identity can be lost in disease states.
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49 Application and evolution of tools to visualize RNA also now make it easier to define the functions of
50 condensates in mRNA regulation. Researchers can now monitor RNA in living cells and in real time.
51 For example, stem loop binding proteins, such as MS2, have been used to fluorescently label single
52 mRNAs *in vivo* and to track their transcription, movement, and degradation within the cell, or to
53 monitor protein translation from an mRNA (Wang et al., 2016). Using these techniques, it is now
54 possible to assess how condensates affect RNA localization, stability and translation. Additionally,
55 optogenetics now allow researchers to generate and dissolve condensates experimentally allowing
56 new ways to test for functional relevance (Shin et al., 2017).
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Concluding remarks

The field of biological phase separation has exploded in the last decade and may provide a unifying explanation for many phenomena in biology. Large multinucleate cells such as syncytial fungi provide opportunities to discover new and surprising mechanisms of cytoplasmic organization, and ultimately may provide insights applicable to other cell types like neurons. As the field continues to mature, it will be critical to move from phenomenology to mechanistic descriptions of condensates accompanied by *in vivo* measurements and phenotypes. New technologies and modeling approaches make these goals feasible and ensure an exciting future for the field of biological phase separation.

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The role of mechanics in a “post-molecular” conception of morphogenesis

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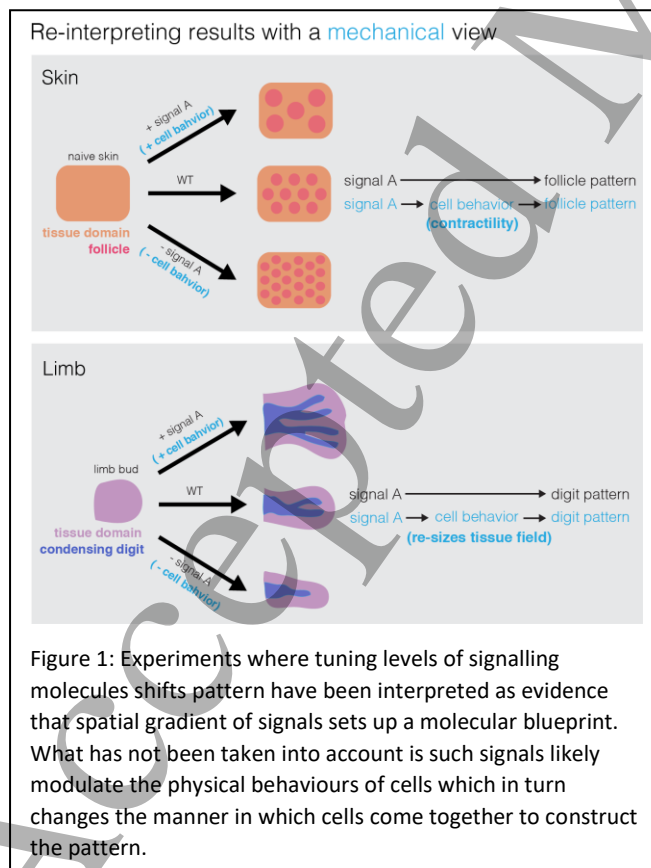
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Status

In recent decades, the study of morphogenesis has been dominated by molecular interrogation. Nobel Prize-winning work in *Drosophila* conducted in the early 1980s used mutagenesis screens to identify individual genes that were essential for proper morphogenesis.¹ To the surprise of many who believed that higher organisms built their complex morphologies with genes not present in simpler organisms, the genes discovered in flies turned out to be conserved across animals. The development of mouse genetics in the 1980s and onward allowed for investigation of the essential roles of these genes in vertebrate development. More recently, the exponential improvement of sequencing technologies has allowed for an expansion of molecular characterization culminating in current day single-cell sequencing.



This molecular focus has highlighted the importance of two classes of genes that have been viewed through distinct conceptual frameworks. The first are transcription factors, which are sufficient to bring about widescale changes in gene expression states as tissue morphology increases in complexity. Remarkably, the forced expression of small numbers of transcription factors can reprogram adult cells to an embryonic stem cell state.² Transcription factors are central to gene regulatory networks, which have been the main framework for explaining the successive emergence of cell fates.³ The second class of genes codes for signalling molecules and their pathway components, which allow cells to communicate with each other. The dominant frameworks, most notably reaction-diffusion systems and morphogen gradients, posit that

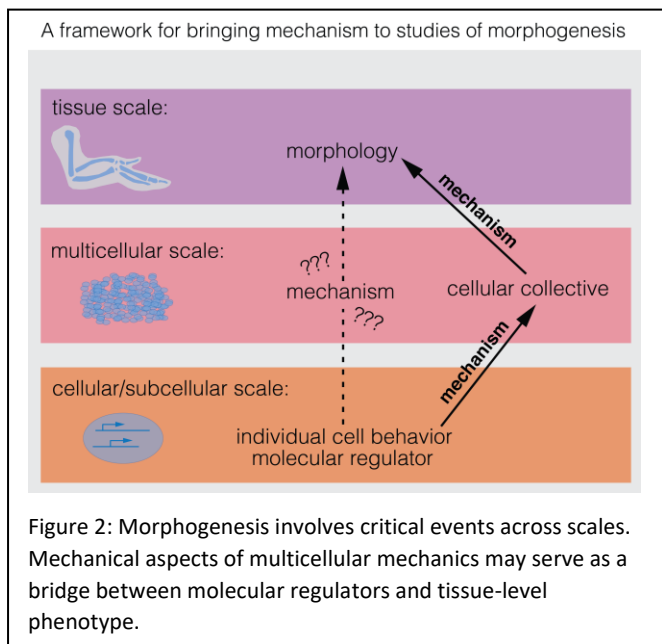
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3 diffusion of such signalling molecules across tissues create molecular patterns that serve as a
4 blueprint for morphology.^{4,5}
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6 Despite a saturation in the identification of the molecular components critical for morphogenesis, a
7 mechanistic explanation of how tissues are physically constructed still remains beyond our grasp.
8 Such a gap is made apparent in organoid cultures, where appropriate fates can be reproduced but
9 control over morphology remains primitive. Studies focused on mechanics are now appreciated as
10 essential to addressing this gap. A fundamental challenge is to integrate mechanical perspectives
11 with existing molecular frameworks that have shaped inquiry for decades. Here, we propose a path
12 forward to achieve this goal and recommend that the integration of mechanics will require some
13 revision of molecular conceptions of morphogenesis.
14

15 **Current and Future Challenges**

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17 While mechanical processes are crucial to morphogenesis, it is currently unclear if key mechanical
18 processes will be conserved across model systems, as in the case of master genetic regulators. For
19 instance, do mechanics in *Drosophila* embryos map to those necessary to generate human tissues,
20 the latter of which occurs at a scale orders of magnitude larger than the former? Given this
21 uncertainty, it may be critical to select contexts in which the study of mechanical processes have a
22 strong likelihood to uncover principles generalizable up to humans. In vertebrates, the most
23 morphologically conserved stage of development is the onset of organogenesis, known as the
24 phylotypic stage. Here, the remarkable similarity in embryonic shape and size suggests the presence
25 of mechanical processes that are so constrained that evolution cannot develop alternate ways to
26 generate such morphologies. Any elucidation of phylotypic stage mechanics has the power to unlock
27 a deeply foundational aspect of morphogenesis.
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30 Molecular characterization of the onset of vertebrate organogenesis has revealed a striking array of
31 signalling molecule and transcription factor expression patterns across developing tissues. Molecular
32 patterning models have typically assumed that these patterns determine the arrangements of
33 emergent morphological features ranging from the digits of the limb, branches of the lung, and the
34 follicles in the skin.^{6,7,8} For example, the vertebrate limb is a system in which morphogen gradient
35 models have framed an understanding of digit pattern formation. Removing or blocking Sonic
36 hedgehog (Shh) results in a loss of digit number whereas an ectopic source of Shh opposite to the
37 endogenous source causes mirror image digit duplication. These results, seen through morphogen
38 models, have been taken as evidence that a spatial gradient of Shh concentration sets up a
39 molecular blueprint for digits. However, a broader perspective that takes mechanics into account
40 during these perturbations reveals that the entire limb field changes size depending on Shh
41 exposure. Ablation of Shh shrinks the size of the limb paddle (hand region) whereas ectopic sources
42 double its size. Thus the impact of Shh may be to modulate the physical size of the limb which in
43 turn changes the resulting limb pattern (Fig1).
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Much like the limb, studies of the developing skin have focused on the patterning role played by diffusing signalling molecules. In the mouse, experimental characterization of Wnt and DKK signals were interpreted via reaction-diffusion models.⁸ Based on these models it was assumed that periodic spatial patterns, after being generated, conferred information to cells to generate properly sized and spaced follicles. Thus, the addition of ectopic signals to skin cultures was assumed to have the primary effect of tuning the molecular pattern landscape. An alternate interpretation when mechanics is taken into account, is that changing signals directly alters the physical

behavior of cells, which in turn shifts the collective dynamics to change the follicle pattern (Fig1).

Moving forward, a central challenge in the incorporation of a mechanical view is that it necessitates a reconsideration of the conclusions of prior molecular studies, many of which may have neglected to account for the mechanical changes that may result from molecular perturbations.

Given that physical events occur at every length scale from the atomic to the tissue, another challenge is to select the length scales at which mechanics shape emergent morphological patterns. While the mechanics of individual cells may be the most tractable scale, due to the wealth of insight and technology from the field of cell biology, it is unclear whether such insights can be extrapolated to understand the mechanics of collectives of cells inherent to tissue formation. Instead, new tools and concepts must be developed to understand mechanics at the scale between single cells and a whole tissue – that of the multicellular collective.

Advances in Science and Technology to Meet Challenges

While more effective measurement and perturbation of physical interactions across collectives of cells will be essential, the fundamental bottleneck is the need for a conceptual framework that can account for the molecular observations made over the past several decades but also incorporate the role of mechanics. Such a conceptual approach is illustrated in a recent study on the initiation of follicles in the avian skin.⁹ This work suggests a process of morphogenesis that is dependent on the multicellular self-organization of skin cells into dense clusters in order to initiate follicle morphology. The formation of these clusters also directly activates a mechanotransductive pathway that initiates the gene expression state of follicles.

A number of potentially generalizable features can be taken away from this mechanism. First, symmetry can be broken in the absence of a molecular pattern through mechanical events that occur in an *emergent* manner at the multicellular scale.¹⁰ Second, due to their emergent character, these processes play a *regulatory* role in determining morphology and are not merely an elaboration of molecular instructions. These features suggest that molecular pre-patterns need not be necessary to initiate tissue morphologies. Instead, [many of] the molecular patterns that have been observed in tissues for decades may be the direct consequence of mechanical impulses communicated from the multicellular scale to the genomic level. Such communication could be one of the critical roles played by increasingly appreciated mechanotransductive pathways. Therefore, patterns of gene

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3 expression must be considered as modulators of cell mechanics that then feed upward to tune
4 multicellular mechanical processes that are already in play through their own emergent accord.
5

6 7 **Concluding Remarks**

8 If vertebrate organogenesis is governed by mechanical processes at the multicellular scale that are
9 not reducible to molecular level regulation, cellular and molecular level analysis alone will be
10 insufficient to fully grasp morphogenesis. Instead we anticipate that a “post-molecular” conception
11 of morphogenesis that focuses on multicellular mechanics will be the key to link the decades long
12 analysis of molecular regulators to tissue level morphology. As has been shown in the case of
13 theoretical studies of cellular automata, where very simple local rules when iteratively deployed are
14 capable of generating tremendous richness of pattern, multicellular mechanical processes may be
15 driven by simple cellular behaviors that nonetheless generate complex final forms. Despite the
16 importance of mechanics for the future of studies in morphogenesis, it is critical to maintain sight of
17 molecules and regulation of the genome. In this new conceptual regime, however, molecules, rather
18 than “encoding” for morphological patterns in any direct sense, are likely to allow for a more robust
19 deployment of the emergent mechanical forces that sculpt tissues at the multicellular scale.
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Dynamic interplay between cell shape and division positioning in the morphogenesis of early embryos

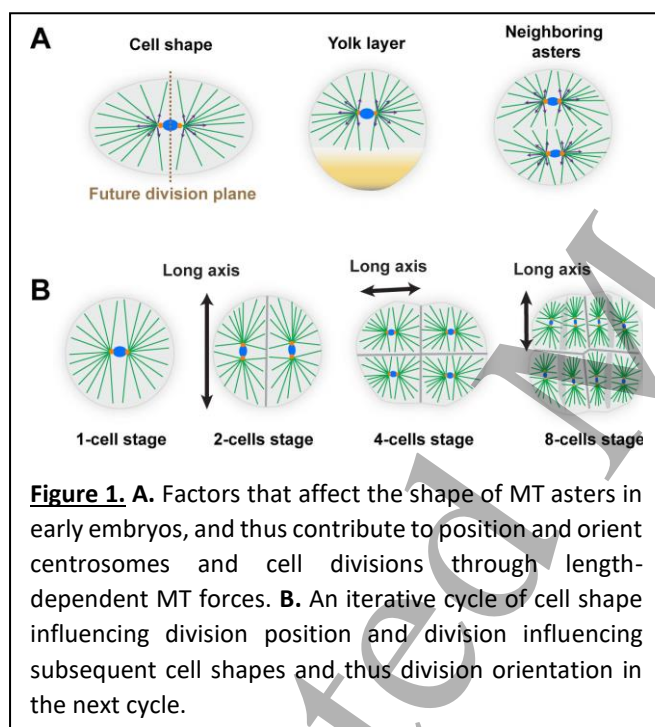
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Status

Cell division is pivotal to tissue morphogenesis, cell size control and fate specification. Even when embedded in complex tissues, cells divide with a predictable position and orientation. This has long fascinated life scientists, with the formulation of empirical rules for division site specification dating from as early as the end of the 19th century (reviewed in [1]). The choreography of reductive blastomere divisions that mark the development of early embryos, known as cleavage patterns, is a



striking example of regular cell division geometries. Because blastomeres do not migrate, grow or die, the morphogenesis of the embryo and the position of each cell will solely depend on the history of their divisions, and adhesion forces between cells [2]. Cleavage patterns come with a remarkable diversity, from orthogonal, holoblastic, meroblastic to spiral patterns. To date, however, their regulation remains poorly understood. This is in part because eggs are extremely large, often opaque, and thus difficult to image; and also because genetic manipulations are limited by the large pool of maternal proteins and mRNA. Yet, studying cleavage patterns is of profound interest for the field of cell division and morphogenesis. First, it provides a dramatic physical limit to cell division, with very large cells that must divide rapidly and accurately. Second, the

diversity of cleavages offers a rich repertoire of phenotypes to study division positioning and tissue morphogenesis in 3D.

Current and Future Challenges

1- Mechanisms of microtubule force generation for division specification

The regulation of division positioning has been studied in many model systems, including yeast and adherent vertebrate cells. In most cells, the nucleus and/or mitotic spindles are first positioned and oriented from forces and torques emanating from astral microtubules (MTs), and cytokinesis then bisects the spindle axis in anaphase. In small somatic cells, astral MTs are generally thought to be pulled upon by dynein motors attached to the cell cortex [1]. In contrast, the dominant model in large eggs and early blastomeres is that division may be primarily organized from forces exerted upon MTs directly from bulk cytoplasm. This is best demonstrated by recent work recapitulating the division patterns of frog embryos from bulk cytoplasm extracts, which lack cortex and plasma

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3 membrane [3]. This essay highlights the current view for the regulation of symmetric and oriented
4 divisions in many early embryos including those of echinoderms, ascidians, fishes or frogs [2, 4-6]. In
5 this view, dynein pulling from bulk allows longer MTs to generate more pulling forces, thereby
6 converting aster shape anisotropies into net forces and torques that move and orient centrosomes.
7 Aster shapes may in turn be regulated by cell shape, aster-aster interactions or the presence of
8 asymmetric yolk layers (Figure 1A, [7]). These mechano-geometric mechanisms provide a simple, yet
9 powerful iterative design that accounts for many features of early embryos, such as the typical
10 orthogonal sequences of early blastomere divisions (Figure 1B, [6]).

11
12 Although modelling and cell manipulation support these models [4-6], still little is understood on
13 how dynein may pull in bulk and organize aster forces. Addressing such mechanisms constitute one
14 important challenge. Dynein could, for instance, pull on static or elastic structures in bulk, such as
15 networks of intermediate filaments or actin. Alternatively, dynein could exert bulk forces from the
16 viscous drag of endomembranes and vesicle cargos it transports to the aster center [8]. Tests of
17 these hypotheses will necessitate the manipulation of bulk elements, the proper measurement of
18 flows at multiple scales, as well as the development of modeling frameworks to understand how
19 force-generation events are integrated to produce the measured net forces and torques applied by
20 MT asters [9]. A second feature of this view, is that asters must stop growing in the vicinity of the
21 cortex, yolk layers or neighboring asters. Dynamic regulation of plus end MT growth, through
22 motors, bundlers, end-binding, or depolymerizing factors, is clearly involved, but mechanisms are
23 still partially lacking [7]. Finally, large asters that span hundreds of micrometers, depart from the
24 standard radial organization picture, and feature bushy, branched and poorly connected MTs. How
25 asters organize and grow, and how forces are eventually transmitted to the centrosomes are other
26 fascinating problems to solve in the future [7].

2- The diversity of cleavage patterns

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31 Cleavage patterns are regular and often invariant among large numbers of species, indicating a high
32 level of regulation. Yet, these divisions can reorganize in response to physical manipulations such as
33 *e.g.* egg bisection, centrifugation or shape modulations, suggesting that they rely more on self-
34 organization than deterministic programs. Furthermore, early embryos loaded with maternal
35 material, develop with a near-constant cytoplasm biochemistry [3]. Those considerations raise the
36 question of how and where deterministic cues may compete or cooperate with self-organizing
37 designs such as those based on aster shape and length-dependent forces (Figure 1). Many embryos
38 inherit maternal polar domains that prime cortical dynein pulling for aster de-centration and
39 asymmetric divisions. Yolk layers may also constitute an initial spatial cue to organize aster position
40 and cell division [2, 6]. Although current models typically implement those guiding cues as fixed
41 initial conditions [6], these cues likely mature in strength or even change position through their
42 interactions with growing and moving asters. Fertilizing embryos also feature large-scale acto-
43 myosin driven flows that may move yolk, cortical domains or endomembranes. Finally, asters may
44 also contribute to control surface mechanics by spatially regulating cell-cell adhesion and
45 contractility. Thus, one exciting possibility, is that both the robustness of embryonic cleavage, as well
46 as their diversification, may result from the tuning of different feedbacks, rather than from pre-
47 determined genetic information. The challenge ahead is thus mostly embedded in documenting and
48 studying these interplays, and how they may predict morphological features of multiple embryos.
49 The use of emerging synthetic methodologies to move the division apparatus, create flows or
50 displace cortical domains will be instrumental to achieve this future challenge [9].

3- Relevance to smaller cells in tissues

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56 When studying cell division in small somatic cells, what can we learn from the divisions of large eggs
57 and blastomeres? Small cells are likely dominated by surface effects, and centrosome positions and
58 cell division may rather hinge on cortical MT forces and dynein activity. However mechanisms for
59 dynein pulling implicating endomembranes and bulk hydrodynamics are likely relevant to small cells

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3 as well, because their internal organization and cytoplasm physical properties are similar to that of
4 large eggs and blastomeres. In spite of plausible divergent regulatory mechanisms, adherent and
5 tissue cells also respond to geometrical cues and usually orient their division along the long cell
6 shape axis [1]. Remarkably, recent findings even suggest that the large scale architecture of
7 developing epithelial tissues (e.g. squamous, cuboidal or columnar) may also depend on iterative
8 shape-division interplays [10]. Addressing the titration of these interplays by effectors of surface
9 mechanics and division positioning, certainly constitutes another challenge for future studies
10 addressing the contribution of proliferation to tissue dynamic.
11

12 **Advances in Science and Technology to Meet Challenges**

13
14 One aspect which has limited studies of embryonic cleavages stands in the accurate dynamic 3D
15 imaging of blastomere shapes, internal organization and division. There is thus a need to implement
16 methods enabling high resolution 3D live imaging in large and poorly transparent embryos. Light-
17 sheet, multi-photon microscopy, and advances in electron tomography are promising avenues. In
18 addition, modelling approaches are thus far restricted to mechanical inputs, and the identification of
19 equilibrium division positions and orientations, and cell shapes [1, 9, 10]. The implementation of
20 novel simulation platforms, integrating cytoskeleton dynamics, such as aster growth,
21 rearrangements, endomembranes and hydrodynamics interactions, will be instrumental to derive
22 comprehensive mechanisms. The study of the spatial organization and division of early embryos
23 could serve to motivate the development of these technologies.
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26 **Concluding Remarks**

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28 As discussed throughout this text, studies in the past decade have revived the early interest of
29 studying cleavage patterns, not only from a developmental perspective, but also as a means to
30 understand cell division and morphogenesis as a whole. To date, many patterns still remain mostly
31 enigmatic. Spiral cleavages, for instance, is a highly represented pattern in invertebrates, for which
32 basic documentation and mechanisms are still lacking. The re-emergence of non-model systems in
33 modern biology will certainly help to fill this gap and address how evolution has tuned specific
34 biological and physical parameters to create the astonishing diversity of cleavage divisions and early
35 embryo morphogenesis.
36

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38 Research Council (CoG Forcaster N° 647073).
39

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Building a mechanical atlas of the preimplantation embryo

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Status

During preimplantation development, the mammalian embryo forms the blastocyst, which will implant into the maternal uterus. The blastocyst is initially composed of two distinct lineages: the trophoblast (TE) and the inner cell mass (ICM). The TE, which envelopes the ICM and a fluid-filled lumen called the blastocoel, is responsible for invading the uterus and contributes to placental tissues. On the other hand, the ICM gives rise to all embryonic tissues as well as extra-embryonic derivatives. Decades of research revealed how the architecture of the blastocyst is crucial for the differentiation of the first lineages and for defining the first axes of the mammalian body. However, it is only recently that studies focused on what shapes the architecture of the blastocyst.

The shaping of the blastocyst can be broken down into distinct morphogenetic steps, that occur as cells undergo synchronous cleavage divisions. In the mouse, these take place in the following sequence: compaction, internalization, and lumen formation at the 8-, 16- and 32-cell stages respectively. Compaction consists in a developmentally regulated wetting process during which cells are drawn into close contact. During internalization, precursors of the ICM envelope themselves with the precursors of the TE until they are no longer in contact with the outside medium. Lumen formation results from the accumulation of fluid within the intercellular space, which separates contacts between the ICM and some of the TE cells. This morphogenesis takes place within the zona pellucida, a porous elastic capsule made of glycoprotein, from which the embryo hatches before implantation.

Mechanical changes driving the morphogenesis of the blastocyst have already been identified using several methods, including laser ablation and micropipette aspiration. Such methods can exploit key advantages provided by this experimental system: countable number of large cells that divide and developing slowly enough to perform repeated measurements. This makes the preimplantation

embryo an ideal platform to characterise cell and tissue mechanics extensively in order to understand embryo morphogenesis and compare different methods of mechanical measurements.

Current and Future Challenges

Several methods have been used to characterise the mechanics of the developing blastocyst: laser ablation, micropipette aspiration, atomic force microscopy (AFM) and deformable hydrogels. These methods pose distinct challenges in terms of invasiveness (access and damage to the sample) and in the type of measurement they offer (absolute values in physical units, information about anisotropy, different model assumptions). This roadmap delineates the methods and associated challenges that can be used to build a mechanical atlas of the developing preimplantation embryo.

Laser ablation offers the unique possibility to probe tension anisotropies from the sub-cellular to the tissue scale. After laser ablation of pre-stressed materials, a recoil can be observed and its initial velocity is proportional to the stress and friction within the material. Therefore, measurements of recoil velocity can be used to infer tension anisotropies *in vivo*, provided that friction within the material is unchanged. During the internalization process that will form the ICM, laser ablation revealed tension anisotropies with larger tensions directed towards the internalizing cell **(1)**. This is caused by higher tension in the internalising cell itself **(2)**. Laser ablation was also used to assess the tension at tight junctions before and after the zippering of a ring of actin surrounding the apical domain **(3)**. However, it remains unclear whether measured differences in recoil velocities were due to changes in tension within the actomyosin cytoskeleton or to changes in friction within the maturing cell-cell contacts. One final cautionary note: although laser ablation provides a very practical way to peer into sub-cellular forces, it can be misleading if uncontrolled, as severe damages, for example causing cell fusion **(4)**, can also affect the recoil. It can also be difficult to determine what is being ablated and for example, an attempt at cutting a filopodia at the cell surface can also sever the nearby actomyosin cortex **(4)**.

Micropipette aspiration can be used to exert controlled pressure from sub-cellular to tissue scales, and surface tensions can be calculated using the Young-Laplace equation. This assumes that the sample behaves as a liquid-droplet, which curvature depends on the ratio between its pressure and surface tension. Unlike laser ablation, micropipette aspiration is a contact method, which requires stripping the embryo from its zona pellucida. Applied throughout the 8-cell stage, micropipette aspiration allowed building a spatio-temporal map of surface tension at the sub-cellular level and in absolute value **(5)**. Measuring absolute values was critical to evaluate the relative contributions of the tension changes at cell-medium versus cell-cell interfaces. This revealed that $\frac{3}{4}$ of compaction is driven by an increase in surface tension at the surface of the embryo and $\frac{1}{4}$ by the relaxation of cell-cell contacts. Furthermore, because micropipette aspiration does not damage the sample, it allowed to evaluate the tensions of cells before they internalize to become part of the ICM during the 16-cell stage. This was essential to follow cells and test a theoretical prediction that cell sorting would occur if and only if the tension ratio between neighbouring cells would grow above 1.5 **(2)**. Later at the blastocyst stage, after blastocoel expansion, micropipette aspiration experiments report different surface tensions between the TE lining the ICM and the TE facing the blastocoel **(6)**. The biological significance of this difference remains unclear and will require further investigation. Also, at this stage, cells have become smaller with cleavage divisions, they stretch extensively, and the nucleus becomes too bulky not to be aspirated into the micropipette. This makes the liquid-droplet assumption difficult to justify and the interpretation of micropipette aspiration delicate **(6)**.

In addition to micropipette aspiration, the properties of the TE were also probed using atomic force microscopy (AFM), a rapid and very sensitive methods to measure the mechanical properties of living materials **(6)**. AFM records the bending of a cantilever while pressing on the embryo which in this experiment, had been stripped from its zona pellucida and glued onto a culture dish. This AFM measurement was done by compressing whole blastocysts and the apparent elasticity of the TE

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3 extracted using an elastic shell model. The validity of this model was not tested and it remains
4 unclear how much mechanical resistance to compression is provided by the epithelial shell and by
5 the lumen that it envelopes.
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7 Multiple techniques have been used to evaluate the pressure of the lumen, and these can be directly
8 compared. Introducing a needle into the blastocoel of an intact embryo and equilibrating the fluid
9 flow allowed measuring the hydrostatic pressure inside the blastocoel at ~ 1 kPa (**6**). However, rapid
10 loss of epithelial integrity, and rapid pressure drops, limits the time scale of this measurement to a
11 few seconds. Less invasive micropipette aspiration at the embryo surface were used to measure
12 pressure while following normal development. Using the Young-Laplace equation yielded a
13 hydrostatic pressure of the blastocoel at ~ 300 Pa (**7**). However, similarly to the AFM approach
14 mentioned above, this measurement could not distinguish the contributions of the blastocoel and
15 the overlying epithelium. Also, micropipette aspiration and AFM measurements were performed on
16 embryos without the zona pellucida. Alternatively, placing embryos within their zona pellucida into
17 hydrogels of known stiffness allowed monitoring the gel deformation upon blastocoel inflation. This
18 revealed blastocoel pressures of ~ 1.5 kPa and a key aspect about the pressure of the blastocoel:
19 building up pressure above a few hundreds of Pa requires the presence of the elastic confinement of
20 the zona pellucida (**8**). This method and the associated model also allowed measuring a battery of
21 biophysical properties of the blastocyst such as the elasticity of the zona pellucida or the
22 permeability of the TE to water and osmolytes. Such data will be key for our understanding of
23 blastocoel formation and maintenance.
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28 **Advances in Science and Technology to Meet Challenges**

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30 Surface tension and pressure measurements in the forming blastocyst have established the
31 preimplantation embryo as an ideal system to study the mechanics of embryonic development.
32 Looking forward, it is now possible to apply additional techniques to extensively characterize the
33 biophysical properties of the mouse embryo and build a mechanical atlas of preimplantation
34 development. Such an atlas will constitute a precious blueprint for future studies. On the one hand,
35 it will facilitate comparing and benchmarking techniques as applied to mouse embryos (**9**). On the
36 other hand, it will facilitate comparing mechanical properties across different tissues and species. In
37 addition, it will provide material for theoreticians to build and test increasingly faithful models of
38 preimplantation development. For this to be possible, building the atlas requires rigorous and
39 ambitious standards: absolute measurements in physical units.
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42 Additional mechanical properties can readily be measured with current technologies. For example,
43 cell and tissue viscosity can be assessed using micropipette aspiration. The mechanical stability of
44 cell-cell contacts can be measured using a dual pipette assay or AFM. The mechanical properties of
45 the plasma membrane could be characterized using AFM, optical or magnetic tweezers. These will
46 provide unique insights into the role of these properties in preimplantation development.
47

48 Other biophysical properties will require developing new tools. In particular, the role of intercellular
49 fluid in shaping tissues will benefit from biophysical measurement of cells and junctions permeability
50 to different solutes. This would allow precisely assessing their relative contribution to osmotic
51 pressure. In addition, understanding the contribution of nuclear mechanics to the deformation of
52 the embryo at later stages of preimplantation development when nuclei deform significantly will
53 require rigorous measurements and theoretical interpretations.
54

55 **Concluding Remarks**

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57 Within 5 years, preimplantation development went from mostly uncharted to one of the best
58 mechanically characterized experimental systems for studying morphogenesis. This allows us to
59 envision building a mechanical atlas of preimplantation development in the near future. Such atlas
60

will be a unique tool to compare biophysical measurements in order to benchmark techniques and to understand more precisely the molecular and cellular origin of the emerging biophysical properties that are measured. This atlas of mechanical properties in absolute values will also allow directly comparing preimplantation development in different mammalian species, such as humans, and thereby open up a new field of research: evolutionary mechanics.

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Physical principles of organization of the cell cycle and morphogenesis in early *Drosophila* embryos

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Status

Like the embryos of many other species that develop outside the mother, *Drosophila* embryos are large. Following fertilization, they undergo a series of rapid cleavage divisions to increase DNA content prior to gastrulation [1]. In these early stages the embryo is a syncytium, thus the rapid nuclear divisions generate a large multinucleated cell. Following a cycle of 13 precisely timed and reproducible rounds of division (Figure 1A), the embryo finally undergoes the maternal-to-zygotic transition. At this transition, zygotic gene expression increases, the cell cycle slows down, a specialized form of cytokinesis encloses the nuclei into membranes- a process known as cellularization- and gastrulation begins [2].

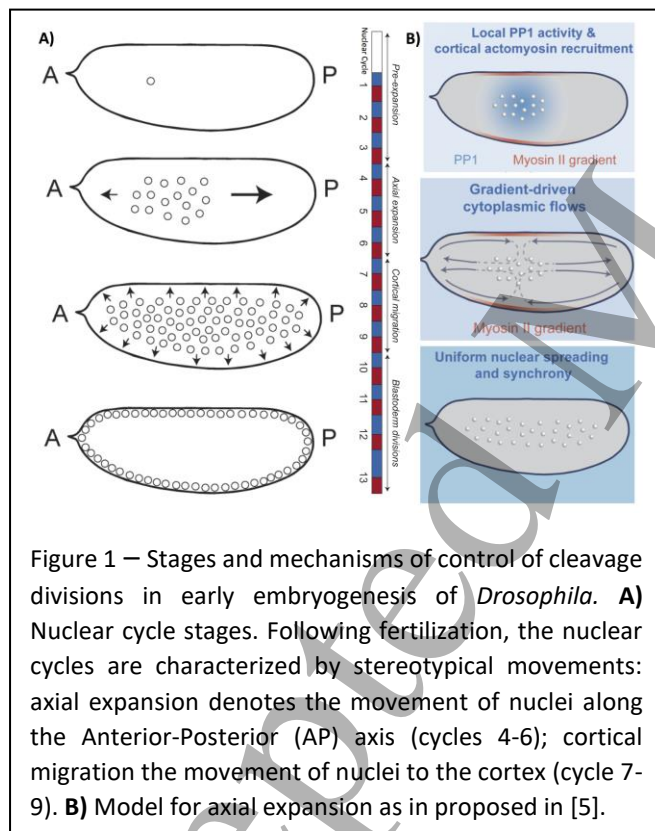


Figure 1 – Stages and mechanisms of control of cleavage divisions in early embryogenesis of *Drosophila*. **A)** Nuclear cycle stages. Following fertilization, the nuclear cycles are characterized by stereotypical movements: axial expansion denotes the movement of nuclei along the Anterior-Posterior (AP) axis (cycles 4-6); cortical migration the movement of nuclei to the cortex (cycle 7-9). **B)** Model for axial expansion as in proposed in [5].

To ensure that these processes occur in a coordinated manner, it is important that the cell cycle in the early cycles is synchronous across the embryo. It has recently emerged that the synchrony of these divisions requires specialized processes [3-5]. Three of the early nuclear cycles (nuclear cycles 4-6) are characterized by rapid nuclear movements, which ensure that nuclei travel along the anterior-posterior axis to uniformly fill the embryo (Figure 1A). These movements are instructed by the cell cycle machinery, yielding an interesting example of integration of biochemical and mechanical signals [5] (Figure 1B). In brief, the activity of one of the major kinases driving the cell cycle – the Cyclin-dependent kinase 1 (Cdk1)- undergoes oscillations restricted to a small region of space surrounding the nuclei. The local decline in Cdk1 activity at mitotic exit, triggers the activation of mitotic phosphatase PP1, within a region of space comparable to the distance between

the nuclei and the cortex (40-50 microns). Thus, at the end of mitosis/early interphase PP1 is active specifically in a region of the cortex overlying the nuclei. Locally active PP1 can trigger the cortical activity of the GTPase Rho, which in turn triggers myosin activation. When myosin activation is not uniform across the cortex, the force imbalances generated by tension gradients induce embryo-wide cytoplasmic flows that have the correct geometry to move the nuclei so as to fill the entire embryo (Figure 1B). Once the nuclei are uniformly placed, the coupling of cell cycle machinery and actomyosin contractility remains active, but the uniformity of myosin at the cortex precludes the generation of further flows. Thus, the mechanisms that drive proper nuclear positioning in the early

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3 *Drosophila* embryos arise from the integration of biochemical (cell cycle oscillator) and mechanical
4 signals (actomyosin contractility and hydrodynamic flows).
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6 Following the process of axial expansion, nuclei migrate towards the embryo surface during cell
7 cycles 7-9 to generate a blastoderm of uniformly spaced nuclei. At the surface, the embryos undergo
8 4 additional rounds of meta-synchronous divisions. These divisions do not occur at the same time
9 across the embryo but display wave-like patterns. *In vivo* measurements of Cdk1 activity,
10 mathematical modeling and theoretical analyses have demonstrated that mitotic waves are
11 controlled by waves of Cdk1 activity [3-4]. It was demonstrated that these waves do not arise
12 through trigger waves, as previously described. Although the Cdk1 regulatory system exhibits
13 bistable dynamics in *Drosophila* embryos, during the very fast early cell cycles, the rapid drive that
14 moves the system out of a bistable regime outpaces the state switching that underlies propagation
15 of a trigger wave. In this scenario, waves arise from the following mechanism. The early dynamic and
16 spatial coupling of Cdk1 activity generates activity gradients. As the cell cycle progresses, bistability
17 is lost almost simultaneously across the embryo. When this happens, the activity of Cdk1 raises at a
18 uniform rate across the entire embryo, which results in a wave-like pattern of mitotic entry.

19 Following this stage, clock-like processes ensure that a wave of mitotic exit follows the wave of
20 mitotic entry [3-4]. Thus, the synchronization of the cell cycle prior to the maternal-to-zygotic
21 transition arises from a reaction-diffusion mechanism generating wave-like spreading of activity in a
22 transiently bistable system [4]. The recent progress described above on the understanding of the
23 cleavage divisions of *Drosophila* embryos highlights the potential of this system to uncover novel
24 physical principles of morphogenesis.
25
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27 **Current and Future Challenges**

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29 Despite much recent progress, several important questions remain open in the field. Novel insights
30 will also be gained by comparing cleavage divisions in *Drosophila* to those in other species. For
31 example, a recent study of the coupling of cell cycle and actomyosin dynamics in zebrafish embryos
32 reveals how this interplay controls the separation of yolk and cytoplasm [6]. In this system,
33 ooplasmic segregation is not driven by cortical contraction, but rather by contractility of bulk
34 cytoplasmic actomyosin. Consistent with previous findings [7], bulk actomyosin contractility is
35 stimulated and not inhibited by Cdk1 activity. The mechanisms and full implications of this different
36 regulation of actomyosin contractility by the cell cycle (stimulated in the bulk and inhibited at cortex)
37 remain to be explored. Furthermore, ooplasmic segregation arises from the opposite movements of
38 yolk and cytoplasm which are controlled by a wave of actin assembly that pulls cytoplasm and push
39 yolk granules. Understanding the hydrodynamic coupling of actomyosin gels and the cytosol (soluble
40 part of the cytoplasm) and how they can be modulated to generate flows of geometrical features
41 desired for different functions remains a fundamental open question in morphogenesis [8].
42

43 Moreover, the study of these flows offers a unique perspective to gain insights on the physical
44 properties of the cytoplasm and to dissect under which conditions the cytoplasm behaves as a
45 simple fluid or a complex one.
46

47 Insect embryos vary in size and shape, but how the coupling of cell cycle, cortical contractility and
48 cytoplasmic flows are influenced by geometry and how they scale with size remains to be explored.
49 The positioning of nuclei does not only rely on actomyosin-driven flows. Following axial expansion,
50 nuclei migrate to the cortex through a microtubule-driven process. Whether this process involves a
51 similar integration of cell cycle and cytoskeletal dynamics remains to be elucidated. Moreover, it is
52 likely that not only nuclei but many other organelles or important molecules (for example
53 morphogens) are transported by cytoplasmic flows.
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55
56 Recent work in both *Drosophila* and other systems [9] has demonstrated the importance of traveling
57 waves of signaling activity as a mechanism to coordinate biochemical dynamics across large scales. It
58 will be interesting to study the ability of chemical waves to propagate through complex multicellular
59 tissues through extracellular ligands or other means of inter-cellular communication.
60

Advances in Science and Technology to Meet Challenges

The recent progress in understanding the cleavage divisions of *Drosophila* embryos was made possible by the use of a biosensor for cell cycle dynamics (a Fluorescence Resonance Energy Transfer biosensor revealing the ratio of activities of Cdk1 and PP1). Moreover, important and definitive insights on the connection between cell cycle oscillations and cortical actomyosin dynamics were established using an optogenetic method to control cortical contractility [5]. We expect that further development of biosensors and optogenetic methods will afford the field a unique ability to monitor and manipulate biochemical and mechanical signals in morphogenesis. These methods could be complemented by physical manipulations to alter cytoplasmic flows, for example by generating artificial temperature differences inside the embryo [10].

The analyses of cytoplasmic flows have been mainly limited to two-dimensional projections. Recent advancements in light-sheet imaging suggest that it might be possible in the future to perform full three-dimensional reconstruction of cytoplasmic flows.

Concluding Remarks

Recent quantitative work in *Drosophila* embryos has revealed novel concepts for the spatiotemporal coordination of the cell cycle during rapid cleavage divisions. These experimental and theoretical results have important parallels and analogies to many other embryonic systems, suggesting shared physical principles for the organization of embryogenesis. Dissecting the generality of these mechanisms and their extension to more complex adult tissues has the potential to provide a new quantitative paradigm for Developmental Biology.

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Dynamic interplay of cell contact geometry and signalling

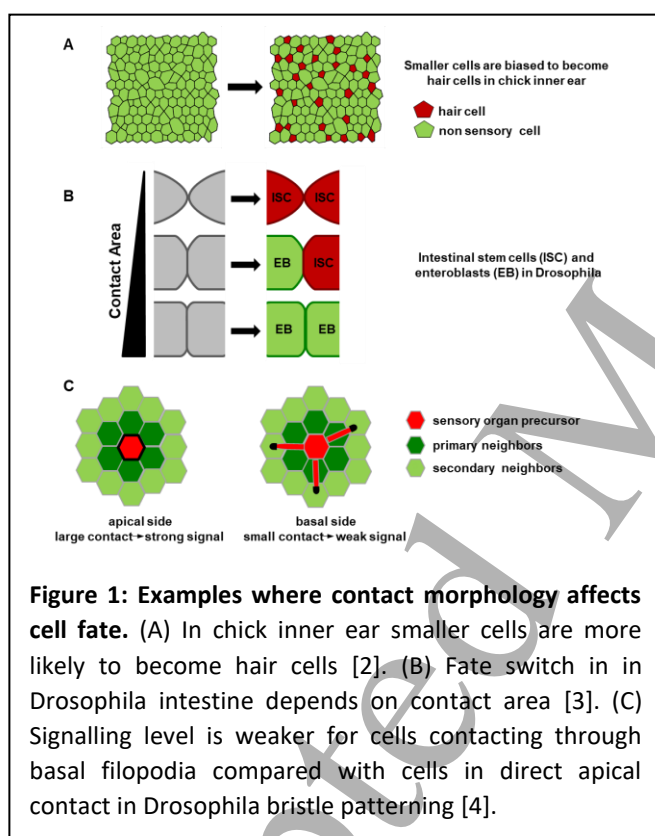
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Status

In recent years it is becoming increasingly evident that cellular and tissue morphology are tightly regulated to achieve specific cellular organization in terms of the number of cells, their types, their positions, and their sizes. Such cellular organization requires feedback from cellular morphology onto the control of differentiation, cell movement, cell death, and more. For example, during epithelial growth in *Drosophila* wing disks, cell competition drives specific cells to go through apoptosis in a process that depends on the number of neighbors expressing high *myc* levels [1].



This example and others raise the question of how morphological information is converted into biochemical signals that regulate differentiation. An appealing possibility is that changes in cellular morphology affect cell-cell signalling through properties of the contact between cells. Several recent works have discussed this possibility in the context of Notch signalling which requires direct cell-cell contact between the sender and receiver cells and hence is likely to be affected by contact morphology.

Shaya et al. have shown that the dependence of Notch signalling on contact area can explain why smaller cells are more likely to differentiate into hair cells during chick inner ear development [2] (Figure 1a). Guisoni et al. showed that Notch signalling strength between pairs of intestinal stem cells correlate with their contact area and influences their differentiation into

different intestinal cell types [3](Figure1b). Finally, Hunter et al. showed that Notch signalling through basal filopodia contacts is weaker than via the larger apical contacts during differentiation of sensory organ progenitors in flies [4](Figure 1c).

Current and Future Challenges

The relation between contact morphology and signalling poses several significant challenges. Establishing causal relation between contact morphology, contact time, and signalling level is difficult because of several factors. First, it is unclear how to uncouple signalling from different sources. Cells are typically surrounded by multiple neighboring cells and receive multiple signals.

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3 This can be solved in some cases, by looking at situations where communication is restricted to pairs
4 of cells. Examples of such situations in vivo can be found during asymmetric cell divisions such as
5 those occurring in the intestinal stem cells mentioned above [3] and in the sensory organ precursor
6 in flies [5]. In these cases, signalling can be correlated with the contact area, however, it is often
7 hard to manipulate the contact area in order to show direct effect of contact on signalling. In-vitro
8 systems may offer better control of the contact area between cells. Shaya et al. used a double micro-
9 well to measure Notch signalling between a sender and a receiver cell where the contact width can
10 be controlled by the width of the neck connecting the two wells [2]. These measurements showed
11 that Notch signalling correlates with the contact width, for contact widths ranging from 1-40 μm
12 (Figure 2a,b).
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16 Another factor is the difficulty to associate intracellular signalling response to events that occur at a
17 specific cellular interface. In a recent elegant work, Trylinski et al. [5] used Notch tagged with a
18 photoconvertible fluorescent protein to show that signalling between p11a and p11b daughter cells
19 during asymmetric cell division of the sensory organ precursor in *Drosophila*, originates from a
20 specific sub-apical compartment between the cells. This was done by converting only the Notch
21 molecules in this sub-apical compartment to red fluorescence, and showing that these converted
22 Notch molecules are the ones that accumulate in the nucleus.
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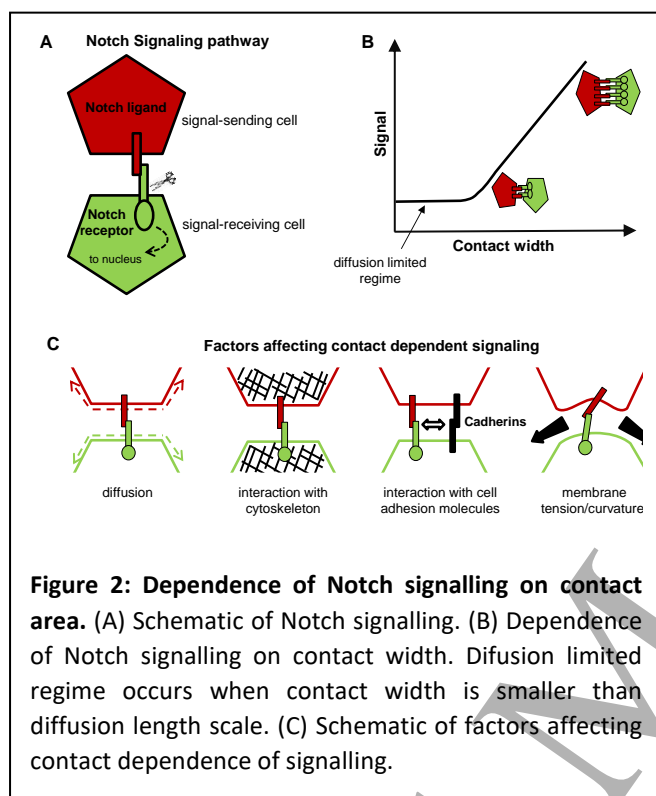
26 Finally, fast signalling readout is needed to allow tracking the dynamic relation between contact
27 morphology and signalling response. Standard transcriptional reporters are often too slow to track
28 dynamic signalling as they often respond within a time scale of tens of minutes to hours. Methods
29 that track faster signalling events include tracking translocation of NICD to the nucleus [5], and
30 tracking mRNA production in active transcription loci using MS2-GFP [6].
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33 A second challenge is to develop methods to identify and characterize which molecular and
34 morphological properties of the contact area, beyond the contact area itself, affect signalling. These
35 include membrane diffusion rates of the receptors and ligands at and around the contact site,
36 interaction with cell adhesion molecules such as cadherins, interaction with the cytoskeleton, and
37 membrane tension and curvature (Figure 2c). For Notch signalling, several recent works provide
38 initial clues to how these properties affect signalling. For example, diffusion rates of Notch receptors
39 and ligands were shown to be important in determining whether Notch signalling would exhibit
40 contact dependence [7]. For contact areas smaller than the membrane diffusion length scale, it is
41 expected that signalling would no longer depend on contact area (see Fig. 2b, diffusion limited
42 regime). This property can potentially explain why signalling mediated by filopodia, where the
43 contact area is very small (of the order of $\sim 0.1\mu\text{m}$ in diameter), can still produce significant signalling
44 output.
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48 The interaction with the cytoskeleton can also significantly affect contact dependent signalling. For
49 example, it was recently shown that activation of the actin nucleator Arp2/3 is required for the
50 activation of Notch in certain contexts, but not in others. It has been suggested that Arp2/3
51 mediated nucleation of actin is important for endocytosis of the Notch ligand which is required
52 during Notch activation [8]. An appealing hypothesis associated with this observation is that
53 membrane tension at the cell boundary may affect Notch activation, however, a direct evidence for
54 the effect of tension on Notch signalling is still missing.
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58 A third challenge is to understand how feedback between signalling and morphology underlies
59 developmental patterning processes. Many studies focus either on the molecular regulatory level
60 ignoring cell morphology, or focus on changes in cell morphology as a downstream consequence of

cell fate decision processes. Yet, it seems that in many developmental processes cell fate decisions occur concurrently with morphological changes. We therefore need to develop methods to experimentally identify such feedbacks, and to theoretically model the developmental processes that rely on such feedbacks. An example for such feedback between signalling and contact properties was recently discussed in the context of Nodal signalling [9]. In that system, a positive feedback between contact duration and Nodal signalling controls a cell fate switch during zebrafish gastrulation.



Advances in Science and Technology to Meet Challenges

To be able to address these challenges we need to develop tools that would enable better spatiotemporal control and readout of signalling. A promising direction is to employ optogenetic tools to spatially and temporally induce signalling, such as those developed for controlling membrane channels and cytoskeleton. Light induced signalling will allow local signalling induction at specific cellular interfaces both in vitro and in vivo. Furthermore, optogenetic tools may also allow controlling the geometry of the cell, by regulating local actomyosin activity. Initial steps towards this goal were recently reported, where optogenetic inhibition of Delta in flies was used to study dynamic signalling responses [10]. One challenge with the currently available optogenetic

tools is how to use these tools and at the same time image the response using fluorescent microscopy. Current optogenetic tools are activated by a broad spectrum of blue/green light (LOV domain based systems) or by red/far-red light (phytochrome based systems) thus limiting the ability to image multiple fluorescent protein reporters. Thus, more versatile light-inducible systems that would allow spatiotemporal control of signalling and the use of the full spectrum of fluorescent reporters for imaging, would be extremely useful.

On the theoretical side **Error! Reference source not found.**, we also need to develop modeling and computational tools that will allow studying the feedback between cell morphology and cell-cell signalling. In recent years, 2D vertex mechanical models have been successfully used to understand how cell and tissue morphology are determined by the mechanical forces acting on each cell and each boundary. These models would have to be expanded to include the regulatory and signalling state of the cells, typically described by rate equations. In such 'hybrid' models the regulatory state of the cell (e.g level of signal or concentration of transcription factor) can affect the mechanical properties of the cell, while the morphology of the cells, determined by the mechanical forces, will affect cell-cell signalling. Generating such models in a way that will be accessible to the community will require development of new modeling and computational platforms that bridge the gap between these two modeling modalities.

Concluding Remarks

The study of the interplay between contact morphology and signalling is still at its infancy. We expect that as new advances in technology and modeling emerge, we will gain further insights into the role of this interplay in various developmental systems. This interplay will likely be expanded to additional signalling pathways both at short and at long range.

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From locally generated forces to global shape and back

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Status

How shapes of organs and organisms emerge from the interaction of cells has been a central question of the past decades. This question has been mainly formulated with the strong assumption that cells are endowed with physical properties and rules of interactions that guide how they arrange and grow to form functional structures. This view often obscures the fact that morphogenesis in animals and plants is the process by which tissues, organs and organisms acquire their shape given boundary conditions that impose geometric, mechanical and biochemical constraints. Recent works have started to emphasize the importance of tissue boundaries and their role in morphogenesis. One prominent example is the extension of the *Drosophila* embryo germband: in this tissue, local forces generated by actomyosin contractile networks remodel cell contacts to produce anisotropic cell rearrangements. While this process occurs across the germband, an external tissue, the forming endoderm, pulls on the germband guiding its extension [1,2].

This example and others point to the necessity of considering morphogenesis as both a local and a global problem. In closed systems with fixed external boundaries such as the *Drosophila* embryo, mechanical models inspired by hydrodynamic theory have shown that local mechanical stresses together with geometric constraints lead to macroscopic flows that pattern the embryo [3, 4]. The extent to which locally produced deformations affect or are affected by boundaries depends on geometry and material properties. For viscous materials subjected to external friction, one can define a hydrodynamic length that describes the length over which the effects of a local force are felt (Fig 1A).

When the hydrodynamic length is of the same order as the embryo size, the boundary conditions strongly affect the pattern of tissue flows. Boundaries are usually defined by an external envelope (in the case of the embryo) or neighbouring tissues (in the case of organs). For instance, the presence of the cephalic furrow (characterized by a local fold of the tissue separating the head from the tail) in the *Drosophila* embryo acts as a barrier to tissue deformation (Fig. 1B).

Understanding the origins of these flows requires to disentangle the local and global scales, and therefore to independently perturb the locally generated forces and the global geometric constraints to which the tissue is subjected. In model systems such as *Drosophila*, local forces can be controlled by acute genetic perturbations, optogenetic approaches, and direct optical manipulation [5]. The spatiotemporal variations of tissue mechanical properties can be experimentally measured in vivo with magnetic and optical manipulation [6].

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3 It is also possible to create artificial boundary conditions by laser cauterization [1] to mechanically
4 uncouple the interactions of different tissues and understand their respective contributions to flows
5 and deformation.
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8 **Current and Future Challenges**

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10 Challenges in this field are both conceptual and technological. Current studies mainly address the
11 two-dimensional dynamics of epithelial tissues in contact with rigid boundaries. The current
12 challenge is to extend these approaches into three dimensions and to develop new concepts to
13 study systems whose global geometry is not fixed (deformable boundary conditions). How do 3D
14 tissue flows within an organ shape its boundaries? In turn, how do these deformable boundaries
15 affect the internal flows?
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19 Tackling these questions requires a strong dialog between experimental and theoretical approaches.
20 Ideally, the experimental system should be amenable to perturbations that affect boundary
21 conditions, system size and system geometry. There have been successful attempts in this path to
22 perturb boundary conditions in vivo but this remains limited to a few examples, and proves to be
23 extremely difficult in mammals due to their lack of accessibility post-implantation.
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26 In parallel, the emergent study of embryonic organoids has revealed the capacity of in vitro
27 multicellular systems to self-organize into structures mimicking organs and parts of embryos. For
28 instance, mouse embryonic stem cells, are able to assemble 3D aggregates, dubbed gastruloids, that
29 undergo gastrulation-like events and elongate. They are able to organize a post-occipital pattern of
30 neural, mesodermal and endodermal derivatives, recapitulating the embryonic spatiotemporal
31 pattern of gene expression [7]. They can be prepared in controlled conditions (size, confinement
32 geometry, matrix stiffness, chemical environment). The effect of fixed boundary conditions has been
33 proven to play a key role in gene patterning of 2D gastruloids confined on micropatterns [8]. In
34 contrast, the boundaries of 3D gastruloids are freely moving, and their shapes evolve through a
35 combination of growth and elongation. The variability in shape and patterning of gastruloids (for a
36 fixed initial number of cells) suggests that they lack certain external mechanical constraints, that are
37 present in embryos and that are critical to robust morphogenesis (Fig. 1C). Thus, they offer a unique
38 opportunity to quantitatively understand the role of mechanics (local and global) to ensure robust
39 morphogenesis. For that, it is necessary to generate quantitative maps of tissue flows and gene
40 expression, while imposing various mechanical constraints to the gastruloids.
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46 **Advances in Science and Technology to Meet Challenges**

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48 Long-term imaging of 3D multicellular systems such as gastruloids is a challenging task, due to light
49 scattering and phototoxic effects. Multiphoton microscopy enables to image deep (a few 100 μm)
50 into tissues, with moderate phototoxicity and allows combining fluorescence with label-free
51 imaging. However, as a point-scanning technique, it is rather slow to image multiple thick specimens
52 in 3D at the minute resolution, which is essential to collect data sets representative of specimen
53 shape, size and patterning variability. The advent of light sheet microscopy provides a means to
54 image fast 3D specimens with low phototoxic effects. For instance, in toto imaging of fast developing
55 embryos like *Drosophila* can be achieved with a time resolution on the order of 1 sec with
56 subcellular resolution. We expect that the use of multiphoton light sheet microscopy will be
57 instrumental to image in parallel large scattering specimens at subcellular resolution.
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3 To connect the collective cell dynamics with the cell-scale events, including mechanical behaviours
4 (cell rearrangements, shape changes, division and death) and fluctuations in gene expression, it is
5 necessary to identify and segment individual cells, track them and reconstitute cell lineages. This
6 task is challenging in densely packed 3D tissues with rapid cellular movements and will highly benefit
7 from the recent advances in deep-learning segmentation approaches. Such approaches will also be
8 instrumental to handle and analyse high-throughput data sets.
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11 Physical modeling is needed to bridge the cell with the tissue scale and understand the feedback
12 between 3D cell collective flows and the global geometry of tissue boundaries.
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15 One can envision two complementary theoretical and computational approaches. On one hand,
16 particle-based models are efficient to relate the stochastic cellular events with the tissue
17 macroscopic behaviour. On the other hand, continuum models considering tissues as active
18 complex materials offer a powerful means to identify phases, instabilities and transitions in gene
19 expression and mechanical behaviour. However formulating continuum models that couple the
20 dynamics of a 3D active material with the deformations of its boundaries will require the
21 development of new theory and new numerical schemes.
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24 Testing and refining the theoretical predictions of such models will require methods to perturb the
25 boundary conditions imposed on developing multicellular systems. A promising approach is to
26 embed these systems in synthetic gels whose geometry, and mechanical and chemical properties
27 could be controlled in space and time. This could be achieved by combining available technology of
28 light-based mechanical engineering of materials [9] with two-photon chemical patterning [10].
29 Integrating experimental (imaging, microfabrication and micropatterning) and theoretical
30 approaches will require the collective effort of physicists, mathematicians, biologists and
31 computational scientists.
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35 36 **Concluding Remarks**

37 We expect a mutually beneficial interaction between in vivo and in vitro systems: in vivo systems
38 give invaluable insight into the evolution of functional structures and gene pathways across species;
39 in vitro systems enable to explore a wide range of perturbations and experimental conditions,
40 mostly inaccessible in vivo. Combining these two approaches is thus an important step to
41 understand morphogenesis from basic principles.
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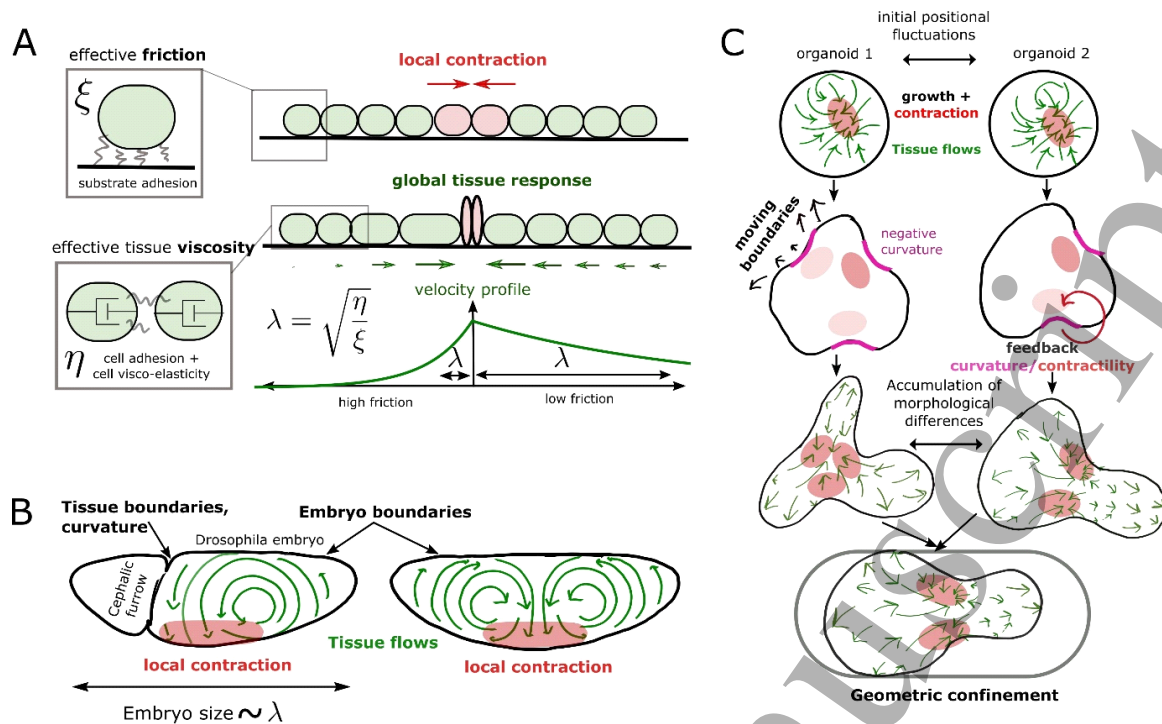


Figure legend A: Local tissue contractions can produce deformation/tissue flows over a length scale λ which is dependent on material properties. In the case of a viscous tissue in interaction with an external substrate, this length scale varies with the tissue viscosity and the effective friction coefficient between the tissue and the substrate. **B:** Geometric boundaries guide flow patterns. Simulations of tissue flows during *Drosophila* morphogenesis [3] demonstrate how a local tissue contraction on the germband can generate a global tissue flow whose geometry is influenced by the embryo geometry. In the absence of the cephalic furrow which is a highly curved interface separating the anterior part and the posterior part of the embryo, the global tissue flow is highly affected. **C:** Coupling between the tissue moving boundaries and the bulk tissue flows in an organoid system. Mechanisms coupling the tissue boundary geometry (here its curvature) with the generation of local active stresses could generate instabilities leading to the shape variability observed in organoids systems. Local mechanics and global geometry are coupled to give rise to shape: this coupling could be perturbed by imposing geometrical constraints to the organoid using micro-patterning for example

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