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A toolbox to explore the mechanics of living embryonic tissues

Otger Campàs[a,b,c]

[a] Department of Mechanical Engineering, University of California, Santa Barbara, CA 93106, USA
[b] Department of Molecular, Cell and Developmental Biology, University of California, Santa Barbara, CA 93106, USA
[c] California Nanosystems Institute, University of California, Santa Barbara, CA 93106, USA

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ABSTRACT

The sculpting of embryonic tissues and organs into their functional morphologies involves the spatial and temporal regulation of mechanics at cell and tissue scales. Decades of *in vitro* work, complemented by some *in vivo* studies, have shown the relevance of mechanical cues in the control of cell behaviors that are central to developmental processes, but the lack of methodologies enabling precise, quantitative measurements of mechanical cues *in vivo* have hindered our understanding of the role of mechanics in embryonic development. Several methodologies are starting to enable quantitative studies of mechanics *in vivo* and *in situ*, opening new avenues to explore how mechanics contributes to shaping embryonic tissues and how it affects cell behavior within developing embryos. Here we review the present methodologies to study the role of mechanics in living embryonic tissues, considering their strengths and drawbacks as well as the conditions in which they are most suitable.

Contents

1. Introduction

It is a constant in science that the appearance of new measurement tools enables numerous discoveries and often steers science in new directions. In addition to one of the most celebrated techniques, the light microscope, examples of techniques that have pushed forward the limits of developmental biology abound. These include advances in imaging, such as the development of confocal [1,2], two-photon [3,4] or light-sheet microscopy [5,6], but also tools to reveal the molecular mechanisms underlying embryonic development, such as the discovery of Green Fluorescence Protein [7] or the recently developed CRISPR/Cas system for gene editing [8]. These and many other techniques have helped reveal numerous biochemical signals that are essential to orchestrate cell behavior during development [9].

While biochemical signals are known to play a fundamental role in the control of tissue morphogenesis [9], it is now clear that mechanics also critically affects the vast majority of cell behaviors required to properly sculpt tissues and organs [10–35]. A large number of *in vitro* experiments have shown that mechanical cues affect the coordination of cellular movements in absence of instructive biochemical signals [36–41], the rate of cell proliferation [42–46], the orientation of the cell division axis [47–49], and even cell differentiation [50–54]. Tissue-like 3D cell culture experiments have also shown the relevance of mechanics in guiding cell proliferation [55], branching morphogenesis [56–59] and tumor progression [60–62]. These discoveries were possible thanks to a vast array of *in vitro* biophysical techniques to apply controlled forces on cells, quantitatively measure cellular forces, and also tune the mechanical properties of the cellular microenvironment, both

E-mail address: campas@engineering.ucsb.edu

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in 2D and 3D geometries [63–70]. Given the relevance of the findings obtained by *in vitro* experiments, it is apt to ask if mechanics does affect cell behavior *in vivo*, within developing tissues and organs, to the extent it does *in vitro*.

There exist many important works, detailed below in the context of different measurement techniques, that highlight the relevance of mechanics in embryonic development. However, compared to our knowledge of the biochemical signals involved in tissue morphogenesis [9], our understanding of mechanical signals *in vivo* is still in its infancy. This is mainly because of specific limitations in the current techniques to measure mechanics within developing 3D tissues. Recent efforts to create new tools and adapt *in vitro* techniques to measure cell and tissue mechanics *in vivo* and *in situ* (i.e., locally within developing embryos) promise to reveal how mechanical cues affect morphogenetic processes and individual cell behaviors within living embryos.

In this review we aim at providing a comprehensive overview of the techniques used today to measure and/or perturb mechanics in living embryonic tissues of animal species. Here, the terms *in vitro*, *ex vivo* and *in vivo* are defined as follows: *in vitro* refers to studies of cells in culture conditions, such as standard 2D cell culture, 3D cell culture using hydrogels as scaffolds, as well as multicellular aggregates; *ex vivo* refers to dissected portions of tissue that keep, at least partially, the original tissue architecture; *in vivo* refers to the intact developing embryo. The discussion presented below on the strengths and limitations of the different techniques needs to be understood within the framework of *in vivo* (and *ex vivo*) measurements. Indeed, many of the techniques discussed below have a long and successful history related to *in vitro* experiments, where some of the limitations mentioned below associated specifically to their use *in vivo* do not exist. Before describing the existing techniques, we first discuss the cellular structures that control cell mechanics within living embryonic tissues, and also the different (and independent) mechanical quantities that can potentially affect cell behavior *in vivo*.

2. Mechanics at cell and tissue scales

Whenever a movement is observed in a cell or an embryonic tissue, there is necessarily an underlying force generating it. This is because inertia is irrelevant in these processes and no movement can exist in the absence of a force generating it, highlighting the essential role of the distribution of forces in a tissue to drive morphogenetic flows and tissue deformations. These forces have their origin in the cell [12,13,16,25,27,29,71], where a relatively small number of cellular structures contribute to their generation (Fig. 1). Typically, cells within tissues generate forces via actomyosin contractility at the cell cortex and also through traction forces [13,16].

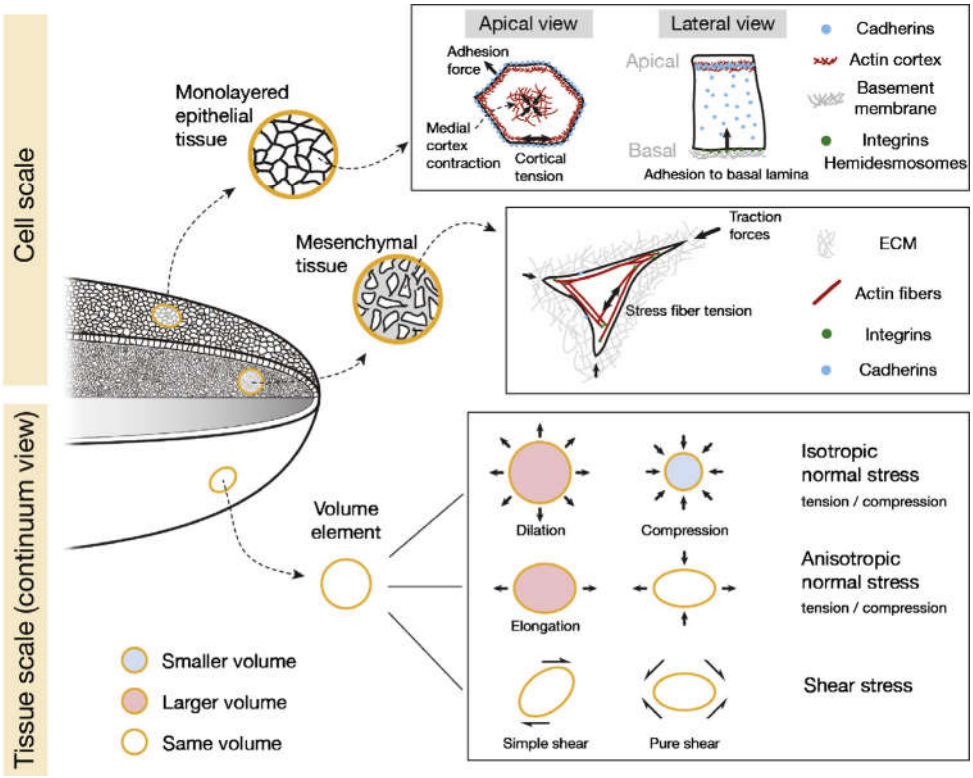

Fig. 1. Schematic representation of a developing embryonic tissue from cell and tissue scale (continuum) perspectives. From a perspective centered at the cell scale, there exist specific forces generated by several cellular structures that contribute to the shaping of tissues at larger scales [12–14,17,19,29]. In the case of monolayered epithelial tissues, forces generated in the medial apical cortex, as well as cortical tension and adhesion to neighboring cells, are known to contribute to morphogenetic processes [13,29]. Whenever epithelial cells are attached at their basal end to a basement membrane, substantial adhesion (and traction) forces may also be exerted there. In mesenchymal tissues, cells exert traction forces on the extracellular matrix (ECM) via focal adhesions, connecting the ECM polymers to integrin receptors at the cell surface [14,17,19,21]. Stress fibers inside mesenchymal cells generate tensile forces that are transmitted to focal adhesion sites and contribute to cell traction forces on the ECM. Cadherin molecules in mesenchymal cells mediate their adhesion and affect their collective movements [21,229,230]. From a tissue scale perspective, volume elements that contain several cells and average locally their properties constitute the basic unit [76,80,81]. In this view, which follows the language of continuum mechanics, deformations of these volume elements by mechanical stresses in the tissue describe quantitatively morphogenetic deformations and flows. In general, the stresses deforming the volume elements do not have a specific origin in a particular cellular structure, but rather reflect the local value of the mechanical stress arising both from local forces as well as forces transmitted from other portions of the tissue. Only a handful elementary deformations of a given volume element exist, and are generated by very specific mechanical stresses acting on the volume element. Uniform normal stresses on the volume element can lead to its dilation or contraction if the material is not strictly incompressible, whereas anisotropic normal stresses lead to elongations and contractions of the reference volume element along specific directions. Shear stresses can lead to elongations (pure shear), but also to combined elongations and rotations of the volume element (simple shear). The spatial variations in mechanical quantities (such as stress or mechanical properties) throughout the tissue, represented here by a graded gray scale, can be described as spatiotemporal maps in the continuum view.

These cell-generated forces are transmitted to other cells in the tissue by the adhesion-mediated connection of the cytoskeleton in adjacent cells or the cytoskeleton in a cell and the neighboring extracellular matrix. Given their relevance in the generation and transmission of cellular forces, cortical tension and cell adhesion have been the focus of many mechanics studies in living embryos (Fig. 1). In addition, other cellular processes, such as cell proliferation, apoptosis or changes in cell volume (controlled by changes in osmotic pressure [72]), generate forces in the tissue and can substantially contribute to the mechanics of tissue morphogenesis.

While it is possible to identify the specific structures and molecules involved in the generation and transmission of force at the cellular level, the measured forces at both cell and tissue scales contain, in general, *in situ* contributions as well as forces generated far away and transmitted through the tissue [12,73–80]. As a consequence, when measuring forces *in vivo* and *in situ* it can be difficult to disentangle these contributions given that both of them are present and may be under similar molecular control. Regardless of their origin, the measurement of forces at cell scales *in vivo* and *in situ* reveals the mechanical cues that cells perceive, whereas measurements of supracellular, tissue scale mechanics help explain the origin of large scale tissue flows.

In tissues composed of large numbers of cells, the mechanics at supracellular, tissue scales can be described using a continuum approach, where every element of volume contains several cells and provides an averaged representation of the local mechanics (Fig. 1; [76,77,80–83]). Unlike many common inert materials, living tissues may feature spatial and temporal variations of several mechanical quantities, such as the stresses (or forces) and mechanical properties (e.g., their elasticity and/or fluidity). When describing the mechanics at tissue scale we follow the language of continuum mechanics (described in several reviews [11,84–88] and also in specialized textbooks [89–93]), where an element of volume can be subject to normal stresses and shear stresses that lead to different deformations (Fig. 1). Specifically, uniform normal stresses on the reference volume can lead to its dilation or contraction (unless the material is strictly incompressible), whereas anisotropic normal stresses lead to elongations and contractions of the reference volume element along specific directions. Shear stresses can lead to elongations (pure shear), but also to combined elongations and rotations of the volume element. The combined deformations of the different volume elements throughout the tissue [94,76,80] quantitatively describe large scale morphogenetic movements [73,76,80,95–97].

3. Mechanical stresses, material properties and tissue deformations

Force is a key concept in mechanics, but so is stress (force per unit surface) and even different components of the stresses, namely shear stress (the stress applied along a tangential direction of a surface or volume element) and normal stress (the stress applied along the normal direction of a surface or volume element) [11,85,87,90,91,93] (Fig. 1). An example that demonstrates the relevance of this distinction is that cell traction forces, but not traction stresses, increase in response to increasing substrate stiffness [98–100], or the fact that some cell types respond specifically to applied shear stress [101,102]. Equally important than force and stress are the mechanical (material) properties of the system which, in some cases, can be simplified to the viscosity (characterizing its resistance to flow; inverse of fluidity) and elasticity (or compliance, characterizing its resistance to deformation). Specifically, the compliance of the cellular microenvironment has been shown to act as a cue for stem cell differentiation [53] and tumor progression [62]. Finally, the strain, which characterizes local relative

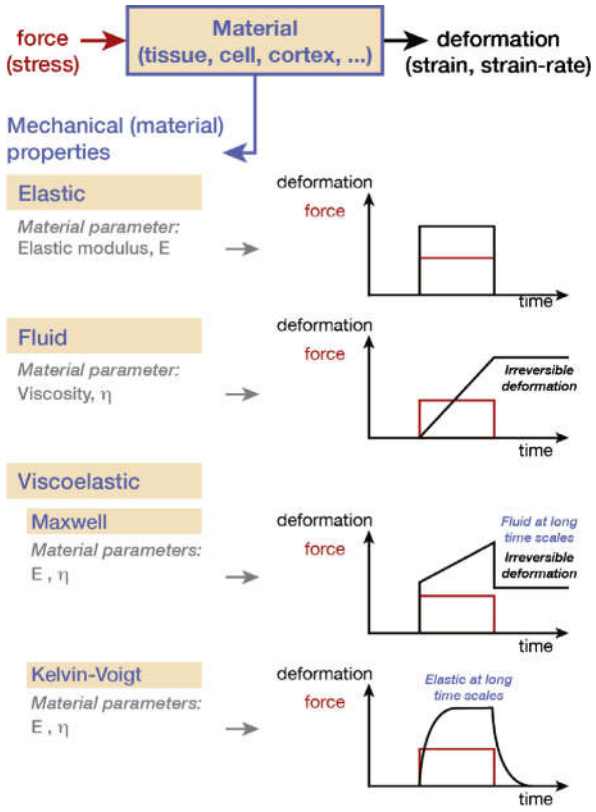

Fig. 2. Generation of cell/tissue deformations and flows from the underlying mechanics. The forces (or stresses) generated within or applied to a biological structure (e.g., cell cortex, the cell itself, a cell aggregate or a portion of an embryonic tissue) result in its deformation or flow, quantified by the level of strain or strain-rate. However, the specific response of the structure to the applied force depends strongly on its mechanical (material) properties. The application of a force (shown in red) during a finite time interval to a given structure leads very different deformations (shown in black) of such structure if this behaves as an elastic material, a fluid or a more complex viscoelastic material. Only Maxwell and Kelvin-Voigt viscoelastic materials are shown for simplicity [88].

deformations, and strain-rate, which characterizes the rate of change of local relative deformations, quantify the local tissue deformations/flows arising from cell rearrangements, changes in cell shape and cellular movements [81,76,79,80]. All these mechanical quantities can, a priori, affect cell behavior in an independent manner, and is therefore important to distinguish them, specially when performing quantitative measurements. However, it is also important to keep in mind that several mechanical quantities can be affected/controlled at the molecular level by the same players (actin, myosin, adhesion molecules, or upstream regulators) and their variation can be correlated. For instance, both the mechanical properties and stresses in actomyosin networks have been shown to be myosin-dependent [103–105].

The morphogenetic flows and tissue deformations observed during embryonic development result directly from two distinct mechanical factors: the spatial distribution of stresses generated by the cells in the tissue (or by neighboring tissues), and the map of mechanical properties (fluidity and/or elasticity) that dictates how the tissue responds to the local stresses. Even with exactly the same distribution of mechanical stresses in a tissue (or cell), its flow or deformation (i.e., its response to the applied force) can be completely different depending on its mechanical properties (Fig. 2). If the tissue is elastic, it will deform upon the application of force, but return to the original configuration once the force is removed. If it is fluid, the deformation in the tissue induced by the applied force will increase over time and the tissue will not go back to the

original configuration after the force is removed: the tissue flows, relaxing the internal stresses induced by the applied force.

In vitro experiments have shown that cellular aggregates behave as viscoelastic materials (Fig. 2), displaying both elastic and fluid properties when probed at different time scales [106–110]. If a force is applied over short time scales (seconds), aggregates deform and bounce back to the original configuration after removal of the force. However, if the force is applied over long times scales (from minutes to hours depending on cell type), cell rearrangements occur and the aggregate flows, remaining deformed after removal of the applied force. Eventually, the aggregate surface tension restores a (roughly) spherical aggregate shape [107–110]. This time-dependent response may be physiologically relevant because cellular and morphogenetic processes occur at different timescales, meaning that the material properties cells sense may be different than the material properties guiding large scale morphogenetic flows.

4. Methodologies to probe the mechanics of living embryonic tissues

The independent measurement of all the different mechanical quantities described above is not possible today, neither *in vivo* (or *ex vivo*) nor *in vitro*. Most of the existing techniques able to measure mechanics *in vivo* were developed to measure different aspects of cellular or tissue forces (or stresses) [111,112]. While some techniques can measure the material properties of tissues *ex vivo*, *in vivo* and *in situ* measurements of local tissue mechanical properties remain extraordinarily rare.

In order to perform a quantitative measurement it is necessary to use a calibrated probe, regardless of the mechanical quantity to be measured. In the case of force measurements, the probe typically consists of a material with well-known (calibrated) response to applied forces (e.g., a cantilever), in such a way that the deformation of the probe due to cell or tissue forces can be translated into a measure of force. In contrast, the measurement of mechanical properties requires monitoring the time evolution of the system deformation in response to a known (calibrated) applied force. Only techniques that have been used in living embryonic tissues (*in vivo* and/or *ex vivo*) of animal species are discussed in what follows (see also Table 1).

Laser Ablation (LA). Initially used to perform controlled microdissections of subcellular structures *in vitro* [113], LA is now perhaps the most widespread technique to probe mechanics *in vivo* and *in situ*, both at the cellular and tissue scales [114]. It consists of using a nano- or femto-second pulsed laser to ablate a portion of a structure in the system, creating a mechanical disturbance (as well as other physical changes [115]) that leads to a measurable response [115–119]. The response of the system to this perturbation is recorded using fluorescence microscopy (typically, confocal or multiphoton microscopy) and quantified by the initial retraction speed of the ablated structure. The main idea behind laser ablation is that the measurement of the initial retraction velocity of the ablated structure provides information about the tension state of the structure before ablation. Additionally, if the measured retraction velocity is assumed to be proportional to the tension in the structure, and the constant of proportionality does not change spatially or temporally in the tissue, then relative variations in the retraction velocity at different locations of the tissue report relative tension differences. Given that LA relies on the retraction of the structure after ablation, it can probe tension in the structure, but not compressive loads.

Strengths – LA is a very attractive technique mainly because it requires just a pulsed laser to perform a mechanical perturbation. This ablation laser can be part of the optical system used to image the specimen (e.g., multiphoton microscopy) or a new ablation pulsed laser can be added to an existing imaging system. Given that the response of the system in terms of the retraction velocity is obtained by imaging the specimen during or just after ablation, no additional equipment is required. In addition, the high accuracy of ablation allows both versatile and precise mechanical perturbations. LA has been primarily used to study the mechanics of epithelial monolayers, mainly because the laser beam used to ablate the system is affected by the amount and heterogeneity of penetrated material.

Limitations – The main drawback of LA is that the relation between the existing tension in the structure before ablation and its dynamic response after ablation depends on the material properties of such structure ([117,116]; Fig. 2). As a consequence, no quantitative measurement of tension can be obtained unless the material properties can somehow be estimated. In some cases, the dynamical response of the system allows an estimation of the type of material (type of viscoelastic response, etc.; e.g., [83]). While values of the tension cannot be estimated even in these conditions, the ratio force/viscosity can be obtained [83]. If the estimated material properties are spatially homogenous and isotropic, then relative changes in tension, as well as their anisotropy, can be measured [120–122]. Given that no quantitative *in vivo* measurements of the mechanical properties of the ablated structures exist, LA results must be interpreted with care, as observed spatial variations in the retraction speed after ablation can be explained by changes in tension states, in the material properties, or in both. Quantitative analysis of the dynamic response of the system after ablation can help estimate whether or not the mechanical properties can be considered isotropic and homogeneous in space and time [120].

Examples – *In vivo* and *in situ* experiments to study mechanics at cellular and tissue scales have been done using LA [114]. Specifically, LA has been extensively used to estimate cortical tensions in different spatial directions (anisotropy) and their spatial and temporal variations during *Drosophila* axis elongation [123–126], dorsal closure [127,116,118], wing development [128–130] and tracheal formation [131], as well as during zebrafish epiboly [132,133,121] and early development in mouse [134] and C. elegans [122,135]. At larger scales, the ablation of tissue portions has been used to estimate its tension state at the tissue scale, including its anisotropy and spatial variations, for instance in the *Drosophila* notum [83], during gastrulation [77] and dorsal closure [72,136], as well as in the wing imaginal disk [82].

Tissue Dissection and Relaxation (TDR). This is a simple, but powerful technique to assess the mechanics of tissues. Originally developed to reveal global stresses driving large scale morphogenetic movements [137,138], it has been more recently refined to measure the tissue surface tension and the mechanical properties of large tissue explants *ex vivo*. The technique consists of dissecting a portion of the tissue of interest from the embryo using a blade, and obtains different mechanical quantities by observing its response (deformation) after its dissection. If the tissue explant can be assumed to behave like a liquid drop, it is then possible to obtain both the value of tissue surface tension and its viscosity by monitoring the shape changes of the dissected tissue over time after deforming the tissue using centrifugation [139,140].

Strengths – The method requires minimal equipment and is relatively straightforward. Yet, it can provide a considerable amount of information about global tissue mechanics for different kind of dissected tissues, including the average tension state of the tissue, the average mechanical properties and its surface tension.

Limitations – Perhaps the main limitation is that it can only be performed on relatively large explants and provides a global measurement for the entire explant. Therefore, this technique does not provide local information or maps of mechanical quantities. Importantly, after tissue dissection, the cells exposed at the surface of the

explant reorganize and, as a consequence, the values of both tissue surface tension and viscosity could potentially be affected.

Examples – As mentioned above, this technique was used to qualitatively assess the level of mechanical stresses in amphibian [137] and sand dollar embryos [138]. *In vitro* experiments using aggregates of cells isolated from chick embryonic tissues provided measurements of cell cohesiveness [140]. More recently, the average mechanical properties and tissue surface tension of different *Xenopus* embryonic tissues were obtained *ex vivo*, using tissue explants [141,142,139].

Force inference (FI). As the name indicates, FI methods infer forces, but do not measure them. The main idea is to infer some of the mechanical quantities in the tissue by analyzing the shapes of the cells, as these shapes result directly from the forces cells apply on each other [143–146]. The relations (equations) between the shape of the cells and the mechanical quantities of each cell are provided by local force balance. Inference methods have been developed for 2D static systems so far and applied to the study of monolayered epithelial tissues, where tissue forces are thought to be dominated by the apical (essentially 2D) surface of cells. FI methods infer the tension of each cell–cell junction in the tissue as well as the internal pressure of all cells, up to a constant. The inferred tensions at cell–cell junctions contain information about the cortical tension of the cells coming together at the junction as well as about their adhesion strength. Only recent versions of FI methods that use both the balance of forces at junctional vertices and the curvature of cell junctions have enough constraints to infer the mechanical quantities without additional assumptions [146].

Strengths – The main advantage of FI methods is that the inference of several mechanical quantities at the cell scale throughout a tissue can be simply done from an image of the cell geometries in the tissue, without any measurement probe (except for a fluorescent label to image cell shapes) or mechanical perturbation of the system. Adaptations of this technique to 3D, dynamic scenarios, accounting for forces along all cellular surfaces instead of only at the apical surface, may be very helpful in the future, as these would waive some of the current assumptions and limitations that restrict the use of FI methods today.

Limitations – Inferring forces from 2D images of cells requires several assumptions to hold in the system being analyzed. Given that cell shapes are used to infer forces, it is assumed that all the (dominant) forces controlling the shape of cells are occurring at the imaging plane. Inferring forces from images of the apical region of monolayered epithelia where cells may not be exerting considerable forces along their sides or at their basal ends, such as during cellularization and onset of gastrulation in *Drosophila* embryos, is an optimal scenario. However, in many monolayered epithelia cells may exert substantial forces at the basement membrane, potentially rendering FI inaccurate. Due to the various assumptions involved in FI methods, it is important to validate the inferred quantities with other techniques. Promising tests in *Drosophila* wing development show positive correlation between the inferred tensions at cell junctions and the retraction velocity after ablation of such junctions [144]. A direct test of the inferred forces with quantitative force measurement techniques would help considerably the validation of FI methods.

Examples – FI has been used to infer the cell–cell junction tensions and cell pressures in the *Drosophila* notum [81,147], and also during dorsal closure [146] and wing development in the fly embryo [147,148,144], as well as in the chick cochlear epithelium [145].

Micropipette aspiration (MA). First developed in 1953 to study embryo mechanics *in vivo* [149], MA was quickly adapted and extensively used *in vitro* with individual cells [150–152]. More recently, it has been used *in vitro* to measure the surface tension and mechanical properties of cellular aggregates [106]. The technique uses a micropipette to locally aspirate a portion of a cell, a cellular aggregate or a tissue and monitors the resulting deformation (length of aspirated portion) over time [152,106,149]. By controlling the aspiration pressure it is possible to measure both forces and material properties using the static and dynamic response of the system, respectively.

Strengths – The major strength of MA is its ability to perform quantitative measurements of force (stress) and mechanical properties, at both cellular and tissue scales *in vivo* (as well as *in vitro* and *ex vivo*). In addition, it can be used to apply controlled forces to perform mechanical perturbations. The set-up needed to perform these experiments is relatively simple.

Limitations – The major limitation of MA is that it requires constant contact with the sample to perform any kind of measurement, as a portion of the structure of interest needs to be aspirated in a micropipette. In the case of a tissue, the aspirated portion may be heterogeneous, including different tissues or cell types, leading to measurements that may be difficult to interpret. In addition, the aspiration of a substantial portion of tissue may disrupt morphogenetic flows and normal development if large portions of tissue are aspirated or the experiment takes longer than typical timescales of tissue flows. Lastly, in order to use MA it is necessary to remove the protective layer that embryos of animal species have, which can be challenging in some cases (e.g., *C. elegans*, *Drosophila*) without perturbing normal development.

Examples – MA is perfectly suited for *in vivo* and *in situ* measurements of cell surface tension (including cortical tension) in oocytes [153] and blastomeres of early embryos [154]. Adaptations of MA using single or double micropipette set-ups [152] have allowed quantitative *in vitro* measurements of cortical tension and cell adhesion strength between isolated cells of different germ layers in zebrafish [155]. Recent experiments have modified typical MA set-ups to follow tissue deformations in 3D and apply controlled ectopic forces to developing mouse limb buds [156]. Finally, a different aspiration technique has been used to study the mechanical properties of the *Xenopus* blastula [157].

Cantilevers and Atomic Force Microscopy (AFM). Both cantilevers and AFM are based on the concept of using a calibrated beam, or cantilever, that deflects when in contact with the surface of a material. By measuring such deflection and knowing the mechanical properties of the cantilever, it is possible to apply controlled forces as well as quantitatively measure forces and mechanical properties. Measuring mechanical quantities at tissue scales has typically been done with relatively simple cantilevers [85], while measurements at single molecule level [158] and subcellular and cellular scales have taken advantage of AFM [159,160]. While AFM works on the same principles than larger cantilevers [161], it has been adapted to perform a wide range of measurements by operating in different modes [162,161]. Indeed, AFMs have been one of the most widely used techniques to apply controlled forces as well as measure forces and mechanical properties of a variety of biological structures, including cells and tissues [159,160,162].

Strengths – Both simple cantilevers and AFMs can perform quantitative measurements of forces and mechanical properties *in vivo* and *in situ* (as well as *in vitro* and *ex vivo*). While AFM is particularly well-suited for molecular, subcellular and cellular scales, it has been also been adapted to measure tissue elasticity at larger (tissue) scales by attaching a large (several microns) bead to the AFM cantilever tip [162]. Additionally, the scanning mode of AFM allows spatial mapping of cell/tissue elasticity, as long as the sample does not change over the time of the scan. For both large cantilevers and AFM it is possible to change the cantilever mechanical properties as well as the cantilever tip to measure different range of forces at various length scales.

Limitations – The main limitations of cantilevers and AFMs are the same as for MA (see above), as all of them require contact with

the surface of the sample to perform a measurement. In the case of AFM, the time required to spatially map the surface elasticity of the sample depends on its extension and may typically be of several minutes. This fact limits its applications to systems that changes over timescales shorter than the AFM scan. AFM spatial mapping requires relatively flat samples, a constraint that may limit its applications in developing 3D embryos. Finally, obtaining the elastic modulus from the AFM response curves requires fitting the data to models of such response (e.g., Hertz model) that involve several assumptions [161].

Examples – Some of the global forces driving gastrulation [163] and blastopore closure [164] in *Xenopus* have been recently measured *in vivo* using calibrated cantilevers. Besides forces, the mechanical properties of embryonic tissue explants of several amphibian species (*Xenopus* [165] and newt [166]) have been measured *ex vivo* using cantilevers under controlled applied forces. At a cellular scale, AFM has been used *in vitro* to measure the differences in adhesion strength and cortical tension in cells isolated from the three germ layers of zebrafish embryos [167,168], as well as the membrane-to-cortex attachment in isolated zebrafish mesendoderm progenitors [169]. Finally, adapting the tip of the AFM cantilever to measure length scales larger than single cells, it has been possible to measure *ex vivo* the spatial variations of tissue elasticity in thin slices of brain tissue [170–172]. Spatial differences in tissue elasticity have also been observed in developing limb buds of cultured mouse embryos [173].

MicroIndenters (MI) and MicroPlates (MP). Both techniques use the application of a controlled force (or strain) at the surface of a cell or tissue while monitoring its response (deformation/force) over time [85,87]. As the names indicate, the MP technique uses a plate to deform the sample whereas the MI technique uses an indenter. Typically, the MPs used are larger than the sample, providing a global measurement. MIs are typically smaller than the sample and can therefore provide spatial mapping capabilities. MPs have been extensively used to measure the surface tension and mechanical properties of cell aggregates *in vitro* [107] and *ex vivo*.

Strengths – Both MIs and MPs allow quantitative measurements of forces and mechanical properties. The spatial mapping capability of MIs, similar to that of AFMs, may prove very useful to scan the mechanical properties of relatively flat embryonic tissues.

Limitations – As was the case for previous quantitative measurement techniques (see above), the major limitation is that any measurement requires contact with the surface of the sample. MPs can measure global (average over the entire sample) mechanical properties and forces. MIs can probe different length scales by changing the size of the MI tip. However, the extension of the mechanical perturbation introduced by indentation is larger than the tip size, meaning the measured mechanical parameters correspond to the average of a relatively large volume, which limits the potential spatial mapping resolution of the technique.

Examples – Microindenters have been used in conjunction with cantilevers (cantilever-based microindenters) to apply controlled deformations and measure the stiffness of embryonic chicken heart tissue *ex vivo* and *in vivo* [174–176]. More recently, this technique has been extended to measure spatial variations in the stiffness of chicken embryonic tissues [177]. Microplates have been extensively used for *in vitro* measurements of surface tension and mechanical properties of aggregates of cells isolated from various chick [110,178,109] and zebrafish [179,108] embryonic tissues. Finally, both MPs and MIs have been used to apply ectopic forces on early *Drosophila* and zebrafish embryos [180–182].

3D Traction Force Microscopy (3D TFM). TFM was initially developed to measure the forces generated by single cells and cell monolayers in culture conditions [38,183], and has been extended to 3D systems to measure the forces of cells embedded in gels [56,184,185]. More recently, it has been adapted to measure the forces generated by growing tissue explants *ex vivo* [186]. In most versions, the technique uses a gel of known elastic modulus containing dispersed fluorescent microbeads. When the cells or tissues in contact with the gel apply forces on it, the positions of the microbeads embedded in the gel change. By tracking the displacements of the microbeads, it is possible to measure the strain field in the gel. Knowing the elastic modulus of the gel allows a direct mapping of mechanical stresses.

Strengths – The major strength of 3D TFM of living tissues is that it enables a quantitative map of the stresses generated by the growing tissue throughout its surface. Moreover, it is relatively easy to implement, as embedding embryos or tissues in hydrogels is relatively easy.

Limitations – Similarly to 2D TFM, obtaining the stresses in the gel from bead displacements is not straightforward. As the technique is typically calibrated only in the linear elastic regime of the hydrogel, only small deformations of the gel should be permitted, limiting the measurements to relatively small tissue growth. It is also unclear whether the mechanical constraint imposed by the surrounding gel on the growing tissue affects tissue growth and the values of the forces measured. This method requires surface contact with the sample and, as a consequence, the limitations described above for techniques requiring surface contact with the sample also apply here.

Examples – 3D TFM has recently been adapted to perform *ex vivo* measurements of the stresses generated by convergent extension in *Xenopus* tissue explants [186].

Optical tweezers (OT). First used in *in vitro* studies of mechanics at single molecule level [187,158], as well as subcellular and cellular scales [188], OTs have recently been extended to the study of intracellular mechanics *in vivo* and *in situ*. The technique uses a focused laser beam to apply controlled forces on an object (typically a bead) with refractive index larger than that of the surrounding medium. The focused beam generates a optical trap for the bead, leading to a spring-like restoring force on the bead from the trap center. The length and force scales (0.5–2 μm and 1 −−100 pN, respectively) probed by OTs are optimal for the study of molecular and sub-cellular processes. OTs apply controlled forces and measure simultaneously the response of the probe (bead), allowing the quantitative measurement of both mechanical properties and forces.

Strengths – Calibrated OTs can perform quantitative measurements of forces and mechanical properties at the sub-cellular scale. Typically, OTs rely on the injection of beads to perform quantitative measurements as these are objects with controlled physical properties (size, refractive index, etc.). However, it is also possible to trap cellular structures that feature a refractive index different enough from their surroundings. In systems where tissue or cellular structures change slowly over time, this technique may enable spatial mapping of mechanical parameters thanks to its ability to perform highly localized measurements.

Limitations – Calibration of OTs *in vitro* is relatively standard [187], but calibration within living embryos may be complex. Indeed, given that the shape of the optical trap and its stiffness depend on the physical properties of the object being trapped, the shape of that object, the homogeneity of the material where the trap is placed, as well as several other factors, it is difficult calibrate the optical trap *in vivo*, even in its linear regime. Moreover, given that several of the mentioned parameters may change from point to point in living tissues, *in vivo* use of OTs requires *in situ* calibration of each trapped object, which may potentially limit its applications. Lastly, in order to apply large enough forces to study subcellular mechanics (up to 100 pN), OTs may need to be calibrated in their non-linear regime, which may be challenging.

Examples – OTs have been used *in vivo* and *in situ* to measure the molecular forces involved in kinesin-mediated transport

in *Drosophila* embryos [189,190]. More recently, OTs have been adapted to measure the tension and mechanical properties of cell–cell junctions in *Drosophila* epithelial monolayers *in vivo* and *in situ* [191], as well as to measure molecular forces and mechanical properties within mouse oocytes [103]. This is a promising set-up to quantitatively measure spatio-temporal variations of cortical tension, as well as the mechanics of other subcellular structures.

Magnetic tweezers (MT). MTs have been long used *in vitro* to apply controlled forces and torques at molecular, subcellular and cellular scales [192,193,158,194,195], and have recently been adapted for use with multicellular aggregates [196]. Initially developed to measure the mechanical properties of the cell cytoplasm [197], MTs have been used extensively to apply controlled forces and torques on magnetic beads (i.e., having a magnetic moment), allowing the quantitative measurement of both forces and mechanical properties. To apply net forces on a magnetic bead it is necessary to place it in a gradient of magnetic field [192,193,195], whereas torques can be applied on the magnetic bead using uniform magnetic fields [158,198,199].

Strengths – In most applications, the forces and torques generated by the magnetic beads have been calibrated and, as a consequence, the different variations of this technique can provide quantitative measurements of force, torque and mechanical properties. The forces generated by MTs are larger than those of OTs and have enabled experiments at molecular, cellular and tissue scales. Moreover, the range of force detection can be varied by changing the size of magnetic beads (force increases with bead size) and the gradient of the magnetic field (larger gradients lead to larger forces), making the technique quite versatile for studies with cells and tissues.

Limitations – Magnetic tweezers have been extensively used with cells *in vitro*. Several constraints associated to the study of living tissues have hindered the use of this technique *in vivo* beyond the application of ectopic forces. MTs require a steep gradient of magnetic field and/or big magnetic beads to apply the necessary forces at supracellular/tissue scales. This fact limits its use *in vivo* in its standard implementation, as it is difficult to generate very steep gradients of magnetic field deep in embryos and the injection of large magnetic beads (20–40 microns) requires large microneedles that considerably damage the tissue upon injection. To overcome this problem, *in vivo* applications typically use a large quantity of magnetic nanoparticles or magnetic liposomes injected inside cells of the living tissue. Calibration of the technique in these conditions is challenging. However, even if not calibrated accurately, the technique can be used to apply ectopic forces that induce tissue deformations qualitatively similar as those observed in normal developmental conditions.

Examples – MTs have been used to apply forces *in vitro* on mesodermal cells isolated from *Xenopus* embryos [200]. At larger, mesoscopic scales, MTs were recently used *ex vivo* to measure the mechanical properties of dissected gut tissue [201]. Finally, application of ectopic forces with MTs using injected magnetic liposomes was used to reveal the mechanically-induced expression of developmental genes and the role of β-catenin in mechanotransduction in *Drosophila* and zebrafish embryos [181,202,182], as well as during tumor progression in adult mice [203].

MicroRheology (MR). Initially developed to quantify the local material properties of complex fluids [204,205], MR has been extensively used *in vitro* to locally measure the mechanical properties of cell cytoplasm [206,207]. Passive MR requires the injection of micron-sized (or submicron) beads in cells. By tracking the motion of the beads subject to thermal noise (as well as active fluctuating forces) inside cells [208], and characterizing the distribution of the bead motion fluctuations, the technique obtains a measure of the material properties of its surrounding material. In contrast to passive MR, active MR requires the application of a force on the particle [209,204]. The technique is best suited for studies at subcellular scales.

Strengths – One of the main strengths of passive MR is that it does not require the application of controlled forces on the beads. Only injection and tracking of the beads are necessary, making the instrumentation necessary for the measurement quite minimal. It can perform quantitative measurements of the local mechanical properties *in situ* and *in vivo* under certain circumstances (see below). Active MR requires the application of controlled forces (for instance, using OTs) and is therefore more difficult to implement, but it provides quantitative measurements *in vivo* and *in situ*.

Limitations – Passive MR assumes that only thermal fluctuations drive the diffusive motion of the bead. As the cell cytoplasm has a considerable number of active forces acting at several time scales, this assumption may lead to erroneous estimations of its mechanical properties. Active MR is not subject to this limitation because it drives the particle with known forces [209,204]. Both techniques can be used at subcellular (micron) scales but cannot be used to probe mechanics at tissue scales.

Examples – Passive MR has been used to measure the intracellular mechanical properties in blastomeres of early C. elegans embryos [210] as well as in syncytial *Drosophila* embryos [211]. Active MR using OTs has recently been used to study the rheological behavior of the cytoplasm of mouse oocytes [103].

FRET-based Tension Sensors. Developed initially *in vitro* to measure tension at the molecular level in living cells [212–215], molecular FRET-based sensors have been used *in vitro* to measure cell-cell adhesion force [216], transmission of traction forces via cell-substrate adhesion [214] and cell surface receptors [217], shear-stress mechanosensing [218], etc. [219–221]. The technique consists in inserting a molecular spacer with known extensional rigidity (spring constant) in between the donor and acceptor of a FRET system. The intensity of the FRET signal depends on the distance between donor and acceptor, which measures the elongation of the inserted molecular spring. After proper calibration of the molecular spring constant, the FRET signal intensity can provide a readout of the tension between the two ends of the sensor.

Strengths – One of the major advantages of FRET sensors is that it can be genetically encoded and, once expressed in cells of developing embryos, tension can be measured by simply imaging the sample. When properly calibrated, it has the ability to provide high-resolution spatial and temporal maps of molecular forces *in situ* and *in vivo*. Given that FRET sensors are associated to a specific molecule or process (e.g., cell adhesion), the tension measurements can help reveal specific molecular contributions to cell and tissue mechanics. Moreover, this technique can take advantage of all existing genetic tools to target the tension sensor to specific tissues.

Limitations – Two major limitations hinder today its widespread use in living embryos. The signal-to-noise ratio of the FRET sensor is not optimal and very sensitive detectors are typically required to visualize the FRET signal *in vivo*. In addition, while single molecule calibration of the FRET sensor is accurate, *in vivo* (and also within cells *in vitro* and *ex vivo*) calibration of the FRET sensor may be challenging. Indeed, the FRET signal and the molecular spring constant depend on the chemical environment to which they are exposed, which may change in space and time when used in living cells. If the chemical environment is not changing strongly spatially and temporally, relative measurements of how tension changes spatially and temporally can potentially be performed. In addition to purely technical limitations, the FRET sensor can only measure tension states, but not compressive loads. The ability of this technique to measure tension in specific molecules may also be a limitation, as generating new molecular FRET sensors and testing them *in vivo* can be complex and time consuming. In most cases, FRET sensors reveal the existence of an average molecular tension, but quantitative measurements of the level of tension remain challenging, not

only for the reasons described above, but also because the measured signal in a given pixel integrates the contribution of many molecules with likely different configurations and tension states. Despite these limitations, the idea of measuring forces with (nearly) molecular resolution *in vivo* and *in situ*, makes this technique very attractive if quantitative measurements can be achieved.

Examples – FRET-based force sensors have been used in living embryonic tissues to study the state of tension at cell–cell junctions in migrating border cells during *Drosophila* oogenesis [222]. In *C. elegans*, FRET sensors have been used for several purposes, including the study of tactile mechanosensation [223,224].

Droplet-based sensors (DS). Initially developed to measure the mechanical stresses generated by growing actin networks *in vitro* [225,226], this technique has recently been adapted to measure mechanical stresses within living embryonic tissues [227,228]. DS use cell-sized, biocompatible oil microdroplets, coated with ligands for cell adhesion receptors, as force transducers *in vivo* and *in situ* (and also *ex vivo* and *in vitro*). A calibrated oil microdroplet (with known interfacial tension) is microinjected between the cells in a tissue. The mechanical stresses applied on the microdroplet by the surrounding cells/tissue induce its deformation. After imaging the microdroplet in 3D using standard fluorescence microscopy techniques (confocal, multiphoton or light sheet microscopy), the reconstruction of its shape in 3D and the knowledge of its interfacial tension allow a quantitative measurement of the cell/tissue mechanical stresses surrounding the microdroplet.

Strengths – Perhaps the main strength of this technique is that allows quantitative measurements of endogenous stresses *in vivo* and *in situ*. By following the droplet deformations over time, it is possible to have spatial and temporal measurements of the stresses within the developing tissue. Changes in the microdroplet size allow probing both cellular and supracellular/tissue scales. In addition, changes of the ligands for cell adhesion receptors at the microdroplet surface allow specific targeting of cells/tissues and the measurement of stresses transmitted via specific molecules.

Limitations – The technique requires the microinjection of a single (or multiple) microdroplet between cells in the tissue. This fact makes it better suited for the study of 3D embryonic tissues, rather than monolayered epithelial tissues. In addition, careful control of the microdroplet surface chemistry is required to precisely specify the microdroplet interfacial tension and the cell-microdroplet interactions. At the cell scale, it can measure normal stress variations around the droplet, but cannot measure the local shear stress applied by individual cells at a given point on the microdroplet surface due to its in-plane fluidity. At the supracellular/tissue scales, it can measure quantitatively anisotropic stresses, but cannot measure the isotropic stress (tissue pressure) because of microdroplet incompressibility.

Examples – DS have been used to measure cell-generated stresses at the cell scale within living mouse mandibles (*ex vivo*) and in cell aggregates (*in vitro*) [227]. These sensors have also been successfully tested *in vivo* within developing chick and zebrafish embryos (unpublished data).

5. Discussion

Despite the variety of existing techniques to explore cellular and tissue mechanics *in vivo*, it remains challenging to perform quantitative mechanical measurements as tissues and organs develop. This is mainly because the sample requires minimal perturbation as it undergoes substantial shape changes in 3D over the time of the measurement. The continuous 3D movement of cells and tissues sets limits to the *in vivo* use of most of the quantitative techniques described above, as these require constant contact with the sample (MI, MPs, MA, AFM, etc.). Techniques based on imaging the sample without an external probe contacting the surface of the tissue or embryo have been often preferred for *in vivo* and *in situ* characterization of cell and tissue mechanics (LA, FI, FRET sensors, OT, DS). From these techniques, only OT and DS allow quantitative absolute measurements *in situ* and *in vivo* (Table 1).

In addition to purely practical challenges, *in vivo* measurements require methodologies with the ability to probe the system at the appropriate length and time scales. While cell size provides a natural length scale for tissue morphogenesis, it is unclear whether a detailed understanding of all mechanical quantities at the cell scale is necessary to understand morphogenetic flows. Indeed, several studies indicate that variations in mechanical quantities within a tissue occur at length scales larger than single cells [81–83,76,77,80]. As a consequence, measurements of the forces driving tissue morphogenesis will require probes adapted to the relevant length scales. In a similar way, the time scale of the measurement is very important, not only because the mechanical

Table 1
In vivo/*ex vivo* measurements of mechanical quantities at cell and tissue scales. The different techniques that have been used to measure each quantity are indicated, together with the type of measurement each technique can perform and some representative studies in each case.

	Mechanical quantity	Measurement methods	Type of measurement	Examples of use
Subcellular to cellular scales	Cortical tension/Cell–cell junction tension	LA	Relative/*in vivo*/*in situ*	[123–127,116,118,128–133,121,134,122]
		FI	Relative (inferred)/*in vivo*/*in situ*	[81,146–148,144,145]
		OT	Absolute/quantitative *in vivo*/*in situ*	[191]
		MA	Absolute/Quantitative/*in vivo*/*in situ*	[154,153]
	Cell adhesion	FRET	Absolute/*in vivo*/*in situ*	[222]
	Cell pressure	FI	Relative (inferred)/*in vivo*/*in situ*	[81,146–148,144,145]
	Mechanical (material) properties	OT	Absolute/Quantitative/*in vivo*/*in situ*	[191,103]
		MR	Absolute/*in vivo*/*in situ*	[210,211,103]
		LA	Relative/*in vivo*/*in situ*	[121]
Cellular to supracellular (tissue) scales	Anisotropic normal stress	LA	Relative/*in vivo*/*in situ*	[83,77,72,136,82]
		DS	Absolute/Quantitative/*ex vivo*/*in situ*	[227]
	Tissue surface tension	TDR	Absolute/Quantitative/*ex vivo*	[141,142,139]
		MIs/MPs	Absolute/Quantitative/*ex vivo*	[110,178,109,108]
	Mechanical (material) properties	LA	Relative/*in vivo*/*in situ*	[83]
		TDR	Absolute/Quantitative/*ex vivo*	[141,142]
		AFM/Cantilevers	Absolute/Quantitative/*ex vivo*	[165,166,170–173]
		MIs/MPs	Absolute/Quantitative/*ex vivo*	[174–176]
		MTs	Absolute/Quantitative/*ex vivo*	[201]

properties display time-dependent behaviors, but also because different mechanical quantities (including stresses) may change over time as a result of biochemical signaling and/or mechanical feedback. Moreover, while it is important to have enough time resolution, measurements at time scales much shorter than morphogenetic processes may not reveal the relevant mechanics underlying morphogenesis.

In the near future, cross-validation of popular, imaging-based existing techniques with quantitative techniques in controlled situations would considerably help studies of mechanics *in vivo*. For instance, quantitative OT measurements of tension at cell–cell junctions of monolayered epithelia [191] could provide a good validation of FI and LA. Also, quantitative measurements of stress anisotropy at cell and tissue scales using DS [227] could be used to validate LA as well as tension measurements with FRET sensors. In addition to cross-validation of existing techniques, the development of new tools to measure mechanical quantities that are not accessible today, such as the local tissue pressure (isotropic normal stress) or shear stress, would also be instrumental.

All these techniques, and others to come, promise to bolster the study of mechanics within living embryonic tissues and organs, and may transform our understanding of many developmental processes, in a similar way that our ability to visualize biochemical signals did in the past.

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