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REVIEW

The complex three-dimensional organization of epithelial tissues

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ABSTRACT

Understanding the cellular organization of tissues is key to developmental biology. In order to deal with this complex problem, researchers have taken advantage of reductionist approaches to reveal fundamental morphogenetic mechanisms and quantitative laws. For epithelia, their two-dimensional representation as polygonal tessellations has proved successful for understanding tissue organization. Yet, epithelial tissues bend and fold to shape organs in three dimensions. In this context, epithelial cells are too often simplified as prismatic blocks with a limited plasticity. However, there is increasing evidence that a realistic approach, even from a reductionist perspective, must include apico-basal intercalations (i.e. scutoidal cell shapes) for explaining epithelial organization convincingly. Here, we present an historical perspective about the tissue organization problem. Specifically, we analyze past and recent breakthroughs, and discuss how and why simplified, but realistic, *in silico* models require scutoidal features to address key morphogenetic events.

KEY WORDS: Three-dimensional cell packing, Cell shape, Scutoid, Apico-basal cell intercalation, Mathematical modeling, Biophysical modeling

Introduction

The invention of the microscope led to the discovery of the fundamental unit of life: the cell. Yet, the collective organization of cells in tissues is far from obvious under the microscope and requires the combination of reliable staining methods and detailed analyses. For example, the neuron doctrine that set the foundations of modern neuroscience was only possible because of the combination of the staining method developed by Golgi (Golgi, 1885) and the histological analyses (and artistic talent) of Ramón y Cajal (de Castro et al., 2007; Ramón y Cajal, 1888; Ramón y Cajal, 1899) who in fact shared, for the first time, the Nobel prize in Medicine and Physiology.

Packed tissues, such as epithelia, pose additional problems for elucidating the cellular organization, because it is difficult to obtain detailed three-dimensional (3D) cellular shapes. This has led to the adoption of diverse reductionist approaches for understanding epithelial tissue organization. The polygonal-like shape of epithelial cells on the apical surface of tissues provides the most important and prevalent simplification: epithelial cells have a prismatic-like shape (Fig. 1A). Thus, textbooks have traditionally schematically depicted the cells of epithelial monolayers as prisms with polygonal bases representing their apical and basal surfaces (Boyle, 2008; Gilbert, 2013). In the case of complex tissue rearrangements (e.g. folding and bending of epithelia), cells have been also represented by prismatic shapes. Still, under those circumstances, the cells reduce one of the polygonal surfaces (apical or basal) to accommodate to the curvature of the tissue (Fig. 1B). The term 'bottle shape' was coined to describe the cell shape that corresponds, geometrically speaking, to a truncated pyramid also known as 'frustum' (Schneider and Eberly, 2003). Epithelial cells with a bottle shape do appear during the invagination processes that occur during embryo development, such as gastrulation or the formation of the neural tube in vertebrates (Davidson, 2012; Lecuit and Lenne, 2007; Pearl et al., 2017). An important implication of the 'prismatic simplification' is that apical and basal surface bases necessarily have the same number of sides (but may differ in size in the case of bottle-shaped cells). Consequently, such representation assumes, tacitly, that it is enough to know the organization of the apical layer to understand the global 3D architecture and the cellular connectivity. Thus, until very recently, most studies have inferred the 3D organizational and biophysical information of epithelia by examining and modeling the apical cell surface alone. However, the natural shape of the epithelial cells is far more complex. In particular, several studies have revealed the existence, predominantly in curved tissues, of apico-basal intercalations (see Glossary, Box 1) that challenge the idea of prismatic epithelial cells (Fig. 1C). This feature appears to be essential to understand dynamical events in different morphogenetic processes and to shed light into the biophysical forces that drive homeostatic epithelial packing. We note that, although cell-cell contacts are far from being straight in a number of well-studied epithelia (e.g. as shown by the curvature of the lateral membranes of columnar cells), here, we discuss efforts to develop reductionist representations of tissues based on 'simple' – yet faithful – representations of cell shapes beyond the prismatic-like paradigm. Thus, we review the study of epithelial organization from a historical perspective and argue that such methodological approaches are particularly required for the implementation of computational models. These models, although still limited, are extremely helpful to unveil the underlying biophysical cues driving morphogenesis.

A historical perspective on the 3D epithelial organization

The structure and cellular organization of developing tissues has been studied since the development of the first microscopes. Interestingly, the term 'cell' was in fact coined in 1655 by Hooke when describing the organization of a tissue rather than an individual entity. Thus, when describing his observations under the microscope of thin slices of cork in 'Observation XVIII' of his celebrated book *Micrographia*, he wrote that this tissue resembled 'much like a Honey-comb, but the pores of it were not regular; […] these pores, or cells […]' (Hooke, 1665). Still, it was not until the

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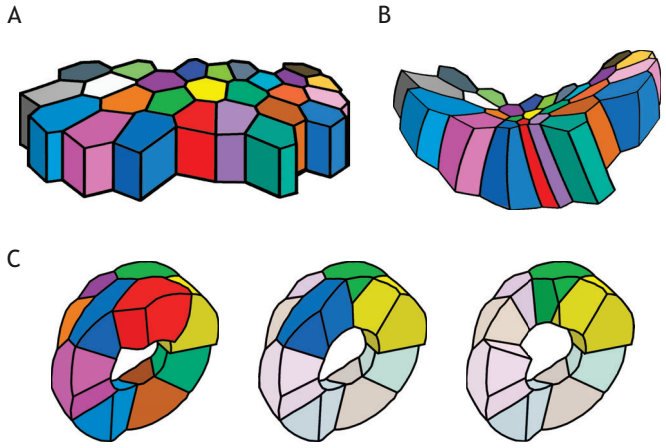

Fig. 1. Schematic representation of monolayer epithelial tissues. (A) Illustration of a planar epithelium where cells are represented as prismatic columns. (B) Cells in A adapt their conformation to the tissue curvature by adopting the shape of a truncated pyramid (i.e. frustum). (C) A Voronoi tubular model mimicking a monolayer epithelial tube, where some cells have been peeled-off (from left to right) to reveal their 3D arrangement. The four-cell motif formed by the blue, red, green and yellow cells undergoes an apico-basal intercalation (T1-spatial transition). Red and green cells are in contact at the basal surface (outer surface), but they are not at the apical surface (inner surface). The opposite happens with blue and yellow cells: they are neighbors in the apical surface but not in the basal surface. All four cells have scutoidal shapes. The colors of the cells in A and B are consistent to track the changes that occur during the transition from a planar to a bent tissue. In the center and right panels in C, the cells that do not belong to the four-cell motif have been shaded to highlight the cells with a scutoidal shape.

Box 1. Glossary

Aboav-Weaire's law: It establishes that, in the surface of an epithelium, cells with a larger number of sides tend to have cell neighbors with few sides, and vice versa (Fig. 3C).

Apico-basal intercalation: The rearrangement of cells along the apico-basal axis in which the cells exchange their neighbors between the basal and the apical surfaces. Roughly speaking, an apico-basal intercalation is similar to a T1 transition, but the neighbor exchange between cells occurs in space (along the apico-basal cell axis) instead of as a function of time.

Euler's principle: The Euler formula relates the number of vertices (V), edges (E) and faces (F) of polygons with the so-called Euler characteristic (2 in convex tessellations of the plane): V-E+F=2 (Fig. 3A).

Flintstones' law: It states that the average number of 3D connections of cells of monolayer tubular epithelia grows as a function of the surface ratio (apico-basal coordinate) following a logistic-like formula (Fig. 3E).

Graph theory: A branch of mathematics that focuses on the study of network properties. Typically, a network is constituted by a set of nodes connected by edges, and these pairwise relationships are the object of analysis.

Lewis' law: It states that, in the surface of an epithelium, the fractional apical cell area increases linearly with the number of neighbors of a cell (i.e. small cells tend to have fewer sides than larger cells) (Fig. 3B).

Vertex models: Off-lattice tissue simulation scheme based on the balance of forces acting on a limited set of points that describe every cell – the vertices that define their polygonal shape.

Voronoi tessellations: Mathematical concept based on compartmentalizing the Euclidean space by proximity in which each one of the compartments is called a Voronoi cell. A set of seeds is necessary for developing a Voronoi diagram. From each seed will emerge a Voronoi cell that fills the surface, preventing gaps among the cells and not allowing overlap between the regions, resulting in a subdivision of convex polygons that follow the rule that a Voronoi cell contains all the points of space that are closer to its seed than to any other seed.

Young-Laplace formula/equation: Given a thin interface that separates two fluids, the Young-Laplace formula evaluates the balance of normal stresses acting on the interface (i.e. surface) and relates the pressure differences with the surface tension and the local geometry (principal curvatures).

19th century that the cell theory was widely accepted and experimental embryology began to flourish, during which the question of how cells collectively organize became paramount. Soon enough, embryologists acknowledged that the cells were under the influence of the physical laws that govern nature. In 1903, Robert thoroughly analyzed the early changes of the development of embryos of the genus *Trochus* (marine univalve mollusc) from this perspective (Robert, 1903). Typically, a four-cell embryo is composed of two lateral cells contacting two central cells, such that the central cells make contact between them and also with the lateral ones. However, Robert found cellular configurations where all four cells were sharing surface contacts (Fig. 2). To understand the processes leading to these configurations, Robert pioneered the use of biophysically-inspired models based on soap bubble experiments. Thus, he studied the 3D structures derived from four-bubble motifs by perturbing the force equilibrium (e.g. in motifs where the bubbles had the same volume) by removing air from the two bubbles at the end of the polar furrow (lateral cells). With these experiments, he was able to reproduce the different configurations observed in real embryos (Fig. 2). He then concluded that surface tension was the most important physical phenomenon underlying the organization of both cells and foam bubbles. Years later, the mathematical biologist Sir D'Arcy W. Thompson, in his seminal book *On Growth and Form* (Thompson, 1917), remarked the presence of the configurations analyzed by Robert and added other configurations found in embryos of different animals, such as the starfish (genus *Asterina*) (Ludwig, 1882) or the freshwater anostracan (genus *Branchipus*) (Spangenberg, 1875), and pollen-grains of orchids (genus *Neottia*) (Goebel et al., 1887) (Fig. 2).

Parallel to these efforts, in 1887 Lord Kelvin proposed a solution to the classic problem of dividing the space with cells with minimum surface area. He introduced the idea of 14-sided shapes, or 'tetrakaidecahedral' cells, and demonstrated their appearance in soap-films (Thomson, 1887) (Fig. 2). Later, Frederic T. Lewis carefully considered the possibility that such shapes were present in the cells of ordinary vegetable parenchyma (specifically, in *Sambucus canadensis*) (Lewis, 1923). Lewis quantitatively examined the cellular 3D contacts and observed cells with a diverse number of sides and conformations. Notably, he found predominantly 14-sided cells as Kelvin predicted, thus validating, indirectly, that surface tension was the main driver of cellular organization. Lewis also observed later the same prevalence of the tetrakaidecahedral shape in human fat cells (Lewis, 1925) (Fig. 2), and in the precartilate tadpole of the common toad (*Bufo lentiginous*) (Lewis, 1933). Marvin confirmed the presence of the tetrakaidecahedron in metal (using compressed lead shots) (Marvin, 1939a), and in the pith of the weed *Eupatorium purpureom* (Marvin, 1939b). Altogether, these studies suggested that similar physical principles led to the same geometric configurations in living tissues, inert froths and even metals.

Subsequent advances in microscopy allowed scientists to dig deeper into the knowledge of 3D cell shapes and tissue organization. Importantly, it became possible not only to study the cell packing of complex organs, but also its relationship to the underlying developmental processes. In 1976, Menton described in detail the cell packing of the parenchymal cells of *Cork cambium*

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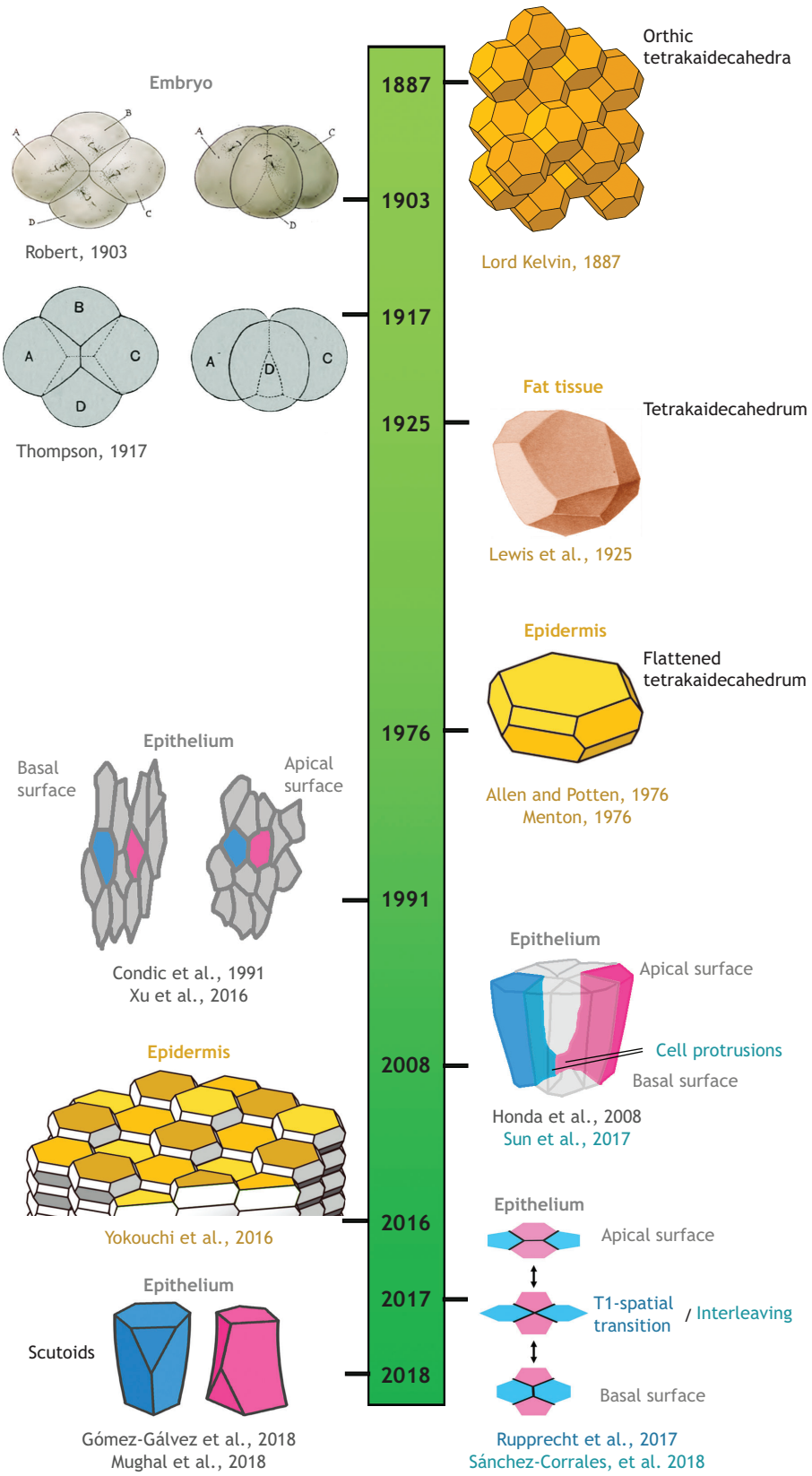

Fig. 2. Historical timeline summarizing breakthroughs in the characterization of 3D cell shapes and their arrangements. A timeline of different realistic descriptions of cell shapes. Yellow-golden colors highlight studies related to solids with 14 faces. The grey-blue-pink colors highlight studies connected to the existence of apico-basal cell intercalations in monolayer epithelia. In 1887, Lord Kelvin proposed the geometrical shape of an 'orthic tetrakaidekahedrum' as a theoretical solution to fill the space optimally. In 1925, Lewis confirmed the existence of tetrakaidecahedra cells, also found by Menton as predominant on the epidermal tissue. In 2016, Yokouchi and colleagues supported Lord Kelvin's tetrakaidekahedrum as a cellular shape, revealing its predominance and important role in stratified epithelia. In a different context, in 1903, Robert found early scutoidal-like cellular configurations later revisited by, D'Arcy W. Thompson in 1917, highlighting its importance. In 1991, Condic and colleagues challenged the 'prismatic approximation' by showing that the cellular organization at the apical and basal layers of an epithelium changed during *Drosophila* development, suggesting the existence of apico-basal intercalations. These intercalations where theoretically postulated by Honda and colleagues as transient cellular configurations to achieve tissue elongation, and were later envisioned as cellular protrusions by Sun and colleagues during *Drosophila* germ-band extension. Recently, two studies emphasized the role of T1-spatial transitions or interleaving in different contexts of developmental biology: in 2018, Gómez-Gálvez and colleagues formally proposed that the spatial intercalations entailed a new cell shape (scutoid) that develops as a consequence of biophysical and geometrical constraints, which were confirmed in a study using soap bubbles.

(from commercial cork bottle stoppers), the pith of shrub stems (*S. canadensis*) and the stratified epithelium of one of the epidermal layers of the mouse inner ear (Allen and Potten, 1976; Menton, 1976). He found, once again, that the cell arrangements of these very diverse organisms were 'universally' formed by columns of flattened 14-sided cells (Fig. 2) (Allen and Potten, 1976; Menton, 1976). In fact, a recent study (Yokouchi et al., 2016) revisited the problem of epithelial cell organization in the mouse ear skin by

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using *in vivo* live imaging and computational models (Fig. 2). The authors corroborated previous experiments and highlighted that the flattened Kelvin's tetrakaidecahedron is indeed the optimal shape to fill the space of this stratified epithelium. In biological terms, the authors suggested that these cell structures promote an accurate barrier to maintain homeostasis and increase the physical strength of this tissue.

The relationship between cell morphology and its primary role in morphogenetic events was also an object of study in monolayer epithelia. In this context, it is worth mentioning the work of Condic and colleagues (1991) (Fig. 2). In their study, the elongation of the *Drosophila* leg imaginal disc was analyzed from the perspective of cellular organization. By comparing the cellular organization of apical and basal surfaces, it was shown that the cells did not preserve the same number of neighbors. These findings thus revealed, indirectly, the existence of apico-basal intercalations that challenged the 'prismatic simplification' in an epithelia monolayer for the first time (Fig. 1B,C; Fig. 2). On the computational side, it was not until 2008 that Honda and colleagues developed the first 3D model, which suggested transient apico-basal intercalations as a way to enable tissue elongation (Honda et al., 2008) (Fig. 2). Additional experimental studies have subsequently revealed – either directly or indirectly – these non-prismatic epithelial shapes in different tissue monolayers. Thus, cell-neighboring changes between apical and basal surfaces (non-compatible with prism-like cells) were also reported on the Wolffian duct epithelium in mouse (Xu et al., 2016). Notably, from a dynamics viewpoint, Sun and colleagues demonstrated that during the *Drosophila* germ-band extension the active tissue elongation was driven by basolateral protrusions and transient apico-basal intercalations among cells (Sun et al., 2017) (Fig. 2). More recently, these dynamical intercalations have been shown to be relevant in different contexts, such as the development of the salivary gland placode in *Drosophila* (Sanchez-Corrales et al., 2018) and during the *Drosophila* embryo cellularization (Rupprecht et al., 2017) (Fig. 2). Finally, the mathematical formalization of a novel geometrical shape in connection to the apico-basal intercalations, the scutoid, uncovered important biophysical consequences for the 3D tissue organization (Gómez-Gálvez et al., 2018) (Fig. 1C). Specifically, it was suggested for the first time that the thickness and curvature of tissues modulates the appearance of apico-basal intercalations. In addition, it was proposed that the underlying motive for this new shape was to minimize surface energy expenditure when tissues are subjected to anisotropic bending. This hypothesis was further confirmed by a study in froth monolayers, which revived the idea of the surface tension as the main driver of cellular organization in the context of epithelial monolayers (Mughal et al., 2018) (Fig. 2).

The mathematics and biophysics of epithelial organization

Mathematical tools/laws to quantify epithelial organization

One important advantage of the 'prismatic' approximation is that it makes it possible to implement common elements of mathematical topology to investigate tissue packing. In particular, the analysis of the topology of the apical surface of epithelia has provided useful information about metazoan development. For example, Reinhart used Euler's principle for convex polyhedrons (see Box 1, Glossary; Fig. 3A) (Euler, 1767), to formally deduce that the average number of sides of the cells in a plane tessellation of convex polygons should be six (Reinhardt, 1918). Later, this conclusion was experimentally confirmed in epithelia by Wetzel (1926).

Lewis further analyzed tissues from a geometrical and topological viewpoint, and established the existence of a linear relationship between the average cell areas and the number of neighbors (Lewis, 1928) (Fig. 3B). Rivier and Lissowski subsequently demonstrated mathematically that the so-called 'Lewis' law' (see Box 1, Glossary) originates from a maximum entropy principle given the constraints of the cellular topology (Rivier and Lissowski, 1982). Lewis' law was successfully confirmed later in a number of biological tissues and two-dimensional (2D) Voronoi tessellations (see Box 1, Glossary) (Farhadifar et al., 2007; Gibson et al., 2006; Sánchez-Gutiérrez et al., 2016). Regarding the similarities between Voronoi diagrams and epithelial tissues, a breakthrough was established in 1978, when Honda and colleagues showed that the Voronoi compartmentalization of a 2D space fitted the pattern of cellular contacts found in epithelial surfaces (Honda, 1978).

Another example of a mathematical principle observed in convex tessellations of the plane is Aboav-Weaire's law (see Box 1, Glossary) that states an inverse relationship between the mean number of sides of the neighbors of a cell and its number of neighbors (Aboav, 1970; Chiu, 1995) (Fig. 3C). This law was first observed in the grains of growing polycrystals, but was also satisfied in 2D Voronoi tessellations (Zhu et al., 2001) and in the apical plane of growing epithelia (Bi et al., 2014; Sánchez-Gutiérrez et al., 2016).

These principles and properties refer to statistical moments (e.g. averages), but the details of the underlying polygonal distribution were also the focus of research. Thus, Lewis quantified for the first time the polygonal distribution of cells in the *Cucumis* epidermis (Lewis, 1928). More recently, seminal work by Gibson and colleagues demonstrated that the origin of a conserved polygonal distribution of cells among Metazoa is a consequence of cell proliferation (Gibson et al., 2006) (Fig. 3D). Subsequent studies introduced elements of Graph theory (see Box 1, Glossary) to analyze the polygonal distribution of cell contacts and, in some cases, to quantify the epithelial topology under physiological and pathological conditions (Escudero et al., 2011; Kursawe et al., 2016; Sánchez-Gutiérrez et al., 2013; Vicente-Munuera et al., 2020; Yamashita and Michiue, 2014). Other complementary methods combined the polygon distribution analysis with the application of *in silico* models, such as vertex models (see Box 1, Glossary) or Voronoi tessellations, trying to reproduce and explain the biological behavior by mathematical/computational means (Aland et al., 2015; Bi et al., 2016; Curran et al., 2017; Farhadifar et al., 2007). These analyses suggested that the conserved metazoan polygon distribution was not exclusively dependent on cell division mechanisms, but a consequence of the physical restrictions found in natural tessellations and of the homogeneous size of the epithelial cells (Sánchez-Gutiérrez et al., 2016).

The mathematical principles and properties described above were assumed to be valid in a 3D context in epithelial monolayers given the 'prismatic simplification'. However, the unveiling of cellular scutoidal shapes challenges some of these organizational principles. We envision further generalizations of the mathematical organizational principles to a 3D context in the future years (see Discussion) (Fig. 3A-E).

Forces and stresses inference

A mechanistic approach towards developmental biology ultimately seeks to elucidate the forces that drive cellular shapes and their collective properties. Such knowledge is required for developing realistic predictive modeling frameworks and, ultimately, to understand the determinants of the organizational and mathematical features displayed by tissues. During the last few

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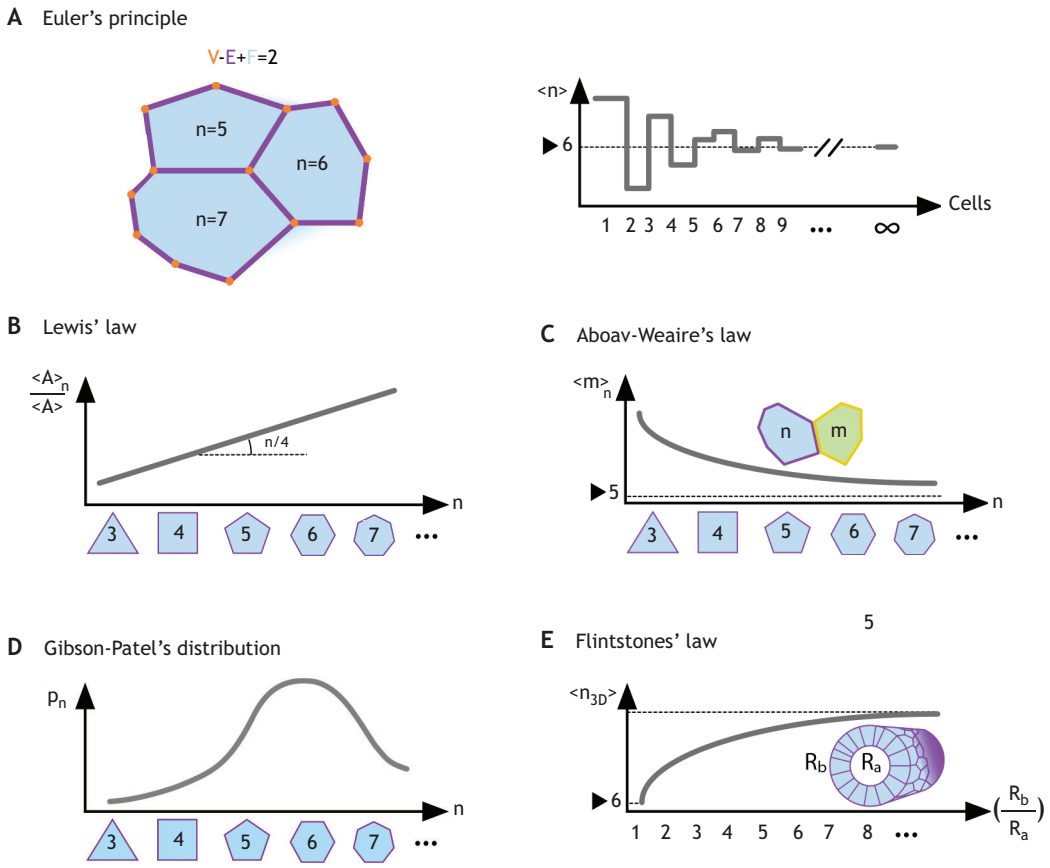

Fig. 3. Mathematical approaches towards the analysis of epithelia. (A-E) The analysis of epithelia from the perspective of space tessellations using convex polygons has led to a number of quantitative laws in morphogenesis. (A) Euler's principle for convex polyhedra implies that Vertex (V)−Edges (E)+Faces (F)=2. In this figure, the labels inside the polygons indicate their number of vertices or edges, *n*. In this case, V=13, E=15 and *F*=4 (note that the external face surrounding all polygons is also included in the face count). Euler's formula implies that in the thermodynamic limit, that is, as the number of cells becomes very large, the average number of neighbors (edges) of a cell in 2D (i.e. $\langle n \rangle$) approaches 6. (B) By denoting by $\langle A \rangle_n$ the average area of cells with *n* number of edges and by $\langle A \rangle$ the average cell area, Lewis' law states that the fractional area of cells, $\langle A \rangle_n / \langle A \rangle$, that belong to a polygonal class (i.e. triangles, squares, pentagons…) increases linearly with the polygonal class (i.e. with *n*). Lewis' law is a consequence of a maximum entropy principle and cellular topological constraints. (C) On the other hand, Aboav-Weaire's law provides an analytical dependence of the average number of neighbors of neighboring cells on the polygonal class. Thus, $\langle m \rangle_n$ indicates the average number of edges of cells that neighbor a cell with *n* edges: the larger the polygonal class, *n*, the smaller the number of edges of neighboring cells. (D) Gibson and colleagues later established the universality of the polygonal distribution of cells due to division events. In agreement with Euler's formula the average of this distribution is 6. (E) More recently, laws are being proposed in the context of the 3D shape of cells. In particular, the Flintstones' law states that, as a function of the so-called surface ratio, R_b/R_a, tubular epithelia increase their 3D connectivity (i.e. the 3D number of neighbors) in a logistic manner as a consequence of apico-basal intercalations.

decades, different approaches have been developed to characterize forces and stresses at the cellular and collective levels, both in 2D and in 3D (Gómez-González et al., 2020; Roca-Cusachs et al., 2017; Sugimura et al., 2016; Xu et al., 2018). In this context, geometric force inference (GFI) methods are of special interest to understand epithelial organization. This methodology is based on either imposing a force equilibrium on the vertices that define either the polygonal (2D) or prismatic-like (3D) shapes of cells, or on applying the Young-Laplace formula (see Box 1, Glossary) to balance the normal stresses (Fig. 4A). Although GFI has limitations (e.g. only relative values of tensions and pressure differences can be estimated), it also has many advantages (e.g. it is non-invasive) and has been instrumental in understanding a number of morphogenetic events (Gómez-González et al., 2020; Noll et al., 2020; Sugimura et al., 2016; Vasan et al., 2019; Veldhuis et al., 2017). Notably, GFI has traditionally focused on the 2D cellular organization of tissues and, to our knowledge, only two recent studies have proposed a 3D extension (Veldhuis et al., 2017; Xu et al., 2018): Veldhuis and colleagues have introduced CellFIT-3D, a tool based on the inference of 3D properties using 2D slides (e.g. confocal images). This approach avoids the methodological bottleneck of cellular reconstruction and has been also proposed to derive statistical properties of the 3D cell geometry in tissues (Sharp et al., 2019). More recently, normal stresses and tensions have also been inferred in 3D to better understand the cellular organization in the early *Caenorhabditis elegans* embryo (Xu et al., 2018). However, as of today, no GFI approach has been used to infer forces in 3D epithelial monolayers where apico-basal intercalations develop. One of the reasons lies in the lack of a precise characterization of the existing lateral cell-cell interactions. Consequently, besides the proposal that line/surface tension plays a key role in determining novel cellular geometries in curved 3D environments (Gómez-Gálvez et al., 2018; Mughal et al., 2018), there is still a gap of knowledge about how the balance of different acting forces (e.g. contractility versus adhesion) leads to a cellular organization in 3D. In this regard, further advances in the implementation of the 'microbulge' technique (i.e. the controlled formation and manipulation of tissue domes and, possibly, of other 3D tissue micropatterns) might shed light on this

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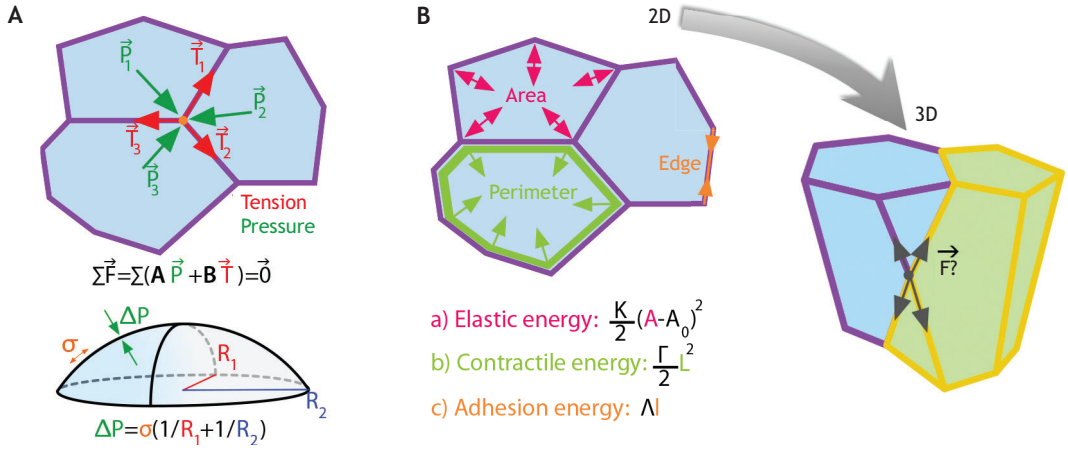

Fig. 4. Non-invasive force inference methods. (A) Force inference methods based on the geometrical analysis of cellular arrangements ultimately rely on applications of a force equilibrium principle on the cellular vertices (top) and/or on the Laplace-Young law (bottom). As for the former, the equilibrium of forces at cell junctions (vertices) implies a balance between pressure terms, *P*, and membrane tensions, *T*. That hypothesis leads to the estimation of parameters (*A* and *B* in this panel), that measure the relative pressure/tension force contributions. On the other hand, the methodology based on the Laplace-Young law is based on the assumption that cells behave mechanically as fluid objects. Thus, it relates the cellular, or the tissue-level, membrane tension, σ, the acting normal stresses, ΔP, that modulate the cell (tissue) shape, and the principal curvatures at a given location, $1/R_1$ and $1/R_2$, that define the local geometry. (B) On the modeling side, the vertex model has successfully reproduced a number of morphogenetic processes. The canonical form of the vertex model includes mechanical contributions at cell vertices due to: (a) the area, *A*, that leads to spring-like forces (volume conservation); (b) the action of the actomyosin ring along the cell perimeter, *L*, that simulate contractile effects, and (c) adhesion terms that mimic membrane tension along cell contacts, *l*. By including non-equilibrium effects, such as cell growth and division, and assuming a fast balance of the mechanical forces, the position of cell vertices can be tracked in space and time and, hence, the cellular motion. Current challenges in the field of epithelial tissue simulation schemes include the development of techniques that reproduce realistically the 3D arrangements of cells and clarify the driving forces underlying apico-basal intercalations.

problem (Latorre et al., 2018). This methodology allows researchers to control the remodeling of cellular and tissue shapes in 3D and to correlate those changes with the acting forces and a compatible cellular mechanics. In particular, by using a 3D biophysical tissue model, the authors of this study have been able to reveal the so-called 'active super-elasticity phenomenon' in bent epithelial monolayers: cells have the capability to deform, reversibly, at a constant tension. This example highlights the importance of developing convincing biophysical tissue models (see below), and the need to develop and implement realistic force inference methods to 3D epithelia in order to unveil the processes that control 3D tissue organization.

Epithelia simulation models: from 2D to 3D

Modern developmental biology is built upon the combined effort of novel biological techniques and computational approaches in order to describe the biological and biophysical behavior of tissues. Following this trend, the field is experiencing a slow – yet steady – progression towards the study of tissues from a more realistic perspective. Specifically, 2D simulations have contributed enormously to the progress of the field, but the next step is the implementation of 3D simulation schemes that make it possible to understand how animals develop in a 4D context (three spatial dimensions and time). Here, we describe major advances in the context of 2D simulation models and elaborate on the challenges for implementing 3D computational approaches.

There is a great number of cell-based computational solutions for the simulation of tissues (Fletcher and Osborne, 2020; Metzcar et al., 2019). Here, we focus on discrete boundary-based models because they are particularly useful for describing epithelia: they are computationally efficient and allow direct comparisons with GFI methods by describing the acting forces on cell connectivity loci. Honda and Eguchi laid the foundations of the 'vertex model' by showing that cell boundary contraction processes in the surface of epithelia could be described by a model of packed convex polygons with an area-conservation property (Honda and Eguchi, 1980). Later, Nagai and Honda formalized the model by proposing a simulation technique that linked the polygonal geometry of cells in epithelial surfaces with the forces acting at cell vertices (Nagai and Honda, 2001). This seminal study showed that a deterministic approach that included line-tension and elastic force terms, together with topological changes (T1 transitions), was enough to describe the organization in epithelia in equilibrium. Further developments of the vertex model have included additional mechanical effects, such as contractility force terms due to the acto-myosin ring (Farhadifar et al., 2007) or to anisotropies in acto-myosin activity (Canela-Xandri et al., 2011) (Fig. 4B). It has also been shown that non-equilibrium contributions due to migration, cellular proliferation and oriented cell divisions can explain transitions from soft to solid phases in tissues (Farhadifar et al., 2007), jamming transitions (Bi et al., 2016), remodeling at the tissue level (Anbari and Buceta, 2020; Mao et al., 2011), the appearance of pathological and mutant conditions deviating from tissue homeostasis (Ramanathan et al., 2019; Sánchez-Gutiérrez et al., 2016) or wound-healing processes (Staddon et al., 2018; Tetley et al., 2019). Recent developments of the vertex model have also included viscoelastic and mechanosensitive effects (Canela-Xandri et al., 2020; Staddon et al., 2019). All these studies have shown that the aforementioned mathematical laws accomplished by real epithelia are satisfied by vertex model simulations, thus providing additional support to this computational method (Figs 3 and 4).

During the last few years, a number of modifications of the vertex model have been proposed, aimed at adapting this simulation methodology to more complex geometries. For example, simulations of 2D cross-sections along the apico-basal axis of curved epithelial monolayers have been used to study *Drosophila* ventral furrow formation (Polyakov et al., 2014) or the buckling and folding of cell cultures (Merzouki et al., 2018). Also, vertex models

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have been modified to simulate either the apical or the basal surfaces of curved tissues. Some examples include the dorsal appendage formation in the egg chamber (Osterfield et al., 2013), epithelial folding (Monier et al., 2015) and the tubulogenesis process (Hirashima and Adachi, 2019).

The generalization of the vertex model to 3D poses some challenges. First, an accurate description of the tissue behavior must account for the mechanical polarization of cells along the apico-basal axis. This implies the need to prescribe distinct mechanical interactions among cells in apical and basal surfaces, and through the lateral contacts. In addition, the possible effects of the extracellular matrix become more relevant. Finally, the computational implementation of some cellular processes that shape tissues, such as growth, division, apico-basal intercalations and/or extrusion/apoptosis, become more complex.

In this context, some attempts have been made to generalize the vertex model to a 3D environment. The first 3D vertex model was proposed by Honda and colleagues to simulate cell aggregates (Honda et al., 2004). Further implementations have been used in the context of epithelial monolayers to simulate proliferation, deformation and invagination during morphogenesis (Bielmeier et al., 2016; Du et al., 2014; Inoue et al., 2020; Misra et al., 2016; Okuda et al., 2015, 2018a; Sui et al., 2018), as well as branching growth (Okuda et al., 2018b), 'microbulge' dome dynamics (Latorre et al., 2018), tubulogenesis (Inaki et al., 2018; Inoue et al., 2016), tumor progression (Messal et al., 2019) and 3D buckling instabilities in epithelial monolayers (Hannezo et al., 2014). For additional information about the foundations of the vertex model, both in 2D and 3D, and other examples about its applicability to morphogenesis we refer the reader to the following studies (Alt et al., 2017; Fletcher et al., 2014). However, regardless of the progress achieved thanks to the vertex model to understand the link between energetic traits (i.e. forces) and epithelial organization, all the aforementioned studies disregard apico-basal intercalations. In addition to the early work of Honda and colleagues (Honda et al., 2008), some recent exceptions include the work by Okuda and colleagues that suggests that scutoids may develop during cell rearrangements owing to fluctuations and asymmetry between the line tension of apical and basal surfaces (Okuda et al., 2019). We argue that these sort of modeling approaches, along with novel hybrid simulation schemes, are crucial to accurately describe the 3D epithelial organization and shed light into the forces involved (especially in the context of curved tissues) (Fig. 4B). By 'hybrid simulation schemes' we mean computational methods that combine the simplicity of the vertex model (that can be easily parametrized by force inference methods, i.e. GFI) with enough complex elements to generate the observed self-organization in tissues containing complex cellular geometries. Some recent promising results have been presented by Ioannou and colleagues, who have proposed a methodology that accounts for the reported asymmetries between apical and basal surfaces, and have applied it to study wound healing (Ioannou et al., 2020).

Discussion and conclusions

For more than a century, the morphology of cells has intrigued researchers from different fields. In *On Growth and Form*, D'Arcy W. Thompson made a unifying, quantitative effort and put together the accumulated knowledge from different fields to understand the basis of shape establishment. In this way, he linked the complex process of morphogenesis to the emergence of mathematical patterns and the physical nature of cells. Notably, in many of these pioneering works that he compiled, there was an exquisite description of the 3D shape of the cells. These depictions included artistic drawings and quantitative approaches that helped to infer the physics underlying the formation of shapes (Fig. 2). Now, researchers have far more microscopy resources than in D'Arcy W. Thompson's day to explore and analyze in depth the form of cells, their dynamic changes and how they integrate within tissues. Interestingly, these advances in microscopy have led to a re-examination of some of the phenomena presented by D'Arcy W. Thompson. In parallel to this experimental progress, different computational tools have been designed to model morphogenetic processes. These tools are based on reductionist approaches that capture the essential biophysical cues and mathematical principles that drive tissue shape and cellular organization. Together, these tools aim to find 'universality' in developmental processes, as D'Arcy W. Thompson aspired to as well.

As reviewed here in the context of epithelial morphogenesis, most studies that have analyzed tissue organization and its biophysics have limited the study to a single epithelial surface. Although informative and extremely useful, these investigations also neglect the realistic 3D cellular shapes in monolayer tissues. Moreover, only a few examples have analyzed the organization of stratified epithelia (Fig. 2). These two aspects are promising research challenges in the field. In this Review, we have particularly focused on single-layer epithelia development, that we identify as the first – and easiest – step in designing realistic *in silico* tools coupled to force inference methods. To that end, further progress is needed to elucidate the forces that determine the epithelial organization in 3D. On the other hand, the parametrization and calibration of *in silico* models must be consistent with those force estimations. Fortunately, recent results seem to suggest that realistic 3D tissue organizational traits, such as the scutoidal shapes, can be reproduced in force-driven models without implementing excessive complexity (Okuda et al., 2019; Ioannou et al., 2020). This will facilitate the exploration of dynamical phenomena in the near future, because apico-basal intercalations also appear to be involved in active cell movements, such as the *Drosophila* germ band extension, egg chamber rotation or the early morphogenesis of salivary glands (Gómez-Gálvez et al., 2018; Sanchez-Corrales et al., 2018; Sun et al., 2017). Another equally important avenue of research is the influence of the global tissue shape on the 3D cellular packing of epithelia. Recent results by Saunders' lab have described a relationship between curvature and the emergence of apico-basal intercalations on the curved tips of the *Drosophila* embryo (Rupprecht et al., 2017). This phenomenon was later generalized through computational models and experiments to show that the appearance of scutoids is directly dependent on the anisotropy of the tissue curvature, and scutoids are more frequently observed in tubular epithelia (Gómez-Gálvez et al., 2018).

Further progress in understanding 3D tissue organization relies on the advances in microscopy to obtain high-resolution imaging of epithelia and provide precise information of 3D and 4D cell conformations. In combination with improvements in machine learning techniques, aimed at performing fixed- and live-tissue segmentation, these methodologies will soon allow realistic elucidation of the cellular changes that drive morphogenesis (Arganda-Carreras et al., 2017; von Chamier et al., 2020 preprint; Falk et al., 2019; Haberl et al., 2018; Lee et al., 2020; Wolny et al., 2020). Moreover, the precise quantification of the 3D tissue structure in epithelia will enable the study of quantitative principles and mathematical laws that, so far, have only been tested in 2D planar epithelia. We stress that the advantage of these quantitative principles lies in their ability to identify biological functionalities in

homeostasis (Escudero et al., 2011; Farhadifar et al., 2007; Gibson et al., 2006) and in pathological conditions (Sánchez-Gutiérrez et al., 2016; Tsuboi et al., 2018). Interestingly, there are already promising 3D approaches modeling cancer disease in tubular geometries (Messal et al., 2019). Unfortunately, the 'prismatic simplification' has led to the (wrong) assumption that some of these principles are automatically satisfied in 3D. However, there are some clear examples that it is not the case. Specifically, the average number of cellular neighbors in 3D (i.e. the average cellular connectivity) cannot possibly be six if apico-basal intercalations occur (Euler, 1767; Reinhardt, 1918). In this regard, recent studies have highlighted the importance of cellular connectivity in different developmental contexts, such as supervising neuroepithelial morphogenesis (Sharma et al., 2019) or controlling cell fate decisions (Guignard et al., 2020). Interestingly, one study has recently uncovered the principle that describes how scutoids modify the 3D cellular connectivity: Flintstones' law (see Box 1, Glossary) (Gomez-Galvez et al., 2020 preprint) (Fig. 3E). We anticipate that there will be additional discoveries of quantitative principles in the context of 3D cellular organization in the years to come, which will help to justify D'Arcy W. Thompson's claim: '*The harmony of the world is made manifest in Form and Number, and the heart and soul and all the poetry of Natural Philosophy are embodied in the concept of mathematical beauty*'.

Finally, some last words referring to promising applications to the field of biomedicine. The possibility of generating human 3D cultures that resemble specific organs (organoids) has opened up enormous possibilities (Rossi et al., 2018; Tuveson and Clevers, 2019); however, recent advances in organoid technology, although highly promising, are hindered by its current lack of reproducibility (Huch et al., 2017). We believe that the combination of an accurate understanding about how the cells self-organize and pack in 3D and advances in knowledge on how substrate curvature guides spatiotemporal cell and tissue organization (Callens et al., 2020) will help to control the growth of organoid cultures. Altogether, the realistic analysis of epithelial packing can also advance the biomedical field, especially in tissue and organ engineering (Hendow et al., 2016; Yin et al., 2016).

Competing interests

The authors declare no competing or financial interests.

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