Mechanics of inflated epithelia

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## Abstract

Epithelial sheets form specialized 3D structures suited to their physiological roles, such as branched alveoli in the lungs, tubes in the kidney, and villi in the intestine. To generate and maintain these structures, epithelia must undergo complex 3D deformations across length and time scales. How epithelial shape arises from active stresses, viscoelasticity and luminal pressure remains poorly understood. To address this question, here we developed a microfluidic setup and a digital twin to engineer 3D epithelial tissues with controlled shape and pressure. In the experimental setup, an epithelial monolayer is grown on a porous surface with circular low adhesion zones (footprint). On applying hydrostatic pressure, the monolayer delaminates into a spherical cap (dome) from the circular footprint. This simple shape allows us to calculate epithelial tension using Laplace’s law. Through this approach, we subject MDCK epithelial cells to a range of lumen pressures at different rates and hence probe the relation between strain and tension in different regimes, while computationally tracking actin dynamics and their mechanical effect at the tissue scale. Slow pressure changes relative to the timescales of actin dynamics allow the tissue to accommodate large strain variations. However, under sudden pressure reductions, the tissue develops buckling patterns and folds with different degrees of symmetry-breaking to store excess tissue area. These insights from experiments and the digital twin allow us to pattern epithelial folds by rationally directed buckling. Our study establishes a new approach for engineering epithelial morphogenetic events.

# # Part I: Introduction

The central focus of this thesis is the amazing epithelial tissue monolayer. From the perspective of a mechanical engineer, these monolayers are endlessly fascinating. They are shape-changing, self-healing, and continuously deforming or jamming depending on the requirement. They are the most basic system in terms of physical understanding of the larger world of biological morphogenesis. Epithelia are everywhere: covering all our body surfaces and lining body cavities and organs. Epithelial monolayers adopt a range of shapes from simple spherical blastocyst to highly branched and folded lungs. These shapes are developed and maintained through constant adaptation of epithelia. In this work, we have explored the physical principles behind the epithelial shape by combining theory and experimentation with simple epithelial monolayers.

The chapters in this part are to be a primer for all the topics relevant to my PhD. Starting with a brief introduction to the epithelial tissue itself and its key components. Subsequently, a summary of mechanobiology of morphogenesis and how we can think of epithelia as an active material, and the historical ways of modeling it. Finally, I will conclude with the emerging field of “bottom-up” morphogenesis, where researchers are reconstructing the biological systems from scratch.

## Epithelial Layers

### Introduction

Figure: The Anatomy Lesson of Dr. Frederik Ruysch, 1670 by Adriaen Backer

A Dutch botanist, Frederick Ruysch, in early eighteenth century coined the term ‘epithelia’ to describe the tissue found while dissecting the lips of a cadaver, combining Greek roots ‘*epi*’ for the top and ‘*thele*’ for a nipple. A few decades later, a Swiss scientist Albrecht von Haller started to use the term epithelium/epithelia to describe fiber(s) of the body. The idea of fibers was an old renaissance theory that the body is made of fibers, which a fundamental building block of living things. The understanding then was that these fibers/tissues arranged in different arrays gave rise to biological structures (maccord2012, zampieri2014). Not far off as epithelial tissues are ubiquitous and cover most of the organs inside out. In the twenty-first century science, epithelial tissues are understood as a type of animal tissue in which cells are packed into sheets. The epithelial cell sheets have strong intercellular bonds that form physical barriers for compartmentalization of the organs and maintains the homeostasis. It plays a key role in developmental stages by supporting growth and driving shape changes of the organs. It protects the organs from external physical, chemical, and microbial onslaughts.

Epithelial cells are polarized, i.e., their apical side, faces the lumen of the organ, which differs in shape and composition from the basolateral side. Its polar organization is reflected in the vectoral functions like creating and maintaining concentration gradients between the separated compartments. Typical examples of these are transporting epithelia such as those of the renal tubule, absorptive epithelia of the intestine, and secretory epithelial cells like hepatocytes. In addition, polarized epithelia guide the developmental process by determining the fate of cells leading to symmetry breaking events in the embryo (kim2018).

### Key components

Epithelial function primarily depends on the tissue’s structure and its microenvironment. It can be described in three parts: first, cell structure; second, microenvironment; and lastly, cell-matrix interactions.

#### Cell structure

In general, cell structure helps cells maintain their shape along with providing mechanical support to perform vital functions like division and migration. This structure is known as the cell cytoskeleton. In particular, the Eukaryotic cell cytoskeleton is mainly constructed out of filamentous proteins that hold the cell and its cytoplasmic constituents. There are three major filaments, which differ in size and protein content. Microtubules are the largest type of filament of the protein tubulin, with a diameter of about 25 nm. Actin filaments are the smallest type, with a diameter of only about 6 nm. Finally, intermediate filaments are medium-sized, with a diameter of about 10 nm. Unlike actin filaments and microtubules, intermediate filaments are constructed from several different subunit proteins. All three types of filaments dynamically alter themselves in reaction to signals from microenvironments and cell networks (Alberts, B., et al., 2013; Fletcher, D.A., and Mullins, R.D., 2010s). Mechanically, actin filaments are stiffer than microtubules in extension, but they rupture at lower extension. It is also reported that the intermediate filaments exhibit an intermediate extensional stiffness at lower extensions, but that the intermediate filament can sustain much larger extensions than the other two types of filaments while exhibiting a nonlinear stiffening response (Janmey et al., 1991; Mofrad, M.R., 2009).

In the case of epithelial layers, the actin cytoskeleton and intercellular junctions make cell-cell contacts stronger and provide integrity to the tissue (Braga V. 2016). The perfect example of these tissue-level structures can be seen in wound healing assays: cells surrounding the wound create a ring of actin to close it (Brugués, A., et al., 2014). One must keep in mind that these structures tend to self-organize as well. It can be seen when cells are confined in a specific shape, like in the case of circular islands of epithelial cells that showed radial patterns in the actin organization (Jalal S., et al.,2019).

Figure: Quilling of human body and then showing apical basal polarity of epithelial and monolayer including all the cytoskeleton and cell junctions and their mechanics

Multiple membrane molecules can mediate adhesion between cells. One of these are cadherins, critical for epithelial cell cohesion through the formation of adherens junctions. In these junctions, cadherins are coupled to the cell cytoskeleton enabling force transmission between cells. It is a key factor in mechanical regulation of division and tissue rearrangement during development and homeostasis (mertz2013). Desmosomes are another type of intercellular junction. They are coupled with intermediate filaments, and the resulting supracellular network confers mechanical resilience on cell layers (Hatzfeld, M., Keil, R., & Magin, T. M. 2017; Latorre, E., et al., 2018). Tight junctions perform a barrier function and enable the transport of ions across epithelial layers to be actively regulated. This plays an important role in the control of fluid pressure in tissues.

#### Microenvironment

Extracellular matrix (ECM) is the cell environment or substrate to which cells adhere; it is also known as the matrix, mesenchyme, or cellular microenvironment. ECM serves many functions: it endows a tissue with strength and thereby maintains its shape; it serves as a biologically active scaffolding on which cells can migrate or adhere; it helps to regulate the phenotype of the cells; it serves as an anchor for many substances, including growth factors, proteases, and inhibitors of such. It also provides an aqueous environment for the diffusion of nutrients, ions, hormones, and metabolites between the cell and the capillary network. On top of that, it is subjected to mechanical forces such as blood flow in endothelia, air flow in respiratory epithelia, or hydrostatic pressure in the mammary gland and bladder (Roca-Cusachs, P., Conte, V., & Trepat, X. 2017; Humphrey, J. D., et al., 2015; Waters, C. M., Roan, E., & Navajas, D., 2012; Paszek, M. J., & Weaver, V. M., 2004; Bross, S., et al., 2003). It is shown that the ECM regulates cell shape, orientation, movement, and overall function in response to these forces.

ECM is a fibrous network of proteins; from a mechanical perspective, the three primary structural constituents of the ECM are typically collagen, elastin, and proteoglycans. Collagen is one of the most abundant proteins in the body. Elastin is the most elastic and chemically stable protein, and proteoglycans often sequester significant water as well as growth factors, proteases, etc. Due to its water content, the deformation of ECM can produce cracks in epithelial layers. ECM acts as a poroelastic material, soaking up water upon stretching (like a sponge) and releasing it under compression, causing a hydraulic fracture effect (Casares, L., et al., 2015). Moreover, collagen network can remodel under the influence of cells aiding in migration or mechanical forces (Shields, M. A., et al., 2012; Humphrey, J. D., 2003). Like most cytoskeletal proteins, most extracellular components turnover. Some continuously and some very slowly. For example, collagen in the periodontal ligament appears to have a half-life of a few days, whereas that in the vasculature may have a normal half-life of several months. In response to altered physical stimuli, disease, or injury the rates of synthesis and degradation of collagen can increase many folds to have a rapid response (Humphrey J. D., 2003).

#### Cell-matrix interaction

Cells and ECM have a symbiotic relationship with each other through various sensors on the cell surface. The biophysical cues are primarily sensed using integrins and focal adhesion complexes in cell-substrate adhesion (kechagia2019). Through these adhesions cells can sense the external mechanical and chemical signals like matrix stiffness, ligand density, or chemotactic gradients (fortunato2022). Recently it has been shown that they could the cells could even respond to viscoelasticity of the matrix (elosegui-artola2022a). The cells have shown to secrete ECM on their own or remodel the substrate to promote growth/invasion of a cancer or reorganize the cytoskeleton all together. Due to recently discovered deeper ties of focal adhesions to the nucleus and in turn transcriptional factors, cell-matrix adhesions could affect the tissue behavior fundamentally. (DuFort, C. C., Paszek, M. J., & Weaver, V. M. 2011; Northey, J. J., Przybyla, L., & Weaver, V. M. 2017). Precise control of cell-cell and cell-substrate interactions enables emergent behaviors in cell sheets that are not observed in single-cell systems (Trepat, X., and Sahai, E., 2018; Ladoux, B., and Mège, R.M., 2017).

Figure: ECM and focal adhesion.

### Role in disease and development

Epithelial integrity and homeostasis are of central importance to survival, and mechanisms have evolved to ensure these processes are maintained during growth and in response to damage (Macara, I.G., et al., 2014). For example, epithelial cells have one of the fastest turnover rates in the body. The entire gut cell lining turns over in 3–4 days. This turnover implies constant cell division and death. The excessive rate of division and death may give rise to tumors. It is known that 90% of cancers emerge in simple epithelia (Torras, N. et al. 2018; Eisenhoffer, G.T., and Rosenblatt, J., 2013). Not only this, but it could easily disrupt the barrier function, as no gaps should emerge around dying or dividing cells. If the fluid compartmentalization goes awry, it has profound implications for epithelial and stromal homeostasis, fluid and/or electrolyte balance, and the generation of inflammatory states. Several bacterial toxins are known to target junctions and cause changes in the tight junction protein ZO1, resulting in compromised barrier function and pathologies such as diarrhea and colitis (Fasano, A. et al., 1991). Cancer cells tend to spread and disperse metastatically by using their very high rate of cell motility and a diminished sense of cell adhesion. This elimination and/or reduction of ZO1 barriers in cancer is essential to allowing metastatic cells to break into and out of blood vessels. The leaky barrier also allows a growing epithelial tumor to access luminal fluids as an additional source of nutrients (Mullin, J.M., et al., 2005).

Moreover, epithelia participate in physiological events such as epithelial–mesenchymal transformation (EMT), a developmental process when epithelial cells gradually transform into mesenchymal-like cells by losing their epithelial functionality. EMT plays a vital role in normal biological function like repair and differentiation; and abnormal pathological activity like organ fibrosis and promoting carcinoma progression. It endows cells with stem cell properties. Thus, the mesenchymal state is enables cell migration to distant organs and allowing their subsequent differentiation into multiple cell types during development and the initiation of metastasis (thiery2009).

Epithelia undergo drastic changes in shape with deformation and reorganization from the embryonic to the adult stage. Unsurprisingly, any improper function would lead to damage and disorder. Epithelial dysfunction is a precursor of diseases such as chronic obstructive pulmonary disease, asthma, cystic fibrosis, or pulmonary fibrosis (carlier2021).

### Different types of epithelia

Epithelial cells have different shapes and may be arranged in single or multiple layers. They are usually classified according to two features: the number of cell layers and the shape of the cells. Simple epithelia are single-cell layers where all the cells contact the underlying basal lamina and have an apical free surface. The shape of the cells can be flat (wider than high), cuboidal (as wide as high), or columnar (higher than wide). However, stratified epithelium contains two or more layers of cells.

The classification in nineteenth century was based on structure and physiological characteristics. In parallel, embryologists expanded the epithelial nomenclature with germ layer theory. During early embryogenesis, endoderm, mesoderm, and ectoderm emerge and maintain separate identities. Ectoderm creates the epithelia lining the skin, mouth, and nervous system. Parts such as digestive tract, respiratory system and liver are from endoderm. Mesoderm develops the endothelia which covers much of the circulatory and lymphatic systems. Our focus is simple epithelia monolayers. In upcoming chapter, we will look closely at the developmental processes of the epithelia.

## The mechanical basis of Morphogenesis

### Complexity of the morphogenesis

During embryonic development, epithelia form transient structures, such as the neural tube, somites, and the precardiac epithelium, that serve as progenitors for the development of more complex organs. Different epithelia acquire diverse morphological forms and performs their specific functions, such as the thyroid follicles, the kidney tubules, sinusoids in the liver, and the complex branching structures found in the lung and salivary glands (Gumbiner, B.M., 1992). Owing to its multifaceted regulation and hierarchical organization, epithelial morphogenesis is a complex phenomenon dependent on coordinated processes at multi spatial-temporal scales.

Some processes appear to be happening fast at local level like series of cells changing their shape by undergoing apical constrictions to create a global change in embryo, the ventral furrow in Drosophila gastrulation. However, at the same time you will find that the chemical signaling events activating this process are slow and at global level. Similar events are happening in vitro systems. For example, a cluster of dissociated mouse embryonic stem cells (ESCs) cultured in an appropriate medium assemble itself in an optic cup. It exhibits all the layers of neural retina (Eiraku et al., 2011; Bedzhov, I. & Zernicka-Goetz, M. 2014). Just like the drosophila embryo, this structure also undergoes local changes creating a global invagination in response to global signals of culture conditions. It gets even more complex when we consider the details of these processes. For example, molecular machinery involved in apical constriction, genes responding to the morphogen gradients, or mechanical stresses at the cells or tissue levels causing flows.

Rudolf Virchow’s third tenet of the cell theory stated ‘*omnis cellula e cellula’* meaning ’all cells come from cells’ (Virchow R.L.K., 1858). All tissues come from cells that contain essentially the same genetic information. Nonetheless, every tissue exhibits a distinct architecture and function. This raises many questions such as what makes cells different from each other? Is it all because of the genes? or environmental factors? What drives the shape changes in tissue morphogenesis? Since the advent of cell theory two centuries ago, the field of developmental biology has answered a lot of these questions alongside has created new debates and unanswered questions. However, the field has been focused on the studies tracking and mapping patterns of cell movements to patterns of gene or protein expression. These studies, while being greatly influential and important to understand morphogenetic patterns, are not enough to explain morphogenesis in itself (gorfinkiel2021, veenvliet2021, odell1981). Because physical understanding is only limited to the kinematic description, which is the deformation of the tissue or motion of the cells. As we know, the cell cytoskeleton and tissues are actively driving these shape changes and movements through generation of mechanical forces. Thus, to have an integrated grasp of morphogenesis, we must consider the role of forces and mechanics. It is a difficult task owing to the technical challenges for measuring forces and observing highly dynamic cellular processes.

### On growth and form

Historically, the form for animate and inanimate objects has been tied to the function. As per the twentieth century architecture principle of “Form Follows Function”; where the organization of a structure should be based upon its intended function. In developmental biology there are many examples of this principle at work as in self-assembling systems like intestinal organoids, cancerous spheroids, and functional kidney tissues (Gjorevski, N, et al. 2016; Ishiguro, T, et al. 2017; Morizane, R. and Bonventre, J.V., 2017). Each emerging out of a set of cells in appropriate environment changing and adapting itself to perform its biological function. However, exactly the opposite design principle is at work in numerous in vitro experiments with controlled cellular environment; illustrating geometric constraints drives biological function. For instance, in a micropatterned collagen scaffold (with structures of intestine) a human small intestinal epithelium was generated that replicates key features of the in vivo small intestine: a crypt-villus architecture with appropriate cell-lineage compartmentalization and an accessible luminal surface (Wang, Y et al., 2017, breau2022a). Or cell reprogramming like in case of fibroblasts turning into induced neurons when supported by specific substrate topography (Kulangara et al. 2014). Advanced microscopy techniques have enabled us to visualize the developmental processes with clarity. We see the cells coming together to organize themselves in an embryo and then radically grow to form an organism. For example, in the gastrulation process, we can see each cell and its motion starting from a spherical embryo to a fly or fish (Gopi Shah video). Cells undergo shape changes and large-scale flows to accomplish the task of morphogenesis. There must be mechanical forces driving these shape changes alongside the biochemical processes of gene patterning. The dichotomy of form and function is incomplete if we isolate it from the physical laws of mechanics. From a mechanical perspective, biology is all about beautiful forms which are growing and are in motion.

More than a hundred years ago, D’Arcy Wentworth Thompson published the classical text “On Growth and Form” (Thompson, 1917), where he tries to unravel the dialectics of morphogenesis by exploring the geometric and physical constraints on living entities during development and across evolution. He tries to ground biological processes in mathematics. Thompson catalogued instances where mathematics connects with biology. He put forth the controversial theory of transformations, which shows the differences between the forms of related species can be represented geometrically. A famous example of this is transforming one type of fish into another: consider *Argyropelecus olfersii* fish mapped onto a cartesian coordinate system can be transformed into *Sternoptyx diaphana* fish by shear transformation of the coordinate system. According to Thompson’s daughter, he used to entertain children by drawing pictures of dogs on rubber sheets and stretching them to make poodles into dachshunds (Wolfram blog). This distortion of the grid is supposed to be representative of significant alterations in various forces or rates of growth throughout the developmental processes of different organisms. The idea was to find generalized principles of different forms and patterns in biology. He illustrated this by comparing growth curves of haddock, trees, or tadpole; or by finding logarithmic spirals in shells, horns, and leaf arrangements. Funnily, He criticized the zoologists and morphologists of the time of assigning shapes to psychical instinct of the organism or some divine interference for creating the perfect shapes by saying

“He finds a simple geometric construction, for instance in the honeycomb structure, he would fain refer it to psychical instinct or design rather than in the operation of physical forces. ... When he sees in snail, or nautilus, or tiny foraminiferal or radiolarian shell a close approach to sphere or spiral, he is prone of old habit to believe that after all it is something more than a spiral or a sphere, and that in this "something more" there lies what neither mathematics nor physics can explain.”

He was also strong critic of the teleology, where the form can be explained because of its function. The intestine has large surface area because of its absorption function, or in evolution wolf has sharp teeth because of wolf’s dietary requirements. Even today in it is challenging to abstract oneself from teleology. Thompson has a special affinity to the physical explanation by saying,

’...to seek not for ends but for antecedents is the way of the physicists, who finds causes in what he has learned to recognize as fundamental properties, or inseparable concomitants, or unchanging laws, of matter and of energy.”

The key takeaways from the book are following:

1. The material forms of living things: cells, tissues, shell, bone, leaf, flowers must follow the laws of physics while undergoing whatever growth and deformation to produce a final form.
2. Quantitative measurement of forms and forces are key to unraveling physical principles of biology.

Thompson’s work has inspired many scientists from Alan Turing to Stephan J. Gould; me and my advisers. In last three decades, there has been a resurgence of interest in physical forces as regulator of development, homeostasis, and disease (Ingber, D. 2005; Barnes, J.M. et al., 2017). This has led researchers across the disciplines to examine the physical mechanisms of tissue formation and its regulation. Unravelling mechanism of Thompson’s mysteriously generated ‘Diagram of the forces’ which governs biological processes (Thompson, 1917; Heer, N.C. and Martin, A.C., 2017). The centenary of the publication was celebrated across the fields of developmental biology and physics. More so by the field of mechanobiology, an interdisciplinary field that studies the role of biophysical forces in cell and tissue functioning. Now more specific questions about shape and function can be answer using advances in bioengineering and microscopy.

### Mechanobiology

The field of mechanobiology has revealed that individual cells can sense their environment and respond by altering their behavior through mechanical or biochemical processes. Cells could change their shape, migrate, differentiate, proliferate, spread, divide, or deform. As mentioned earlier, cells have a symbiotic relationship with their environment to ensure homeostasis or morphogenesis. Many of these functions are tightly regulated. In simplification, one can imagine each cell in epithelial tissue as a mathematical system which integrates several input types to result in an output behavior. Such inputs cues can be chemical like soluble chemicals as in morphogen gradients in embryos, and cell-cell/cell-surface signaling molecules; or be mechanical – involving the generation-modification of intra- or intercellular forces as in apical constriction of ventral furrow formation; or external forces of tension/shear like breathing lungs (reference) and flowing blood through vasculature (Fenech, M., et al., 2019). While outputs could be cells shape change, migration, differentiation, or apoptosis. (Kumar, A. et al., 2017) (Figure reference). The mechanism for sensing the environment ‘mechanosensing’ is mediated by mechanochemical switches at cell-cell junctions or cell-matrix adhesions. This triggers the biochemical cascade leading to a response. This crosstalk between biochemistry and mechanics is ‘Mechanotransduction’.

However, at tissue scale we observe variety of events such as wound healing, collective migrations, oscillations, tissue rearrangement, or even turbulent flows. For example, epithelial tissues exhibit durotaxis in response to stiffness gradients. They sense the matrix rigidity and migrate from low to high stiffness. This occurs during neural crest migration in *Xenopus laevis* in vivo (Alkobtawi, M., et al., 2018). It also perceives cell crowding; and cells delaminate from those sites to ease higher intercellular tensions. It can be observed during homeostasis in the colon epithelia (Eisenhoffer, G. T. et al. 2012). Not just matrix affects the cells, but reverse is also true. In case of Drosophila oogenesis, disorganized matrix is remodeled into a polarized, restrictive matrix, which aligns with the actin bundles in the follicular epithelium through coordinated rotation of the cells (Cetera, M. et al. 2014; Haigo, S. L. & Bilder, D. 2011). These polarized fibers can in turn guide the directed motion of cells (Cetera, M. et al. 2014; Nam, K.-H. et al. 2016). In all these events, there is an interplay between individual cell, its neighbors, and exogenous stimuli. Thus, decoupling various biophysical aspects of environment, such as forces, pressures, matrix stiffness, spatial confinement, porosity, viscoelasticity, material degradability and binding affinity becomes challenging. Therefore, tight control over the experimental subject is needed to understand the biological processes. This has been achieved through continuous technological advancement in fluorescent probes, imaging, microfabrication, and mechanical measurement tools. Experiments are done across the scales from molecular to cellular scale; tissue to embryo scale; and explants to organ level to unveil physical principles for morphogenesis. Here I overview some of the relevant mechanobiological experiments and techniques.

#### Synthetic substrates

Synthetic 2D substrates are the most common ways of controlling material properties, protein density, and porosity (wen2014). Polyacrylamide and soft PDMS (Polydimethylsiloxane) gels have allowed researchers to investigate mechanical interactions at cell-substrate adhesion. For example, just seeding cells on different stiffness hydrogels reveals drastic effect on actin cytoskeleton (yeung2005) or on cell lineage specification (engler2006). Not limited to the control of matrix properties, it enabled us to measure forces through techniques like traction force microscopy (TFM). Every adherent cell exerts forces on its matrix. On sufficiently soft substrate, the force will create measurable deformations. TFM utilizes deformation of soft gels to calculate forces exerted on the substrate. Initially the technique was developed for single cells, but now it has been extended to the multicellular systems. These studies showed that cells and tissues can exert higher forces on the stiffer substrate because of remodeling of the cytoskeleton. Another aspect of these substrates is the ability to control the cell-ECM adhesion through coating specific ECM proteins and ligand density. Higher matrix stiffness has shown to induce Yes-associated protein (YAP) translocation from the cytoplasm to the nucleus. With control over ligand density, it is shown that increasing ECM ligand density alone can induce YAP nuclear translocation without changing substrate stiffness (stanton2019). Moreover, advanced microfabrication techniques like photolithography have allowed to create microscale topographical structures. These structures can be used as stamp to ECM patterns of adhesive or non-adhesive regions; or be used to confine tissues with a stencil. The micropatterning of cells is also possible through photopatterning of ECM protein. It provides precise control for coating multiple proteins on same substrate and establish a viable co-culture system.

Commonly, synthetic substrates like plastics (PET, PEGDA), hydrogels(Polyacrylamide, collagen gels), and elastomers (soft PDMS) are used to study mechanics because one can control rigidity, topography, and mechanical forces. They can be fabricated using basic chemistry and new microfabrication technologies like lithography. For example, 2D substrates are patterned with adhesive or non-adhesive ECM to control tissue/cell shape. This has revealed complex behavior in arrangement of epithelia in confinement (reference) and mechanism of force transmission throughout the tissue (reference). 2D Surface ECM patterning and topography also have helped in engineering 3D epithelial. Moreover, the integration of different techniques and materials further allows even higher levels of complexity in both two and three dimensions (Xia, Y. N. & Whitesides, G. M. 1998; Théry, M. 2010; Alom Ruiz, S. & Chen, C. S). For example, multiple proteins can be patterned on flat as well as 3D surfaces using UV patterning and stenciling (Ruprecht, V. et al.), to form adhesive patterns with spatially controlled adhesive strengths.

Taking one step further, using these microfabrication techniques we could directly create substrates with topographical cues. As most of the epithelia in vivo is three-dimensional, the curvature of epithelia is also key factor to control. Recently Lutolf lab used microfabricated elastomeric stamps to create intestinal epithelial tissues of controlled geometry with villus and crypt regions. They showed that the tissue adopts physiologically accurate patterning just through topographic control (gjorevski2022a).

Using such a techniques, collective cell migration under confinement (Vedula, S. R. K. et al., 2012; Xi, W., et al., 2017; Yevick, H. G., et al., 2015), collective durotaxis (Sunyer, R. et al., 2016), geometrical and stretching-controlled epithelial extrusion (Eisenhoffer, G. T. et al., 2012; Saw, T. B. et al. 2017) and epithelial growth regulation in two dimensions (Nelson, C. M. et al., 2005) and three dimensions (Salomon, J. et al., 2017) is studied, which led to a better understanding of cell-ECM interactions (Ladoux, B. & Mège, R.-M. 2017) and of tissue as active matter (Prost, J., Jülicher, F. & Joanny, J. F. 2015 ). In vitro studies have elucidated the effects of multiple mechanical cues of the microenvironment on cellular behavior, and insights into tissue mechanics have promoted the development of new materials and design methods for bioengineering, in vitro modelling, immunotherapy, and gene therapy.

#### External perturbation

Living systems do not just have spatial control, they also have mechanical control through physical forces emerging out of growth, deformation and remodeling of ECM, and flow or arrest of fluids in closed geometries (reference). In our body, intestinal epithelia are stretched during peristaltic movements in the gut and lung alveoli deform during breathing (Ethier CR, Simmons CA, 2007). Not just tension, compression is known to guide several morphogenetic events involving tissue bending and folding, such as the formation of the optic cup (Sidhaye, J. & Norden, C. 2017), gut villi (Shyer, A. E. et al., 2013), and cortical convolutions in the brain (Tallinen, T. et al. 2016). To understand tissue behavior under external perturbation, cells and tissues are probed at the molecular and subcellular scales using, atomic force microscopy (AFM) (Emad, A. et al. 1998), magnetic beads (Broders-Bondon, F., et al, 2018), optical tweezers (Bambardekar, K., et al., 2015), and micropipettes (Evans, E. & Yeung, A. 1989); and at larger scale using stretching devices, tissue rheometers and force plates (Huh, D. et al 2010; Gudipaty, S. A. et al. 2017). These tools are useful in characterizing mechanical properties of cells and tissues as they provide with kinematic and force data. These experiments reveal viscoelastic behavior at different regimes of deformation involving different parts of cytoskeleton. It has been shown that the cells in a tissue reorganize or stretch depending on the timescale of the stretch (reference). Also, they elucidate the role of signaling pathways in terms of transcription factors like YAP involved in mechanosensing (reference).

In developing embryo, embryonic and extraembryonic fluids generate forces: frictional and tensional stresses when flowing (Freund et al.,2012), or hydrostatic pressures when in confined spaces (Navis and Bagnat, 2015). Micropipette experiments revealed that internal hydrostatic pressure determines embryonic size and directs allocation of fates by affecting allocation to internal or external compartments (Chan et al., 2019). As a fluid filled structure, imagine a balloon, the hydrostatic pressure inside relates to tension in the surfaces. Any changes in luminal volumes are sensed by cells through the increased cortical tension, which in turn induces changes in cell shape and cytoskeletal organization (Chan et al., 2019; Latorre et al., 2018).

As mentioned earlier, the tissue-matrix interaction is playing a critical role in sensing and transmitting forces rapidly (Tambe, Dhananjay T., et al. 2011; Sunyer, R., et al., 2016; Serra-Picamal, X., et al., 2012). However, in early embryonic epithelia where little or no ECM is present, stresses generated by actomyosin contraction of the cells in one tissue are transmitted over long ranges via intercellular adhesions to other tissues. Thus, the system of studying just simple free-standing epithelial monolayer is very appealing in terms of characterizing mechanical response to stretch at different time scales. Harris and colleagues created suspended monolayer by culturing cell monolayer on collagen matrix on two rods; later matrix was removed using enzymatic digestion. These experiments showed that elasticity measurements of monolayer were two orders of magnitude larger than that of individual cellular parts, and monolayer could sustain more than 200% strain before rupture of cell-cell junction. Moreover, imaging of cell cytoskeleton showed that the acto-myosin network and cadherin junctions actively remodel during stretch, also keratin network reinforces the monolayer integrity at higher strains. A significant rearrangement and realignment of cells is observed.

In purview of cell mechanics, microfluidic system—cells on chip— has been proven as an outstanding tool for mimicking in vivo conditions and controlling biophysical cues; enabling us to study cell behavior (Vanapalli SA., et al, 2009). It was a useful way for applying shear forces or create a controlled microenvironment. Recent advances in the culture of cells within 3D contexts has enabled us to begin to model more complex behaviors and tissue functions (Eyckmans, J. and Chen, C.S., 2017). To recreate organ level physical cues, like surface tension at an air-liquid interface in the case of the lungs involved, as well as both fluid flow through the vasculature and cyclic mechanical stretch of the tissue-tissue interface due to breathing motions (Huh D., et al., 2010). In developmental biology, culturing iPSC-derived motoneurons and brain microvascular endothelial cells together on a similar chip produced the neuromuscular unit with significantly enhanced function and in vivo-like maturation of spinal cord neural tissue (Sances et al., 2018). This opens new opportunities to in developing current view of self-organization and embryo functions with controlled physical conditions.

## Active tissue mechanics

Advances in experimental technologies and better-resolved spatial-temporal measurements provide more detail view of tissue function and its complex physical and molecular underpinnings. Similarly, advances in engineering and mathematical models allows for exploration of hidden physical phenomenon; along with aiding experimentalists to perform physically motivated experiments. The strong interdependence between mechanical and molecular signals makes it difficult to interpret the relative contribution of different cues to the shaping of epithelial tissues. Mathematical models can help to disentangle this complex relationship.

### Historical context

There is a history to mathematical modelling of epithelia. Going back to D’Arcy Thompson, looking at cell aggregates he invokes

Alan Turing put forth his theory of reaction-diffusion of two chemicals. It showed that interaction of two chemicals can generate intriguing spatial patterns in his paper “The chemical basis of morphogenesis” (Turing, A. 1952). These patterns, Turing patterns, can be seen in leopard’s spots, butterfly’s wings, or fish’s scales (Figure reference). Well now, with much more information of physical forces involved in biological process one can start thinking about mechanochemical basis (Howard, J., Grill, S.W. and Bois, J.S., 2011).

There are mathematical models describing physics and biology at multiple scale. There are hyperelastic continuum material models at larger tissue scales, for example, describing behavior of cardiovascular system (reference). There are active fluid models at relatively smaller scales explaining epithelial tissue behavior in systems such as active dewetting or drosophila wing development (reference).

#### Actin cytoskeleton and internal force generation

Any response to biophysical cues is mainly manifested as physical change in the cell which in turn shows up in tissue. The change in shape is related to mechanical properties of the cell, which are largely defined by the actin cytoskeleton. Actin cytoskeleton is a highly polymorphic and multifunctional cellular system that consists of actin filaments (F-actin) organized into various higher-order arrays capable of dynamic remodeling (Svitkina, T. 2018). Its most important function is to generate force. For pushing, actin filaments typically assemble into branched networks assembled by the Arp2/3 complex (Svitkina, T.M. and Borisy, G.G. 1999), while, for contraction, they form composite networks or bundles with bipolar filaments of myosin II (Verkhovsky, A.B. et al. 1995). These generated forces can also propagate through the tissue through cell–cell junctions which form dynamic cellular contacts and the substrate (Halbleib, J. M. & Nelson, W. J. 2006; Sunyer, R., et al, 2016). Cell–matrix and cell–cell junctions are under constant mechanical stress (Maruthamuthu, V., et al., 2011), which causes remodeling of the junctions (Mège, R. M. & Ishiyama, N. 2017) and triggers cell signaling events within the tissue (Ladoux, B. & Mège, R.-M. 2017). To summarize, any ‘input cues’ start a cascade of biochemical reactions from ‘mechanosensors’ to cytoskeleton resulting in ‘output response’ through contraction/extension. This process is called as ‘mechanotransduction’ (Harris, A.R., et al., 2018).

#### Continuum models

Fortunately, as anticipated by Descartes and other mechanists, soft tissues respect the basic postulates of mechanics (e.g., conservation of mass, momentum, and energy), and basic concepts such as stress, strain and entropic elasticity apply as well. Hence, much of continuum models focuses on the formulation of reliable constitutive relations and then on the solution of initial-boundary-value problems. Constitutive relations describe the response of a material to applied loads, which depends on the internal constitution of the material. Because of the incredible complexity of both the ultrastructure and microstructure of these materials researchers continue to rely primarily on phenomenological descriptors of the behaviors of interest, descriptors that are often motivated by the knowledge of the underlying structure.

The uniaxial/biaxial tension and compression testing has been able to provide insights into the constitutive relation of the tissues. In these experiments, one measures deformation in terms of strain tensors, like green’s strain tensor. Where, F is a deformation gradient tensor. Here, in the theoretical framework of finite elasticity, one can assume a strain energy function (W) relates to stress (S). The stress-strain data extracted from the experiment allows to predict the form of strain energy function. In case of bladder, heart tissue, skin, and arteries, hyperelastic form has been useful in capturing the material response (Fung YC, 1990; Humphrey JD, 2002). This kind of formulation is also flexible in adding extra physical constraints as anisotropy of the tissue microstructure or its incompressibility. Borrowing from composite materials, transversely isotropic material models have been instrumental in understanding mechanism of myocardium infarction and various aneurysms (add references from Humphrey, Holzapfel and Ogden). Slight modification to these constitutive relations could capture material response, such as explaining strain stiffening, or inhomogeneity in the material like accounting for collagen content and crosslinking in the tissue (Holzapfel, G.A. and Ogden, R.W., 2020). These models are also used in understanding growth and remodeling with using the theory of kinematical growth. It has pointed out existence of residual stresses in growing tissue to make compatible elastic and inelastic growth-induced deformations, which in turn remodel the tissue properties modifying the material into a spatially inhomogeneous and anisotropic one. This process is crucial in solid tumor growth mechanobiology, the residual stresses directly influencing tumor aggressiveness, nutrients walkway, necrosis, and angiogenesis (Carotenuto, A.R., et al., 2019). The nonlinear continuum models have also been used in mechanical modelling of the brain tissue. As an ultra-soft and heterogeneous material, researchers can model its behavior in high strain rate scale of brain trauma to slow growth in developmental stages (Budday, S., et al., 2019).

#### Vertex models

Monolayered epithelial tissue shows a polygonal cellular pattern on its surface. This enables to describe/track cell motion and shape change easily in terms of vertices and edges (Figure reference). Vertex models have been developed to understand the complex interplay between cellular shape, the forces generated inside epithelial cells and mechanical constraints externally imposed on the tissue. Models could be 2D or 3D depending on the system being modelled, but cells are consistently defined as each one has apical and basal surface, and lateral interfaces between neighbours. This enables one to model for cell polarities in either direction. More complexities have been added to describe specific systems like intercalations in 3D epithelia using geometric shape as Scutoid (Gómez-Gálvez, P., et al., 2018) (Figure reference). The molecular and mechanical details are added in formulation of mechanical description. In mechanics to determine the motion of vertex, mechanical forces must be specified. It is done using virtual work function (W); and forces can be obtained by differentiating with vertex position. There are two components to W: internal and external. The Changes in internal virtual work (dWi) can result from changes in the cell volumes, in the areas of surfaces, or in the lengths of bonds. By defining the cell pressure, the surface tension and the line tensions, the differential of the internal virtual work for vertex movements can be written.

Similarly, the external virtual work can be written external forces that arise from external compression or tension applied to the tissue, fluid pressure acting on apical or basal cell surfaces, or attachment of cells to the underlying basement membrane.

The state of the monolayer is estimated by minimizing the virtual work function (Alt, S., Ganguly, P. and Salbreux, G., 2017). Here, molecular details are incorporated in the changes of the surface tension and line tensions (Figure reference). In epithelial layers, the actin cortex plays a huge role in determining tensions along the edges (ref). This has been very useful in capturing physics in systems starting from cell shape distribution and cell packing to appendage formation and growth of epithelial vesicles (Staple DB, et al., 2010; Osterfield M, et al., 2013; Okuda S., et al., 2015).

#### Active surfaces

At cellular scale, we know that the mechanical properties of the tissue are controlled by the biopolymeric system called cytoskeleton. Filaments and their cross-linkers (molecular motors) continuously transduce energy (ATP to ADP) to contract or extend the network. This system can be considered as a physical gel because of its cross-linked actin filament network. But phenomenon like treadmilling, active polymerization-depolymerization of filaments, and mobility of molecular motors like myosin makes the tissue system an active gel. Also, to note that cellular system lacks time reversal symmetry because it is constantly transducing energy. Moreover, these filaments are polar, as constituents can acquire orientational order. Thus, one can model tissues as active gels; are used to model active systems like flocks for birds and schools of fish using hydrodynamics of active matter (Toner. J. et al., 2005). Active matter systems are sub class of continuum models used to describe dynamics of packed active particles; based on liquid crystal theories of soft condensed matter. Like liquid crystals cells too have orientation and ability to move past each other. In this framework, one characterizes the orientation of filaments in cytoskeleton or elongation of cells in the tissue by nematic order parameter matrix (Q). Where n is a unit vector indicating local average orientation axis, S is the scalar order parameter and theta is the angle of each element with the n vector. This formulation aids in defining active forces generated by the network. The stress is divided in two parts: active and passive. Where passive stress will be arising from mesoscopic viscoelasticity of the material and from the bending, splaying, and twisting of the aligned agents. Active stresses are obtained using combination of zeta, strength of activity, and nematic order matrix. Zeta’s sign determines the nature of the force dipole. If negative, system contracts; if positive, system expands along the nematic axis. For example, actomyosin systems are contractile. Active stress is very crucial for the motion of the system. Even in low Reynold’s number systems, motion could get chaotic. In a dense bacterial system of Bacillus subtilis, jet flows and turbulent flow patterns were observed (Wensink, H.H., et al., 2012). Also, independent vortices have been observed in the expanding monolayers (Saw, T.B., et al., 2015). Nematic equations have capture physics very well in 2D confined systems or expanding systems. For 3D, active surfaces are used. The actomyosin cortex near cell membrane or epithelium in the embryo is like a thin sheet of matter, which drives shape changes at cellular or tissue level by causing deformation due to the generation of internal forces and torques. These three-dimensional structures resemble a curved active two-dimensional surface. The framework developed for active matter can be used by applying mathematical tools from differential geometry. The curved surface is defined in generalized coordinates X. The metric tensor g and curvature tensor C are used to describe the kinematics. And forces and torques are defined as, Where, dl is length of the line element with tangential unit vector v normal to the line, and t and m are tension and moment respectively. Total torque is, The force balance expression becomes

These equations can be used with constitutive equations for specific cases. For example, Salbreux and Julicher show that flat active Helfrich membrane with up-down asymmetry have its stability dependent on active tension and active tension-curvature coupling term. They find that the active flat surface undergoes shape instabilities in two cases. First, classical buckling instability occurring when active stresses are compressive and establish a negative surface tension in the membrane. Second, active buckling instability occurs when tension is dependent on curvature, Because of this dependency, a perturbation of the surface shape results in regions of low and high surface tension. These surface tension differences lead to flows towards region of higher tension. These flows generate further in-plane torques causing further deformation of the surface (Salbreux, G. and Jülicher, F., 2017). This tension-curvature dependency can be seen in the pancreas of mice that the morphology of epithelial tumors is determined by the interplay of cytoskeletal changes in transformed cells and the existing tubular geometry (Messal, H.A., et al., 2019). Consistent with theory predictions: small pancreatic ducts produced exophytic growth, whereas large ducts deformed endophytically. Another example shows that curls of high curvature form spontaneously at the free edge of suspended epithelial monolayers. It is pointed that the curling originates from an enrichment of myosin in the basal domain that generates an active spontaneous curvature. It was shown that the extent of curling is controlled by the interplay between internal stresses in the monolayer (Fouchard, J., et al., 2020).

#### Rheological measurements

Epithelial tissues are much complicated materials than that of simple metals or polymers. However, the complex biological behavior can be connected to mechanical response using basic material testing techniques (reference). Typically, it is done by probing the material mechanically and then recording its deformation, or by deforming the material and recording force response. In mechanical terms, one records material behavior with stress (force) strain (deformation) relation, often called as constitutive relation and can be quantified in term of passive and active rheological properties (reference). This depends on the type of deformation as in shear, tension, or compression and is also multiaxial like in composite materials (reference). The rheological properties often correlate with their physiological state and are crucial for their specific functions (Park, J.-A. et al. 2015; Vedula, S. R. K. et al. 2015, Vedula, S. R. K. et al. 2014). For example, Heart failure is often due to the loss of contractility of heart muscle cells; as it is observed in remodeling under mechanical stretch (Fung YC, 1990). So, one must test rheological properties along with different microenvironment. The mechanical information include deformation, rates of deformation or velocity fields, traction forces exerted by cells on the substrate and intercellular mechanical stress. Coupling these parameters with information on cellular architecture, obtained by imaging, can provide a mechanistic understanding of tissue rheology (Roca-Cusachs, P., Conte, V. & Trepat, X. 2017). This has been done at the molecular and subcellular scales using, atomic force microscopy (AFM) (Emad, A. et al. 1998), magnetic beads (Broders-Bondon, F., et al, 2018), optical tweezers (Bambardekar, K., et al., 2015), and micropipettes (Evans, E. & Yeung, A. 1989); and at larger scale using stretching devices, tissue rheometers and force plates (Huh, D. et al 2010; Gudipaty, S. A. et al. 2017). This reveals complex mechanism of strain stiffening and viscoelastic behavior at different regimes of deformation involving different parts of cytoskeleton. Also, elucidated the signaling pathways in terms of transcription factors like YAP involved in mechanosensing (reference).

## Bottom-up morphogenesis

Bottom doesn’t explain the top

Biology and engineering darcy thompson

Biology can help to create new ideas. Biomimicking like velcros and seashells

Spontaneous pattern formation and mechanical instabilties. Splashes of fluids analogous to plants. This has been confirmed through quantitative studies. HE was speculative but now it has been shown quantitively. Gut, brain, shapes of plants and non euclidean plates. This understanding leads to material

Speculation of classification beaks and shells.

What Is to Be Done? Burning Questions of Our Movement

It is very clear that the mechanics and biology of the epithelial tissues is complicated; intertwined by mechano-chemical signaling; and multiscale in its behavior. The lens of active material has been very helpful in providing information on role of molecular elements in performing biological function. These studies have also led to observe emergent behaviors which would be impossible to see in vivo. The mechanistic understanding has been enhanced with newer mathematical tools and advanced microscopy; enabling us to measure forces involved in the tissues.

Biomimetics? What remains?

Engineering in biological systems have pushed our limits in understanding physiological response, morphogenesis, and pathologies. However, engineers are not just attracted to this subject for its application in health and disease, but for its potential to inspire constructions of new material or engineering systems. At many instances, where new materials have been fabricated inspired by biological matter. Also, improved understanding of biological systems has provided new methods for creating organically optimized systems. Biomimetics is a field where nature continuously inspires human innovation: from hydrophobic surfaces to supersonic passenger plane! Here, epithelial tissue has displayed incredible capabilities such as self-assembly, self-healing, and self-replicating. This makes it a very interesting material for engineers as its study provides new ideas in strength, adaptability, flexibility, and functionality aspects of the material.

What we want to do?

The principles which govern tissue form and function are very important; on two fronts. First, to understand fundamental physical rules of the biology, and secondly for inspiration of new engineering tools and design principles. We want to use state of the art technologies such as bioprinting, microfluidics, and 3D cell cultures to control morphogenetic driving factors individually; giving us opportunity to test tissues from material science point of view. This specific probing allows us to comprehend intricate mechanism of generation of forces, and shape change at cellular and tissue level. Using microfluidic setup, we subject tissues to unravel emergent phenomenon at different spatial and temporal scales.

This is a process of deformation or growth of the tissue under the combination of endogenous and exogenous mechanical forces that include contractility of the epithelium itself and of the surrounding matrix as well as hydraulic pressure from the lumen. These stresses are applied on different material components of the tissues, such as cells and the extracellular matrix, that display distinct viscoelastic properties and remodeling time scales. Understanding how the complex interplay between tissue stresses and viscoelastic properties gives rise to specific morphogenetic events in vivo poses outstanding technical and conceptual challenges. These include difficulties to disentangle the relative role of the distinct components involved in a system, the lack of tools for quantitative measurements of stresses and mechanical properties, and the inability to impose controlled stresses over a broad range of amplitudes and rates. As a complementary strategy, bottom-up approaches aim at understanding the role of each component of the system and its morphogenetic potential, with the ultimate goal of building complexity through rational engineering of the building blocks that form a functional tissue. These approaches have been successful at engineering elementary morphogenetic processes such as epithelial bending or buckling. However, despite the emerging success of bottom-up approaches, we still lack tools to simultaneously measure and control the shape and stress of 3D epithelia. In addition, we lack computational models that integrate cellular and tissue shape with the subcellular determinants of epithelial mechanics such as the contractility, turnover and viscoelasticity of the actomyosin cortex.

Here we present a microfluidic-based technique to impose a controlled deformation on an epithelial monolayer while continuously monitoring its state of stress. With this technique we probe the active viscoelasticity of epithelial layers over a range of physiological time scales. We present a 3D model of the epithelium, which shows that the observed phenomenology can be explained by the active viscoelastic properties of the actomyosin cortex. Finally, we show that these viscoelastic properties combined with adhesion micropatterning can be harnessed to engineer epithelial wrinkles of predicted geometry.