

## **Part I**

# **Introduction and motivation**



# Chapter 1

## Epithelial Layers

### 1.1 Introduction



**Figure 1.1:** The Anatomy Lesson of Dr. Frederik Ruysch, 1670 by Adriaen Backer (Adriaen Backer Wikipedia, 1670)

The term “epithelia” was first introduced by Dutch botanist Frederick Ruysch in the early 18th century (see fig 1.1). He used it to describe the tissue he observed while dissecting the lips of a cadaver, and the word is derived from Greek roots “*epi*,” meaning top, and “*thele*,” meaning nipple.<sup>1</sup> A few decades later, Swiss scientist Albrecht von Haller began using the term “epithelium/epithelia” to describe the fibers of the body, following the old Renaissance

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<sup>1</sup>Ruysch is referred to as a “Artist of death” because of his famous anatomical collection. He was the first to use arterial embalming, which allowed for visualizing and dissecting smallest arteries. He also was part of the macabre practice of public dissections (Halley, 2019).

theory that the body was made of fibers, which were believed to be a fundamental building block of living things.<sup>2</sup> It was thought that these fibers and tissues arranged in different arrays gave rise to biological structures (MacCord, 2012, Zampieri *et al.*, 2014). This theory was not far off, as epithelial tissues make up more than 60% of the cells in a vertebrate's body and are found ubiquitously, covering the organs both inside and out (Alberts, 2015).

Epithelial cells are polarized, i.e., their apical side (typically facing the lumen of the organ), which differs in shape and composition from the basolateral side. Its polar organization is reflected in the vectorial functions like creating and maintaining concentration gradients between separated compartments (Marchiando *et al.*, 2010). 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 (Alberts, 2015). In addition, polarized epithelia guide the developmental process by determining the fate of cells leading to symmetry-breaking events in the embryo (Kim *et al.*, 2018).

## 1.2 Key components

The function of epithelia primarily depends on the tissue's structure and the surrounding microenvironment. It can be divided into three aspects: cell structure, microenvironment, and cell-matrix interactions.

### 1.2.1 Cell structure

The cell cytoskeleton plays a crucial role in maintaining cell shape and supporting vital functions such as cell division and migration (Alberts, 2015). The Eukaryotic cell cytoskeleton is composed primarily of filamentous proteins, including three main types of filaments that differ in size and protein composition: microtubules, actin filaments, and intermediate filaments (see fig 1.2). Microtubules, with a diameter of approximately 25 nm, are the largest and made of the protein tubulin. Actin filaments, with a diameter of only 6 nm, are the smallest. Intermediate filaments, with a diameter of around 10 nm, are composed of several different subunit proteins and have a diameter intermediate between the other two types (Mofrad, 2009). All three filament types dynamically respond to signals from the microenvironment and cell networks.

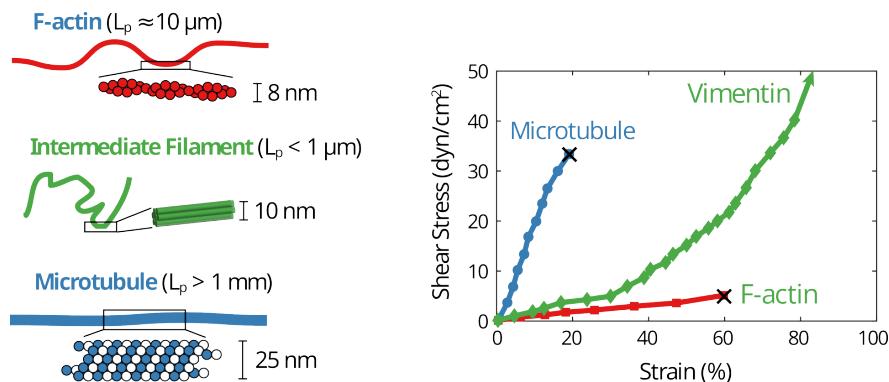
Mechanically, actin filaments have higher extensional stiffness than microtubules but break at lower extensions. Intermediate filaments have intermediate extensional stiffness and can sustain larger extensions while showing a nonlinear stiffening response (Wen and Janmey, 2011). Differences in strength and stability arise from the properties of individual subunits. The persistence length can range from 1 $\mu$ m for intermediate filaments to 1mm for microtubules

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<sup>2</sup>Finding a fundamental unit of living entities comes from the philosophy of Gottfried W. Leibniz. It was based on the idea of "monad". Thanks to progress in microscopy and philosophy, naturalists were able to put together ideas for cells, fibers, and even cytoskeleton!(Zampieri *et al.*, 2014)

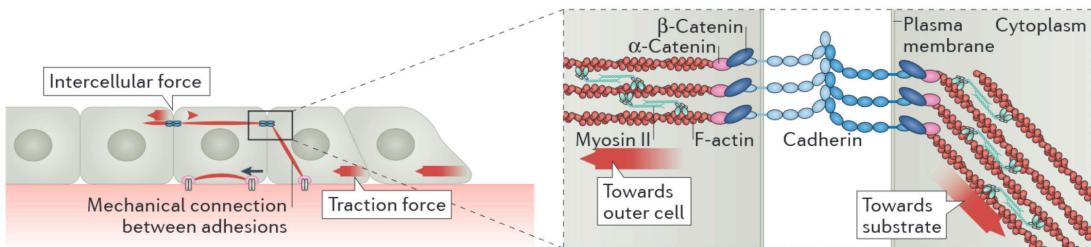
(Fletcher and Mullins, 2010). Actin filaments, most relevant to this thesis, have a persistence length of a few microns.

The assembly and disassembly of these filaments are dictated by the dynamics of their macromolecular components and accompanying proteins. The combination of actin filaments and myosin motors forms the actomyosin cortex, which is essential in producing intra- and intercellular forces. In an epithelial tissue, the actomyosin cortex and intercellular junctions make cell-to-cell contacts stronger and provide tissue integrity (Braga, 2016) (see fig 1.3). A good example of these tissue-level structures can be observed in wound healing assays, where cells surrounding the wound create a ring of actin to close it (Brugués *et al.*, 2014). In Chapter 3, we will delve into the actomyosin network in more detail.



**Figure 1.2: Mechanics of cytoskeletal filaments:** Schematic and sizes of actin filaments, intermediate filaments and microtubules; along with the strain response to shear stress. *Adapted from (Leggett et al., 2021)*

Multiple membrane molecules can facilitate cell adhesion, including cadherins. Cadherins are a crucial component for epithelial cell cohesion and the formation of adherens junctions, which transmit forces between cells. This key factor is involved in the mechanical regulation of cell division and tissue rearrangement during development and homeostasis (Godard and Heisenberg, 2019, Mertz *et al.*, 2013). Desmosomes, another type of intercellular junction, are coupled with intermediate filaments and provide mechanical resilience to cell layers (Hatzfeld *et al.*, 2017, Latorre *et al.*, 2018). Tight junctions serve as a barrier and regulate the active transport of ions across epithelial layers, playing an important role in controlling fluid pressure in tissues (Chan and Hiiragi, 2020, Marchiando *et al.*, 2010).



**Figure 1.3: Intercellular forces through actomyosin cables and cadherins:** Schematic showing mechanical connections between adhesions and tissue force transmission with actomyosin cytoskeleton and adhesion proteins. *Adapted from (Ladoux and Mége, 2017)*

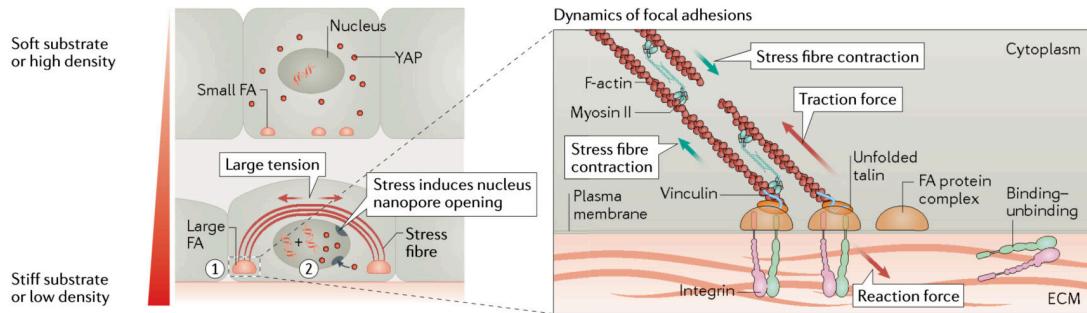
### 1.2.2 Microenvironment

The extracellular matrix (ECM) is the substrate or cell environment to which cells adhere. It is also referred to as the matrix, mesenchyme, or cellular microenvironment. The ECM serves many functions. It endows tissues with strength, thereby maintaining their shape. Additionally, it serves as a biologically active scaffolding that allows cells to migrate or adhere. The ECM also plays a role in regulating the phenotype of cells. It provides an aqueous environment that facilitates the diffusion of nutrients, ions, hormones, and metabolites between the cell and the capillary network (Alberts, 2015).

Moreover, the ECM 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 (Walma and Yamada, 2020, Waters *et al.*, 2012). It has been shown that the ECM regulates cell shape, orientation, movement, and overall function in response to biophysical forces (Alberts, 2015).

The ECM is a fibrous network of proteins, consisting of collagen, elastin, and proteoglycans as its primary structural components. Collagen is one of the most abundant proteins in the body, while elastin is the most elastic and chemically stable protein. Proteoglycans can sequester significant water as well as growth factors and proteases. The water content of the ECM allows it to deform as a poroelastic material, absorbing water upon stretching and releasing it under compression, causing a hydraulic fracture effect (Casares *et al.*, 2015). The collagen network can also remodel under the influence of cells and mechanical forces (Humphrey *et al.*, 2014).

Most ECM components undergo continuous turnover, some quickly and some slowly. For example, the half-life of collagen in the periodontal ligament is a few days, whereas that in the vasculature may be several months (Humphrey *et al.*, 2014). In response to altered physical stimuli, disease, or injury, the rates of collagen synthesis and degradation can increase many times, allowing for a rapid response.



**Figure 1.4: Cell-matrix interaction with respect to matrix stiffness and cell density:** In higher tension condition, the nucleus is deformed triggering mechanotransduction and causing alterations in cytoskeleton and tractions. *Adapted from (Xi et al., 2018)*

### 1.2.3 Cell-Matrix interaction

The cells and the extracellular matrix (ECM) are in a dynamic relationship, constantly exchanging information and influencing each other. The cells sense the biophysical cues in the ECM through sensors such as integrins and focal adhesion complexes, which are responsible for cell-substrate adhesion (Kechagia *et al.*, 2019) (see fig 1.4). These adhesions allow cells to respond to various stimuli such as matrix stiffness, ligand density, and chemotactic gradients (Fortunato and Sunyer, 2022). It has also been shown that cells can respond to the viscoelasticity of the matrix (Elosegui-Artola *et al.*, 2022).

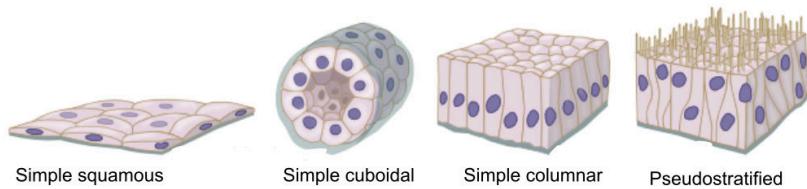
In addition to sensing the ECM, cells also contribute to its composition by secreting ECM components or remodeling the substrate (Malandrino *et al.*, 2018). This interplay between the cells and ECM can impact the tissue behavior fundamentally, as the connections between focal adhesions and the nucleus can affect the expression of transcriptional factors (Lomakin *et al.*, 2020, Venturini *et al.*, 2020). The precise control of cell-cell and cell-substrate interactions enables cells to transform into intricate shapes, such as curved forms in cell sheets (Schamberger *et al.*, 2022).

## 1.3 Forms of epithelia

The structure and arrangement of epithelial cells are crucial for maintaining the integrity and homeostasis of tissues and organs (see fig 1.5). Simple epithelia are single-cell layers where all cells are in contact with the underlying basal lamina and have a free surface on the apical side. The shape of the cells can vary, ranging from flat to cuboidal to columnar. Stratified epithelia, on the other hand, have two or more layers of cells. Additionally, there are pseudostratified epithelia, which appear to be stratified, but are monolayers where the cell nuclei are positioned in a manner that gives the appearance of a stratified epithelium.

The classification of epithelia was first established in the XIXth century based on their structure and physiological characteristics. Germ layer theory, developed by embryologists, further expanded the epithelial nomenclature (MacCord, 2012). During early embryogenesis, three layers emerge: endoderm, mesoderm, and ectoderm. The ectoderm forms the epithelia lining the skin, mouth, and nervous system, while the endoderm gives rise to the digestive tract, respiratory system, and liver. The mesoderm, in turn, develops the endothelia covering much of the circulatory and lymphatic systems.

It is important to note that not all tissues classified as epithelia, mentioned in this thesis, are purely composed of epithelial cells. They may be a mixture of different cell types that have epithelial-like characteristics. The focus of this thesis is on packed cell monolayers, which can form and self-organize into various 3D shapes, ranging from simple spheres to complex branched tubules. The thesis will explore the role of mechanics in epithelial morphogenesis.



**Figure 1.5: Forms of epithelial tissues:** Simple squamous, cuboidal, columnar epithelia and pseudostratified epithelia. *Adapted from (zot)*

## Chapter 2

# The mechanical basis of Morphogenesis

### 2.1 The complexity of the morphogenesis

Epithelial cells play a crucial role in the formation of transient structures during embryonic development, such as the neural tube, somites, and precardiac epithelium, which serve as the precursor for the development of complex organs. During this process, different types of epithelia acquire distinct morphological forms and perform specific functions, including branched lungs, looped gut, kidney tubules, thyroid follicles, and sinusoids in the liver. The regulation of epithelial morphogenesis is a complex and hierarchical process that involves coordinated events at multiple spatial and temporal scales (Trepat and Sahai, 2018).

Some processes appear to be happening fast at the local level, such as cell shape changes through apical constrictions, which lead to global changes, such as the formation of a ventral furrow in a *Drosophila* embryo (Martin *et al.*, 2009). At the same time, chemical signaling events that activate these processes are slow and occur at a global level. The same complexity can be seen in *in vitro* systems, where a cluster of dissociated stem cells can assemble into an organoid or gastruloid and undergo global folds in response to appropriate culture conditions (Collinet and Lecuit, 2021).

The underlying mechanisms of epithelial morphogenesis are intricate and involve multiple factors, including genes responding to morphogen gradients, molecular machinery involved in apical constriction, and mechanical stresses that cause tissue-scale deformations. To fully understand the phenomenon of epithelial morphogenesis, it is essential to study these processes in detail, at multiple levels of complexity (Lecuit *et al.*, 2011, Schöck and Perrimon, 2002).

Rudolf Virchow's third tenet of the cell theory states that "omnis cellula e cellula," meaning

“all cells come from cells” (Virchow *et al.*, 1860). <sup>1</sup> Although all tissues originate from cells that contain essentially the same genetic information, each tissue has a distinct architecture and function. This raises several questions, such as: what makes cells different from each other? Are differences due to genes, environmental factors, or both? What drives shape changes in tissue morphogenesis? Over the last two centuries, the field of developmental biology has addressed many of these questions, but it has also raised new issues and left others unanswered.

Until last decade, the focus of the field had been on tracking and mapping patterns of cell movements to patterns of gene or protein expression (Gorfinkel and Martinez Arias, 2021). While these studies are influential and important for understanding morphogenetic patterns, they fall short in explaining how cells and tissues are physically shaped (Odell *et al.*, 1981, Veenvliet *et al.*, 2021). This is because the physical understanding of tissues has been limited to kinematic descriptions, which only describe tissue deformation or cell motion. However, we know that cells and tissues actively drive shape changes and movements through the generation of mechanical forces (Lecuit *et al.*, 2011). Thus, to have an integrated understanding of morphogenesis, we must consider the role of forces and mechanics.

## 2.2 On growth and form

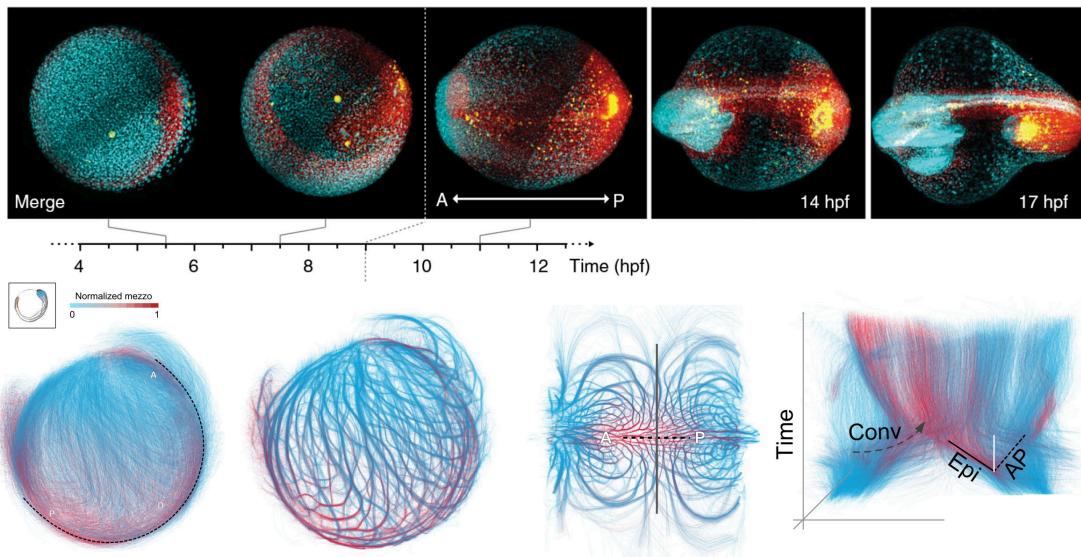
Throughout history, the form of both animate and inanimate objects has been closely linked to their intended function. In fact, the XXth century architecture principle “form follows function” highlights the idea that the organization of a structure should be based on its intended purpose. Similarly, in developmental biology, self-assembling systems such as intestinal organoids, cancer spheroids, and gastruloids are perfect examples of this principle in action, as each structure emerges from a set of cells in a suitable environment, adapting to perform a specific biological function (Gjorevski *et al.*, 2016, Ishiguro *et al.*, 2017, Morizane and Bonventre, 2017, Vianello and Lutolf, 2019).

However, the opposite design principle appears to be at work in numerous *in vitro* experiments that involve a controlled cellular environment. In such experiments, geometric constraints appear to drive biological function (Xi *et al.*, 2018). For instance, seeding stem cells in a bio-printed three-dimensional geometry of the gastrointestinal tract led to the production of functional tissues with physiological characteristics of the intestine. The curvature of the structure can even control the formation of villus-like structures (Brassard *et al.*, 2021).

In a way, assembly of biological systems treads the line between self-organization and programmed material. Advanced microscopy techniques have allowed us to witness the intricacies of developmental processes with unprecedented clarity (see fig 2.1). We can now

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<sup>1</sup>The famous epigram was coined by François-Vincent Raspail. Virchow is regarded as influential biomedical scientist of 19th century, but more interesting part is as a radical who took part in the March revolution of 1848. He was one of the first to advocate for the social origins of illness (Brown and Fee, 2006, Wright and Poulsom, 2012).



**Figure 2.1: Multiscale imaging and tracking of embryo cell dynamics:** Top panels show *in toto* imaging of germlayer specification; red is mesendoderm, blue is epiblast, and yellow is endoderm. Bottom panel shows data analysis of long term pan embryo cell dynamics (Shah *et al.*, 2019)

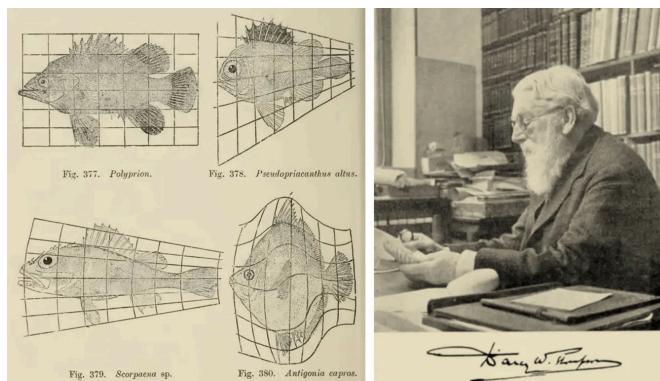
observe cells and their motion throughout the morphogenetic process, from the formation of a spherical embryo to the creation of a complete organism (Shah *et al.*, 2019). Cells undergo shape changes and large-scale flows as they undergo morphogenesis, driven by mechanical forces in concert with biochemical processes (Labernadie and Trepat, 2018, Lecuit *et al.*, 2011, Trepat and Sahai, 2018). Thus, the dichotomy of form and function is incomplete without considering the physical laws of mechanics.

Over a century ago, D’Arcy Wentworth Thompson wrote the influential book “On Growth and Form” (Thompson, 1979), in which he explored the relationship between geometry, physics, and biology in the context of morphogenesis. Thompson used examples to show how mathematical principles can explain biological phenomena, such as his theory of transformations, which demonstrates how related species can be represented geometrically (see fig 2.2). According to Thompson’s daughter, he even used to draw pictures of dogs on rubber sheets and stretch them to show children how poodles could become dachshunds (wol). This distortion of shape represents significant alterations in various forces or rates of growth throughout the developmental processes of different organisms.

Thompson’s approach was highly speculative, but his goal was to identify general principles behind the diverse forms and patterns found in biology. He compared growth curves of haddock, trees, and tadpoles, and found logarithmic spirals in shells, horns, and leaf arrangements.<sup>2</sup>

<sup>2</sup>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: “He finds a simple geometric construction,

Essentially, this book emphasized two points: first, all material forms of living things—cells, tissues, and organs—must obey the laws of physics, and second, quantitative measurements are necessary to unravel the physical principles of biology.



**Figure 2.2:** D'Arcy Thompson's fishes and his theory of transformation. (Thompson, 1979, Wolfram, 2017)

Thompson's work continues to inspire researchers even today. Right as I began my Ph.D., the centenary of the book's publication was being celebrated in the fields of developmental biology and biophysics (nat, 2017a,b, Heer and Martin, 2017). Even more so by the field of mechanobiology, an interdisciplinary field that studies the role of biophysical forces in cell and tissue functioning.

### 2.3 Mechanobiology

The cells within epithelial tissue can be viewed as mathematical systems that integrate multiple input cues to result in an output behavior. These inputs can be mechanical or chemical, such as the stretching of lungs or the presence of morphogen gradients during embryonic development. The outputs can include cell deformation, migration, differentiation, or proliferation (Kumar *et al.*, 2017). Some outputs can even feedback into the system as an input, such as when cells remodel the matrix (Malandrino *et al.*, 2018). Mechanochemical switches at the membrane, cell-cell junctions, or cell-matrix adhesions mediate the sensing of the environment, triggering a biochemical cascade that leads to a cellular response (Roca-Cusachs *et al.*, 2017). This interplay between biochemistry and mechanics is known as mechanotransduction.

During morphogenesis, mechanotransduction occurs at various scales, ranging from a single cell to complex multicellular tissue. To understand the role of different variables, experiments

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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

at different scales are necessary. It has been observed that individual cells can sense their environment and respond by altering their behavior through mechanical or biochemical processes. Whereas, multicellular systems can transmit forces and information at a longer length scale, allowing for emergent characteristics such as collective migrations, oscillations, rearrangements, and even turbulent flows (Heer and Martin, 2017, Lecuit *et al.*, 2011, Trepat and Sahai, 2018).

An excellent demonstration of the interaction between tissues and their environment is provided by the phenomenon of durotaxis. Epithelial cells can detect changes in the stiffness of the extracellular matrix and migrate towards areas of higher rigidity. This migration towards stiffer regions has been observed both *in vitro*, where cells in a monolayer collectively expand and relocate to stiffer areas, and *in vivo*, such as during the migration of neural crest cells in *Xenopus laevis* (Shellard and Mayor, 2021, Sunyer *et al.*, 2016). It is worth noting that the migration of neural crest cells themselves generates the durotactic gradient. In another example, during Drosophila oogenesis, the disorganized matrix is remodeled by cells to create a polarized matrix that aligns with the actin bundles in the follicular epithelium. This alignment is achieved through the coordinated rotation of cells and can guide the directed motion of cells along the polarized fibers (Cetera *et al.*, 2014, Haigo and Bilder, 2011).

The interplay between individual cells, their neighbors, and exogenous stimuli makes it difficult to decouple various biophysical aspects of the environment, such as forces, pressures, matrix stiffness, spatial confinement, porosity, or viscoelasticity. Direct force measurements in and out of tissues are also challenging. To address these challenges, researchers from various disciplines have attempted to recreate experimental systems with precise control over the biochemical and mechanical environments of cells (Xi *et al.*, 2018). This has been made possible through continuous technological advancements in fluorescent probes, imaging, microfabrication, and force measurements (Roca-Cusachs *et al.*, 2017). In the following section, I will provide an overview of relevant techniques and experiments in the field of mechanobiology.

### 2.3.1 Synthetic substrates

The use of Polyacrylamide and soft PDMS gels has enabled researchers to investigate mechanical interactions at cell-substrate adhesion (see fig 2.3 A). Simply seeding cells on hydrogels of different stiffnesses reveals a significant impact on the actin cytoskeleton, cell shape, and lineage specification (Engler *et al.*, 2006, Yeung *et al.*, 2005). These substrates, because of their known elastic response, are also utilized in techniques like traction force microscopy (TFM) to measure the forces exerted by cells and tissues on the substrate (Gómez-González *et al.*, 2020, Harris *et al.*, 1980) (see fig 2.3 D). TFM studies have shown that cells and tissues can exert greater forces on stiffer substrates as a result of the remodeling of the cytoskeleton (Elosegi-Artola *et al.*, 2016). Higher matrix stiffness has also been found to induce the translocation of Yes-associated protein (YAP) from the cytoplasm to the nucleus, which is considered a sensor

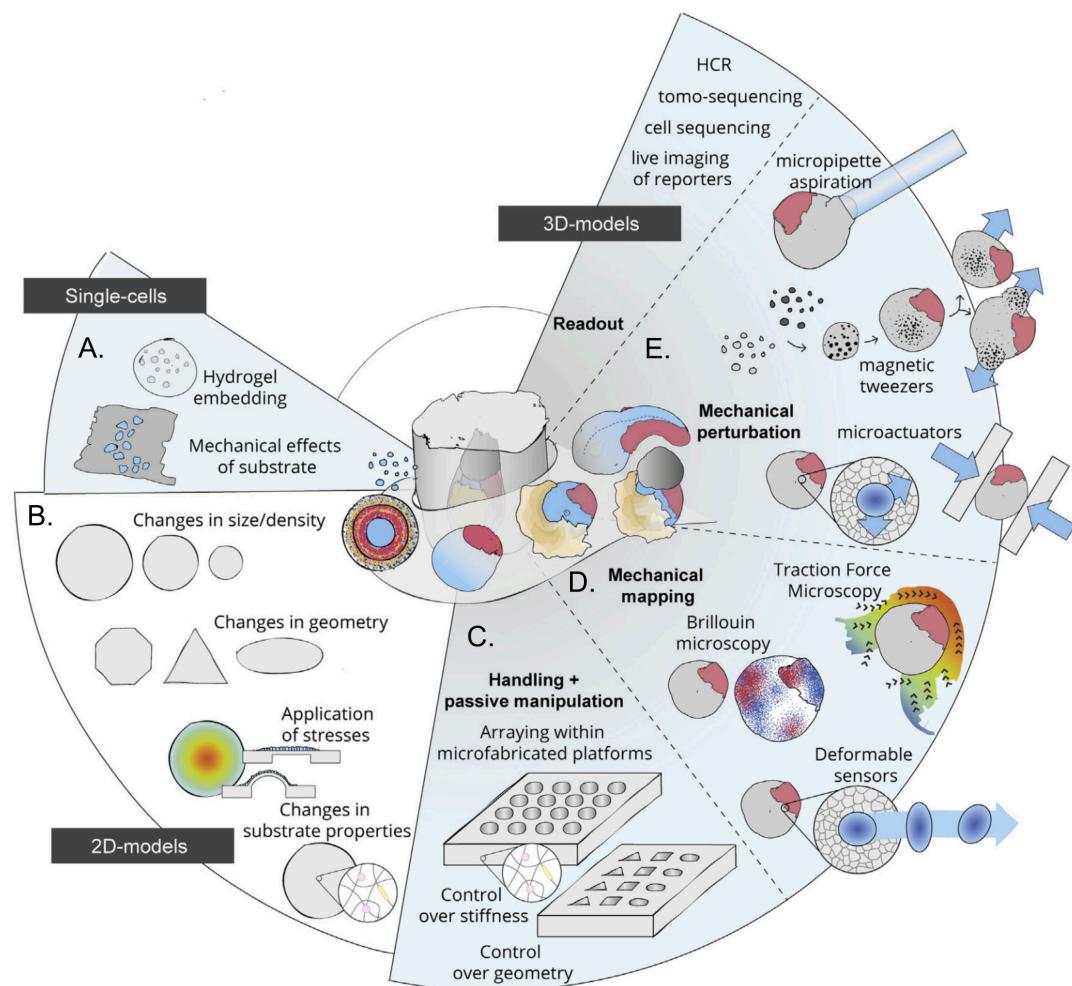
for mechanotransduction (Elosegui-Artola *et al.*, 2017). However, increasing extracellular matrix (ECM) ligand density alone can induce YAP nuclear translocation without changing substrate stiffness (Stanton *et al.*, 2019).

### 2.3.2 Geometric control

The shape of cells or tissues on 2D substrates can be controlled using micropatterned adhesion proteins or microfabricated stencils. Protein patterning techniques are used to pattern adhesion promoting proteins and control cell attachment and spreading, while microfabricated stencils physically confine cells in a particular geometry (see fig 2.3 B C). When cells are confined, they respond by reorganizing their actin cytoskeleton and focal adhesion complexes to match the shape imposed on them (Vignaud *et al.*, 2012). Confined tissues undergo larger-scale rearrangements, leading to the formation of fascinating topological defects or oscillations (Balasubramaniam *et al.*, 2021, Guillamat *et al.*, 2022, Tlili *et al.*, 2018). Through these experiments, we can uncover the mechanisms of force transmission and regulation of collective cell migration and epithelial growth in two dimensions (Deforet *et al.*, 2014, Nelson *et al.*, 2005, Vedula *et al.*, 2012).

Embryonic stem cells subjected to 2D confinement have been shown to differentiate based on the shape and size of the confinement. For example, a circular monolayer of stem cells can reproduce the tissue patterning of a 3D gastruloid (Warmflash *et al.*, 2014), and confinement in a triangular shape can lead to high tension at the vertices and activate Wnt signaling, promoting differentiation to mesoderm (Muncie *et al.*, 2020). Moreover, advancements in photopatterning technologies allow for precise control of multiple proteins on the same substrate (Guyon *et al.*, 2021, Prahl *et al.*, 2022), enabling the establishment of complex co-culture systems that mimic *in vivo* events.

Not just 2D shape, epithelial monolayers are also able to respond to curvature by regulating cell migration, orientation, cell/nucleus size, and shape (Marín-Llauradó *et al.*, 2022, Schamberger *et al.*, 2022) (see fig 2.3 C). For example, an epithelial monolayer on hemispheres of elastomers acts as a fluid with increasing curvature (Tang *et al.*, 2022). On a smaller scale, cells attached to corrugated hydrogels show variations in lamins, chromatin condensation, and cell proliferation rate in response to curvature (Luciano *et al.*, 2021). Bio-printing of three-dimensional tissue architectures can also create functional tissues (Brassard *et al.*, 2021, Breau *et al.*, 2022).



**Figure 2.3: Mechanobiological strategies for studying morphogenesis** Adapted from (Vianello and Lutolf, 2019)

### 2.3.3 Mechanical control

Living systems have mechanical control in addition to spatial control, as physical forces emerge from growth, deformation, and remodeling of the extracellular matrix (ECM) and fluid pressure in closed geometries. For example, the intestinal epithelia are stretched during peristaltic movements in the gut and lung alveoli deformations during breathing. Compression can also guide morphogenetic events that involve tissue bending and folding, such as the formation of the optic cup, gut villi, and cortical convolutions in the brain (Okuda *et al.*, 2018, Shyer *et al.*, 2013, Tallinen *et al.*, 2016).

To study tissue behavior under external perturbation, cells and tissues are probed at the molecular and subcellular scales using techniques such as atomic force microscopy, magnetic beads, optical tweezers, and micropipettes (Bao and Suresh, 2003) (see fig 2.3 E). At a larger scale, various types of stretching devices, tissue rheometers, and force plates can be used (Xi *et al.*, 2018). These experiments reveal that cells exhibit complex viscoelastic behavior at different levels of deformation and different regions of the cytoskeleton (Mofrad, 2009). The response of tissues to stretching can vary depending on the timescale of the stretch and the reorganization of cells within the tissue (Guillot and Lecuit, 2013). Rheological experiments also help to uncover the role of signaling pathways, such as YAP transcription factors, in mechanosensation (Wagh *et al.*, 2021).

The microfluidic system, also known as “cells on a chip,” has emerged as a valuable tool for investigating cell behavior under controlled biophysical conditions that mimic *in vivo* conditions (Ingber, 2018). This system allows for the application of stretch or shear forces, as well as the creation of a controlled microenvironment that mimics the organ-level cues present in the body. For instance, the surface tension at the air-liquid interface in the lungs and the fluid flow through the vasculature, as well as the cyclic mechanical stretch of the tissue-tissue interface due to breathing, can be replicated using this approach (Huh *et al.*, 2010).

In the context of developmental biology, the use of microfluidic systems has allowed for the study of self-organization and embryo functions under controlled physical conditions. The co-culture of iPSC-derived motoneurons and brain microvascular endothelial cells in a microfluidic system has produced the *in vivo*-like maturation of spinal cord neural tissue, representing a new avenue for exploring the complex interplay between physical and biological factors in development (Samal *et al.*, 2019, Sances *et al.*, 2018).

As mentioned earlier, the tissue-matrix interaction plays a critical role in sensing and rapidly transmitting forces (Serra-Picamal *et al.*, 2012, Sunyer *et al.*, 2016, Tambe *et al.*, 2011). 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, studying a simple free-standing epithelial monolayer is very appealing in terms of characterizing the mechanical response to stretch at different time scales.

Only two techniques are available for this: first, Harris and colleagues created a suspended monolayer by culturing a cell monolayer on a collagen matrix on two rods, and later removed the matrix using enzymatic digestion (Harris *et al.*, 2012). Second, epithelial domes, where MDCK cells pump ions to form fluid-filled blisters, have been used (Lever, 1979). Recently, my colleagues, Ernest Latorre and Ariadna Marín-Llaurado, have enhanced control over the curvature, shape, and size of the domes (Latorre *et al.*, 2018, Marín-Llauradó *et al.*, 2022), details on this system in the next chapter. These experiments showed that elasticity measurements of the monolayer were two orders of magnitude larger than those of individual cellular parts, and

the monolayer could sustain more than 200% strain before the rupture of cell-cell junctions. The cell cytoskeleton, particularly the actomyosin network and cadherin junctions, actively remodel during stretching, while the keratin network reinforces monolayer integrity at higher strains (Duque *et al.*, 2023, Latorre *et al.*, 2018). With sustained stretching, the tissue undergoes significant realignment and rearrangement via division (Wyatt *et al.*, 2015). Experiments on tissue devoid of the matrix also revealed epithelial actions such as superelasticity and buckling (Latorre *et al.*, 2018, Wyatt *et al.*, 2020).

#### 2.3.4 3D systems

*In vitro* experiments with 2D or 2.5D cell systems have improved our understanding of cell mechanics in morphogenesis by allowing us to measure deformations and forces and control environmental conditions that are inaccessible *in vivo*. However, to gain a deeper understanding of cell mechanics, systems closer to the *in vivo* environment must be probed.

Cell aggregates are a promising *in vitro* system for probing cell mechanics, where synthetic matrix and mechanical measurement tools can be used. The response of cell clusters to the matrix, while similar to planar tissues, is more complex and includes sensitivity to matrix stiffness, confinement, and ECM concentration, as well as the ability to undergo 3D shape transformations (see fig 2.3 E). Our lab has demonstrated that cell aggregates perform durotaxis and exhibit wetting behavior dependent on stiffness (Pallarès *et al.*, 2022, Pérez-González *et al.*, 2019). Additionally, cell aggregates in suspension behave like viscous droplets and can be used to measure rheological properties, such as when squeezed between plates or probed with AFM or a micropipette (Xi *et al.*, 2018). The viscoelastic properties of cell aggregates can even be measured by coalescing two aggregates (Oriola *et al.*, 2022).

In recent years, the use of hydrogel systems for the culturing cell aggregates has gained significant attention. Hydrogels, such as polyethylene glycol (PEG), polyacrylamide, collagen, or Matrigel, serve as a supportive environment for cell growth. Naturally extracted hydrogels like Matrigel provide a similar architecture to the native ECM. When embedded into a hydrogel, polarized epithelia tend to form a spherical structure with a hollow lumen, which can be induced to form branching morphogenesis by hepatocyte growth factor (Bryant and Mostov, 2008).

Cell-driven self-assembly in organoids leads to tissue formation that mimics organ features, but achieving reproducibility in shape and composition is often challenging (Hofer and Lutolf, 2021, Nelson *et al.*, 2008). Synthetic hydrogels with control over ligand presentation, crosslinking, and degradability have proven useful for epithelial organoids, allowing for control over cell fate (Gjorevski *et al.*, 2022, 2016).

3D gel-based culture systems with spatiotemporal control over the mechanical properties corresponding to *in vivo*-like functional structures have also been developed (Torras *et al.*, 2018). Interestingly, recent publications show tissue transformation from planar to complex organ-resembling tissue without fine environmental control. For example, intestinal epithelium

mechanically compartmentalizes itself, and 2D stem cells transform into a 3D neural tube (Karzbrun *et al.*, 2021, Pérez-González *et al.*, 2021).

In developing embryos, both embryonic and extraembryonic fluids generate frictional and tensional stresses when flowing, or hydrostatic pressures when confined within spaces (Chan and Hiragi, 2020, Vianello and Lutolf, 2019). The challenge of measuring these forces has led to the use of various techniques, including micropipette aspiration. Micropipette experiments, where a needle is inserted into the embryo to control pressure, have revealed that the internal hydrostatic pressure determines the embryonic size and dictates cell fate allocation (Chan *et al.*, 2019) (see fig 2.3 E). As a fluid-filled structure, the hydrostatic pressure inside the embryo corresponds to tension in its surfaces, and changes in luminal volumes are sensed by cells through increased cortical tension, inducing changes in cell shape and cytoskeleton organization (Chan *et al.*, 2019, Choudhury *et al.*, 2022b). Micropipette aspiration has also been effective in measuring the surface tension of individual cells or whole blastomeres (Dumortier *et al.*, 2019), thus providing insight into the role of the actin cortex in regulating preimplantation embryonic contractility (Firmin *et al.*, 2022, Özgürç *et al.*, 2022).

The measurement of forces within embryos has also been approached through the insertion of deformable probes, such as hydrogels, oil, or magnetic droplets (Campàs *et al.*, 2014, Dolega *et al.*, 2017, Serwane *et al.*, 2017). The shape changes of these probes allow for measurement of local forces and osmotic pressures (Mongera *et al.*, 2023).

In addition to embryos, explant systems have been utilized to study organogenesis in the brain, gut, and lungs. Lung explant research has been particularly useful in understanding different aspects of shape formation, which occurs under the influence of pressure and growth factors. The explant system allows for direct control over the chemical and mechanical environment at specific stages of development. Work with mouse airway epithelium has shown that pressure and matrix stiffness impact the number of lung branches (Nelson *et al.*, 2017, Palmer *et al.*, 2021, Varner *et al.*, 2015).

Other tools such as optical tweezers, laser ablation, and optogenetic excitations have been used at different levels to probe the mechanics of development (Gómez-González *et al.*, 2020, Lecuit *et al.*, 2011). However, independent control over multiple factors remains difficult and force measurement remains indirect.

In conclusion, epithelial tissues are highly sensitive to various biophysical forces and constantly undergo remodeling at different scales and timeframes. There are multiple techniques available to manipulate and study these tissues, from single cells to embryos, with controlled forces and deformation. Due to its dynamic behavior, epithelial tissue can be considered an active material. The focus of this thesis is to develop a system that can control and measure physical forces to understand epithelial behavior as an active material. In the following chapter, we will delve into the molecular machinery responsible for driving these active tissues.

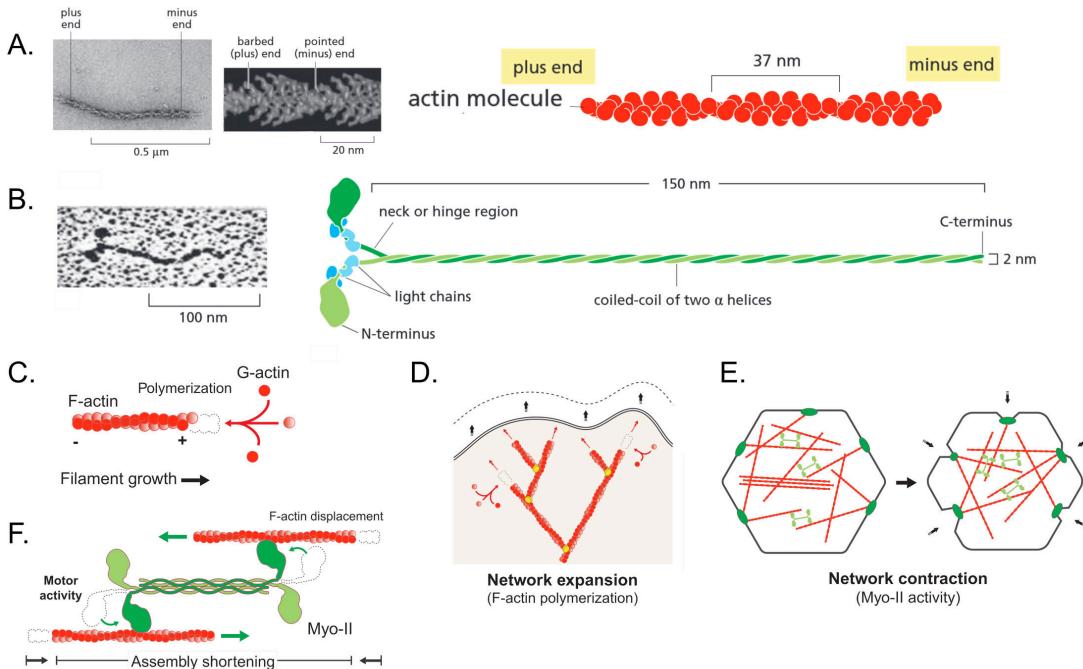
## **Chapter 3**

# **Active tissue mechanics**

### **3.1 Force generation with actin**

In the field of morphogenesis, cells are central to the formation of specific structures through changes in their shape. Early embryologists posited the existence of a mysterious external vital force that guides the morphogenesis of individual cells in tissues (Thompson, 1979). However, as research progressed, particularly experiments by Wilhelm His and Wilhelm Roux, it became clear that the physical forces generated within the cell itself (Clarke and Martin, 2021). In the present day, we now understand, what was unknowable in the XIXth century, that the machinery responsible for generating these physical forces is the actin cytoskeleton.

Specifically, the actomyosin cortex forms a mesh containing actin filaments and myosin motors just beneath the plasma membrane of a cell (Alberts, 2015). This mesh is organized into various higher-order arrays capable of dynamic remodeling, giving rise to the complex shapes and structures we observe in the world around us. We can understand the actomyosin cortex step by step, starting from its basic organization of single actin filaments to higher-order supracellular actomyosin cables.

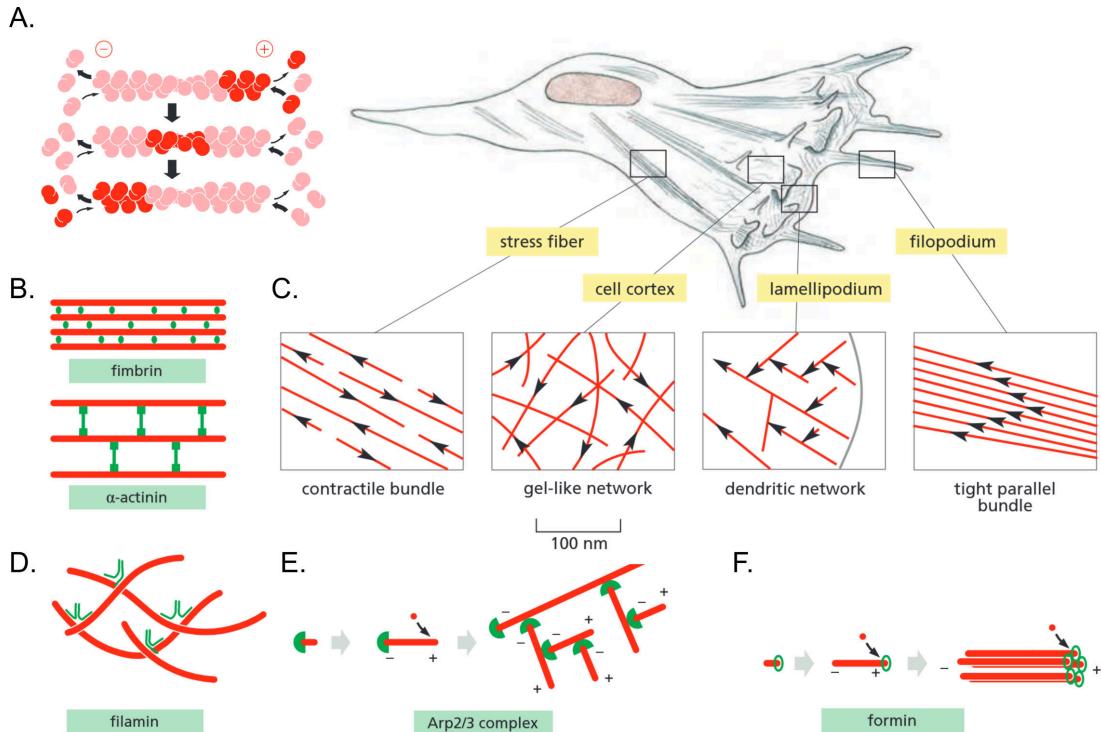


**Figure 3.1: Actin and Myosin:** (A) Electron micrograph of Actin filament with zoomed in images of barbed and pointed end. (B) Same for Myosin II minifilament with clearly visible two globular heads and a long tail. (C-D) Actin network can apply pushing force through polymerization of single filaments or network expansion. (E,F) While myosin activity would lead to contraction of the networks. *Adapted from A-B (Alberts, 2015) and C-F (Clarke and Martin, 2021)*

### 3.1.1 Actin filaments

The actin filaments are helical polymers composed of G-actin proteins (see fig 3.1 A). The asymmetrical nature of these proteins leads to the development of two distinct ends, referred to as the barbed and pointed ends, that can be differentiated based on their appearance in electron micrographs. The actin filaments are known for their dynamic assembly and disassembly processes, where the distinct ends have different rates of kinetics. This results in growth in the direction of the barbed end, with the length of the filament can be maintained by a constant flux of subunits from the pool of monomers in the cell and nucleotide hydrolysis. This process is referred to as *treadmilling* (see fig 3.2 A). However, if one end of the filament is capped, it will continue to grow and apply a pushing force in the outward direction.

### 3.1.2 Actin networks



**Figure 3.2: Forms of actin networks:** (A) Actin treadmilling: where highlighted actins move from positive end to negative end as the filament polymerizes and depolymerizes from both ends. (C) In an adherent cells, there are many different kinds of actin structures from contractile network to gel-like cortex. (B,D,E,F) Actin structures can be thought as meshwork of actin filaments (red) with crosslinkers(green). Different crosslinkers produce distinct form of actin network. *Adapted from (Alberts, 2015)*

Actin filaments can also form branched networks, facilitated by the presence of nucleation sites on the filament and proteins containing actin-binding motifs. The actin nucleation can be catalyzed by two primary factors, the ARP 2/3 complex or formins. The ARP 2/3 complex creates a pointed end in the center of a filament, leading to the formation of a new branch from that site. This results in the formation of a tree-like network of branches, capable of generating sufficient pushing forces to move a part of the cell membrane (see fig 3.2 E,F). The formins, in conjunction with profilin, aid in the growth of the filaments, with profilin serving as a staging area for the rapid addition of monomers to the filament. These structures can take the form of dendritic actin networks that enable membrane protrusion at lamellipodia or spike-like projections of the plasma membrane that allow a cell to explore its environment (see fig 3.2 C). The pushing forces generated at the molecular level are of the order of 1 piconewton.

### 3.1.3 Actin cortex

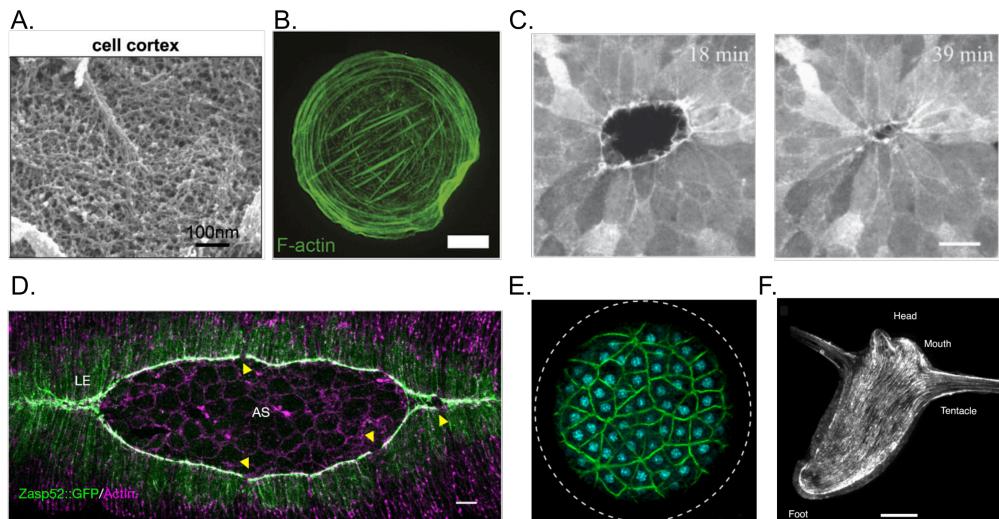
The actin filaments can also form tight or loose bundles, facilitated by crosslinking proteins. Fimbrins enable multiple actin filaments to arrange in parallel, resulting in closely packed bundles that exclude myosin from connecting to the filaments. On the other hand,  $\alpha$ -actinin crosslinks actin filaments with opposite polarity into a loose bundle, allowing myosin to bind and create contractile bundles (see fig 3.2 B). Myosin II oligomerizes into a bipolar short filament that can connect multiple actin filaments and move across them, resulting in a pulling effect (see fig 3.1 B). This movement is driven by ATP hydrolysis making contracting an active process. The loose bundle forms the gel-like network in the cell cortex. Other actin crosslinking proteins can result in different structures. Filamin creates a loose and viscous gel that is essential for migration, while spectrin creates a strong and flexible web-like network of short actin filaments that allows cells to reversibly deform (see fig 3.2 D). The actomyosin bundles in the cortex can generate two orders of magnitude more force than a single filament (Clarke and Martin, 2021).

## 3.2 Actin structures at a larger scale

During epithelial morphogenesis, individual cells can undergo shape changes by modifying their contractility or actin turnover, resulting in the development of tissue curvature. As mentioned previously, epithelial cells exhibit apicobasal polarity, which results in a non-uniform distribution of the actin cytoskeleton that influences cell shape and tissue architecture.

The geometry of columnar or wedge-like cells in a monolayer determines the specific ways in which they can be organized (Gómez-Gálvez *et al.*, 2021). Columnar cells, when arranged together, produce a flat tissue, while wedge-shaped cells with a narrow top result in convex curvature (see fig 3.4 A). Conversely, concave curvature with a narrow bottom can also be created. By observing the actin cytoskeleton, we can determine the specific mechanisms of tissue shaping (see fig 3.4 B). For example, apical constriction with concentrated actin cortex on the apical surface is involved in multiple convexly curved tissues, such as the invagination of the intestinal crypt, the Drosophila mesoderm, and the vertebrate lens placode (Houssin *et al.*, 2020, Lecuit *et al.*, 2011, Pérez-González *et al.*, 2021).

On the other hand, basal constriction results in opposite curvature, as observed in the optic cup and mid-hind brain fold of zebrafish (Gutzman *et al.*, 2018, Sidhaye and Norden, 2017). However, convex curvature can also be produced through basal expansion, as seen in the Drosophila wing disc (see fig 3.4 A). Certain parts of the wing disc can locally relax the basal side without affecting the apical side, leading to basal expansion (Sui *et al.*, 2018). In addition to the apical and basal surfaces, lateral surfaces can also contract or expand due to myosin II activity, which can cause tissue folding in the wing and leg discs of Drosophila (Monier *et al.*, 2015, Sui *et al.*, 2018). Furthermore, cell-cell rearrangements can be produced by altering

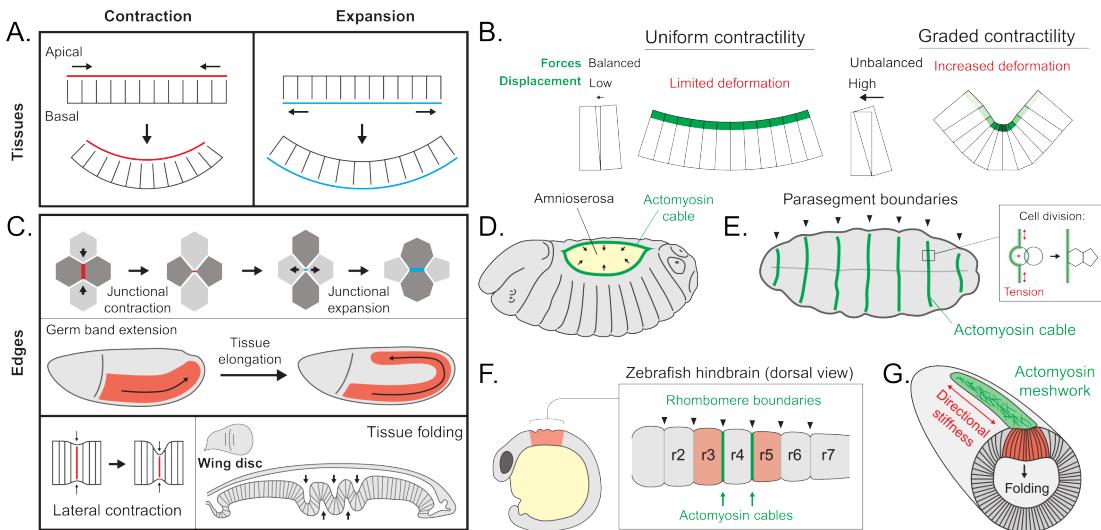


**Figure 3.3: Actin organization at different scales:**(A) Electron micrograph of actin cortex of mitotic HeLa cells (Kelkar *et al.*, 2020). (B) Different forms of actin organization in circular fibroblast cell (Jalal *et al.*, 2019) Scale= 10 $\mu$ m. (C) Supracellular actin ring during wound closure (Brugués *et al.*, 2014) Scale= 20 $\mu$ m. (D) Dorsal closure of amnioserosa with actin network (Ducuing and Vincent, 2016) Scale= 10 $\mu$ m. (E) Supra-cellular organization of actin for cellularization of coenocyte. Circle is 60 $\mu$ m (Dudin *et al.*, 2019). (F) Hydra with actin network, whose nematic defects determines morphogenesis (Maroudas-Sacks *et al.*, 2021) Scale= 100 $\mu$ m.

junction lengths during germ band extension (Collinet *et al.*, 2015, Yu and Fernandez-Gonzalez, 2016) (see fig 3.4 C).

Not only do individual cells undergo coordinated actin reorganization during epithelial morphogenesis, but supracellular actin structures can also emerge at the tissue level (see fig 3.3 A-C). Junctional actomyosin organizes to form bundles connected across multiple cells, allowing for important functions such as wound healing and morphogenesis (Brugués *et al.*, 2014, Clarke and Martin, 2021) (see fig 3.4 D-F). These supracellular networks can exert forces at the scale of the embryo, as observed in cases such as dorsal closure and parasegment boundary formation in Drosophila and epiboly in zebrafish (Calzolari *et al.*, 2014, Ducuing and Vincent, 2016). Additionally, these networks can alter the material properties of specific regions in the embryo, making them more prone to deformation and thus aiding in the formation of folds or invaginations (see fig 3.4 G).

During Drosophila gastrulation, tissue-level actin cortex is altered in the direction of the anterior-posterior axis, providing increased bending strength in that direction. This supports the internalization of the mesoderm by promoting folding in a perpendicular direction (Yevick *et al.*, 2019). Interestingly, highly organized actin bundles are also found in even larger systems such as Hydra, vertebrate smooth muscle, and the heart (Cetera *et al.*, 2014, Helm *et al.*, 2005,



**Figure 3.4: Morphogenesis driven by actin at tissue scale:** (A) Apical contraction or basal relaxation both results in the same curvature. (B) However, amount of deformation will depend on the contractility gradient. (C) Lateral surface of cells can also undergo expansion or contraction leading to cell rearrangements or tissue folding. (D-G) Supracellular actin cables plays vital role in creating boundaries or causing large scale deformations. Adapted from (Clarke and Martin, 2021)

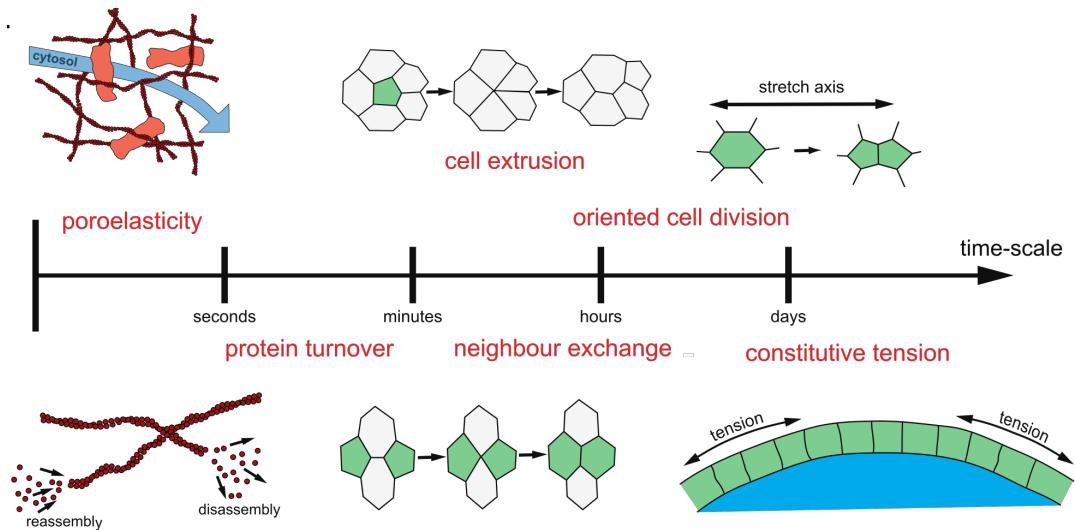
Maroudas-Sacks *et al.*, 2021, Palmer *et al.*, 2021) (see fig 3.3 D-F). These bundles assist in generating mechanical force patterns that create coordinated tissue movements at a global scale.

### 3.3 Timescales of the actin cytoskeleton

Morphogenesis, the process of shaping and forming living structures, occurs at varying timescales and requires the cell cytoskeleton to change its shape accordingly. Rheological and mechanobiological experiments have given us insights into how cells respond to forces and deformations based on their magnitude and rate (see fig 3.5 A; reviewed in (Wyatt *et al.*, 2016)).

For fast deformations (in the range of milliseconds to seconds), cells exhibit predominantly elastic behavior, as there is insufficient time for the actin cortex to respond or remodel (Deng *et al.*, 2006). The cytoskeleton can store elastic energy and release it. At this scale, there is also flow of cytosol through the cortical mesh, resulting in poroelastic behavior(Moeendarbary *et al.*, 2013).

When forces or deformations are applied over longer timescales (seconds to minutes), cells exhibit an increasingly viscoelastic behavior (Kollmannsberger and Fabry, 2011). The actin cortex can flow and is unable to fully store energy. The actin filaments and crosslinkers, such as myosins and actinin, allow the cytoskeleton to remodel in response to mechanical



**Figure 3.5: Timescale of actin network related processes:** Timescales of different actin driven cellular processes, ranging from cytoskeletal fluid deformation to large-scale tissue deformations. Adapted from (Kelkar et al., 2020).

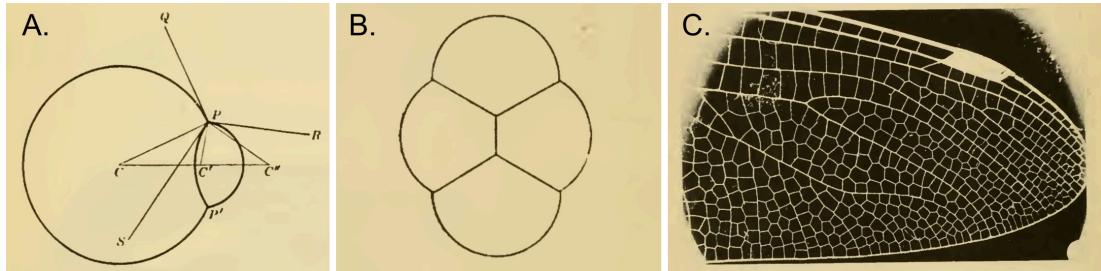
perturbations through turnover in tens of seconds or a few seconds, respectively. Myosin mini filaments, however, can take longer to remodel, up to hundreds of seconds.

At even longer timescales (minutes to hours), cells or tissues may respond through oriented division or rearrangement, allowing them to adapt to persistent forces such as gravity or surface tension. Tissues may resemble a viscous fluid and morph into a sphere, such as a blastocyst. Interactions with the extracellular matrix over hours can lead to adjustments in the constitutive tension of tissues based on biophysical and biochemical forces (Porazinski et al., 2015).

## 3.4 Modeling active tissue dynamics

The advancement of molecular biology and tissue dynamics has increased our understanding of morphogenesis. However, it is becoming increasingly crucial to interpret biological experiments through theoretical models in order to generate new hypotheses and validate them through further experimentation.

Mathematical models at multiple scales are used to describe both physics and biology. At larger tissue scales, hyperelastic continuum material models could be utilized to describe the behavior of the cardiovascular system (Holzapfel et al., 2019). On smaller scales, agent-based models are used to explain epithelial tissue behavior in terms of cell sorting and reorganization (Voss-Böhme, 2012). This section aims to provide the reader with a brief overview of the relevant modeling approaches in this field.



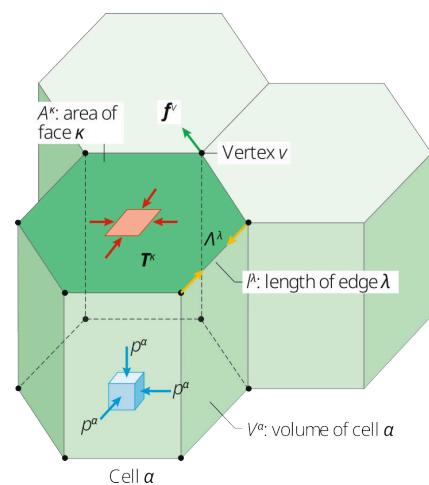
**Figure 3.6: D'Arcy Thompson's forms of tissues:** (A-B) Thompson equates cell aggregates to coalescence of bubbles like in a froth. (C) A dragon fly wing is a clear example of this organization. Adapted from (Thompson, 1979)

### 3.4.1 Vertex models

D'Arcy Thompson, in his chapter on "The Forms of Tissues," presents an intuitive argument regarding the role of surface tension or capillarity in organizing cells into a tissue (Graner and Rivelin, 2017, Thompson, 1979). He observed this phenomenon in a wide range of biological systems, from two connected cells to the organization of cells in a dragonfly wing, which resemble the associations of soap bubbles or foams (see fig 3.6). <sup>1</sup> In the case of monolayered epithelial tissue, its polygonal cellular pattern on its surface enables the easy description and tracking of cell motion and shape change through the use of vertices and edges. Vertex models have proven to be valuable in understanding the complex interactions between cellular shape, the forces generated within epithelial cells, and the mechanical constraints imposed on the tissue from external sources (as reviewed in (Alt *et al.*, 2017)). These models can be two-dimensional or three-dimensional, depending on the system being modeled, but cells are consistently defined as having both an apical and basal surface, as well as lateral interfaces between neighbors. Further complexities have been added to describe specific systems, such as intercalations in three-dimensional epithelia, through the use of a geometric shape known as the Scutoid (reviewed in (Gómez-Gálvez *et al.*, 2021)).

To determine the motion of the vertex, mechanics must be specified. It is often done using the virtual work

**Figure 3.7: Vertex model for cells in a monolayer** Adapted from (Gómez-Gálvez *et al.*, 2020).



<sup>1</sup>"we recognize the appearance of a "froth," precisely resembling that which we can construct by imprisoning a mass of soap-bubbles in a narrow vessel with flat sides of glass; in both cases we see the cell-walls everywhere meeting, by threes, at angles of 120 deg, irrespective of the size of the individual cells: whose relative size, on the other hand, determines the curvature of the partition-walls", writes Thompson

function ( $W$ ). There are two components: internal ( $\delta W_i$ ) and external ( $\delta W_e$ ).

$$\delta W = \delta W_i + \delta W_e.$$

The changes in internal virtual work, ( $\delta W_i$ ) can result from changes in the cell volumes ( $\delta V$ ), in the areas of surfaces ( $\delta A$ ), or in the lengths of bonds ( $\delta l$ ). By defining the cell pressure ( $P$ ), the surface tension ( $T$ ), the line tensions ( $\Lambda$ ), and internal dissipative forces ( $f_i$ ), the differential of the internal virtual work for vertex movements can be written.

$$\delta W_i = \sum_{cell \alpha} (-P^\alpha \delta V^\alpha) + \sum_{surface k} (T^k \delta A^k) + \sum_{edge \lambda} (\Lambda^\lambda \delta l^\lambda) - \sum_{vertex v} (f_i^v \delta x^v).$$

Similarly, the external virtual work, ( $\delta W_e$ ), can be written according to the external forces ( $f_e$ ) that come from external mechanical forces applied to the tissue through the matrix, or fluid pressure acting on apical or basal cell surfaces.

$$\delta W_e = -\sum_{vertex v} (f_e^v \delta x^v).$$

The state of a monolayer is determined by minimizing the virtual work function, taking into account the molecular complexities that contribute to surface tension and line tensions. In the context of epithelial layers, the actin cortex significantly impacts the tensions along the edges. Vertex model simulations in 2D models demonstrate the important role of interfacial tensions in shaping cell orientation, coordinating collective migration, and facilitating tissue rearrangement through cell division.

In contrast, 3D models capture the physics of various morphogenetic processes, such as the formation of appendages on the drosophila eggshell and the mechanical compartmentalization of intestinal epithelia (Osterfield *et al.*, 2017, Pérez-González *et al.*, 2021). These models offer unique insights into cell packing and the transition between jamming and unjamming (Park *et al.*, 2015, Tang *et al.*, 2022). In some cases, phase transitions from a solid to fluid state result from localized proliferation and oriented divisions, showing that the epithelial tissue behaves as an active material (reviewed in (Lenne and Trivedi, 2022)).

### 3.4.2 Continuum models

The viscoelastic properties of tissues are captured in vertex models, which are useful for smaller scale. However, for larger scale deformations or flows, we can model tissues as a continuous material. There are two tactics for thinking about these models: one focuses on the rheological properties of the tissue, and the other on shape transformations. By thinking of a continuous sheet of cells as an active surface, we can capture the physics of single cells to embryos (Khoromskaia and Salbreux, 2023, Salbreux and Jülicher, 2017).

Continuum models focus on developing reliable constitutive relations and solving initial-

boundary-value problems. Constitutive relations describe how materials respond to applied loads, and they depend on the internal constitution of the material. Determining constitutive relations for epithelial monolayers can be challenging because these tissues are much more complex than simple metals or passive polymers (see fig 3.8 A-B). However, their complex material behavior can be understood by characterizing their mechanical response using standard material testing techniques (Humphrey, 2002). Typically, they can be probed mechanically in a biologically relevant manner, such as through biaxial or uniaxial stretching experiments that simulate *in vivo* tissue behavior (Humphrey *et al.*, 2014). These experiments with epithelial tissues have revealed the viscoelastic nature of these materials (Harris *et al.*, 2012, Khalilgharibi *et al.*, 2019).

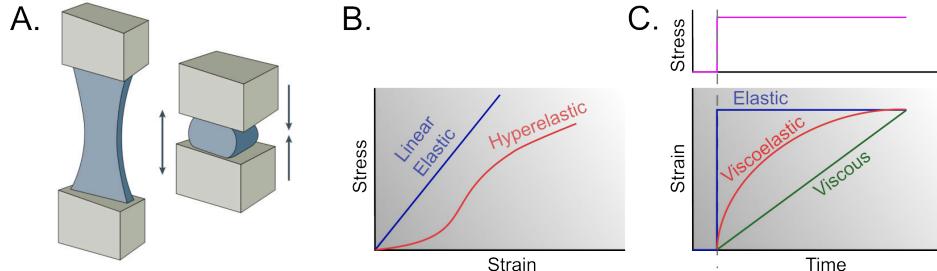
Solids, such as rubber, are considered to have elastic properties, allowing them to deform reversibly when subjected to a force. Conversely, fluids are characterized by their viscosity, meaning they flow in response to an applied force. Viscoelastic materials exhibit both solid-like and fluid-like behaviors (see fig 3.8 C). Simple models can represent these behaviors by combining elastic components, represented as springs, and viscous components, represented as dashpots. The elastic response does not dissipate energy, unlike the viscous response.

$$\sigma = E\epsilon, \quad \sigma = \eta \frac{d\gamma}{dt}.$$

Other material properties like stiffness or Poisson's ratio can be revealed through quasi-static stretching or compression. However, dynamic properties are better understood through frequency sweep, creep, or stress relaxation experiments (Guimarães *et al.*, 2020). Rheological experiments have been extremely valuable in gaining insight into the mechanical response of various biological materials, ranging from reconstituted cytoskeletal proteins to large multicellular aggregates (Cavanaugh *et al.*, 2020, Mofrad, 2009, Xi *et al.*, 2018).

Rheological properties are often linked to physiological state and are crucial for their specific functions (Park *et al.*, 2015, Vedula *et al.*, 2012). For example, many fundamental shape transitions in embryos occur through abrupt change in tissue material properties. (Hannezo and Heisenberg, 2022). Therefore, it is important to assess rheological properties in different microenvironments. Mechanical information such as deformation, deformation rates or velocity fields, traction forces exerted by cells on substrates, and intercellular mechanical stress can provide a more complete picture of tissue rheology when combined with information about cellular architecture obtained through imaging (Roca-Cusachs *et al.*, 2017). These types of experiments shed light on the complex mechanisms of strain stiffening and viscoelastic behavior at different deformation regimes involving various parts of the cytoskeleton.

However, in certain cases like modeling cardiovascular mechanics or the growth of organs, we can rely on hyperelasticity or composite material framework. The basic kinematics assumes a mapping,  $x = \chi(X, t)$ , deformation from reference to deformed configuration. The



**Figure 3.8: Stress strain behavior of materials:** (A) materials being stretched or compressed. (B) Quasistatic deformations yield stress-strain curves. (C) Creep test where strain response is characterized at constant stress.

deformation gradient and Green's strain tensor are defined.

$$F = \nabla_X(\chi(X, t)); \quad E = \frac{(F^T F - I)}{2}.$$

The elastic and growth can be delineated in the deformation gradient through decomposition.

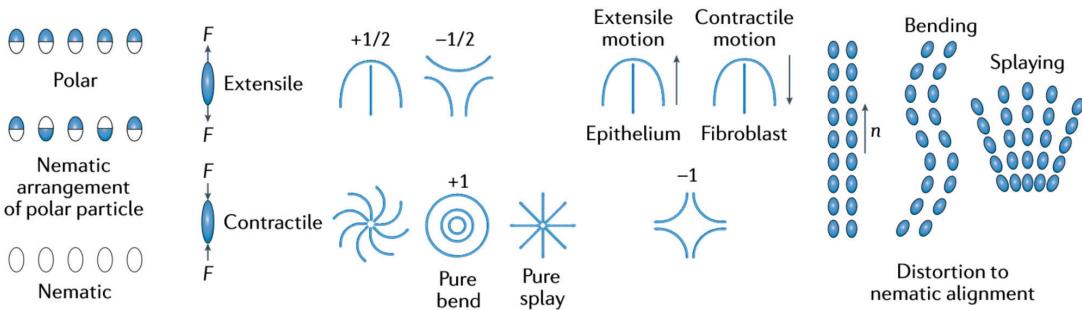
$$F = F_e F_g.$$

Here, in the theoretical framework of finite elasticity, one can assume a strain energy function relates to stress. The stress-strain data extracted from the experiment allows for predicting the form of the strain energy function.

$$S = \frac{\delta W}{\delta E}.$$

The utilization of hyperelastic models has proven to be effective in capturing the material response in various biological tissues, such as the bladder, heart tissue, skin, and arteries (Holzapfel, 2000). This type of formulation provides a degree of flexibility, as it allows for the inclusion of additional physical constraints, such as the anisotropy of the tissue microstructure or its incompressibility. Minor modifications to these constitutive relations can be used to capture the material response, such as explaining the phenomenon of strain stiffening, or accounting for the inhomogeneity in the material, such as the collagen content and crosslinking in the tissue (Holzapfel *et al.*, 2019).

These models are also employed in the understanding of growth and remodeling, through the use of kinematical growth theory (Ambrosi *et al.*, 2019). This theory highlights the existence of residual stresses in growing tissues, which allow for compatible elastic and inelastic growth-induced deformations, leading to a modification of the tissue properties into a spatially inhomogeneous and anisotropic state. This process is of great significance in the field of solid tumor growth mechanobiology, as the residual stresses directly impact tumor



**Figure 3.9: Active nematics:** Schematics of (A) nematic or polar particles, (B) extensile and contractile force dipoles, (C) Various types of defects and related motion of cells *Adapted from (Xi et al., 2018)*.

aggressiveness, nutrient pathways, necrosis, and angiogenesis.

### 3.4.3 Active surface models

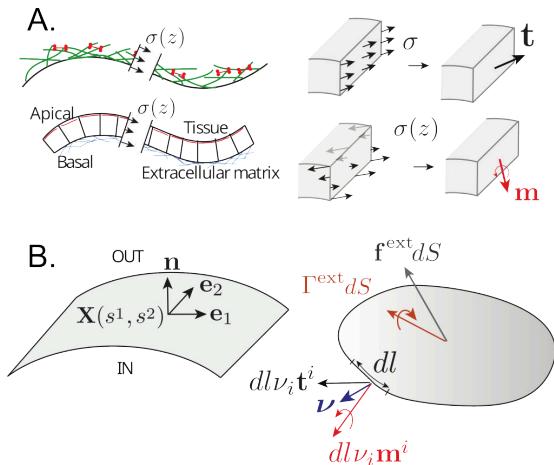
At the cellular level, the mechanical properties of tissues are largely determined by the biopolymeric cytoskeleton, which consists of filaments and cross-linkers and molecular motors. These components continuously convert energy, ATP to ADP, through contractions or extensions of the network, resulting in a physical gel-like system due to its cross-linked actin filament network. However, the presence of phenomena such as treadmilling, active polymerization-depolymerization of filaments, and the mobility of molecular motors, such as myosin, makes the tissue system an active gel that lacks time-reversal symmetry due to its continuous energy transduction.

Additionally, the filaments are polar, which allows for the acquisition of orientational order. This has led to the modeling of tissues as active gels, similar to modeling active systems, such as flocks of birds and schools of fish, using hydrodynamics of active matter (Jülicher *et al.*, 2018). Active matter systems are a subclass of continuum models used to describe the dynamics of packed active particles, which are based on the liquid crystal theories of soft condensed matter. Like liquid crystals, cells also possess orientation and the ability to move past each other. In this framework, the orientation of filaments in the cytoskeleton or the elongation of cells in the tissue can be characterized by a nematic order parameter matrix (see fig 3.9).

$$Q = \frac{3S}{2} \left( n \otimes n - \frac{I}{3} \right), \quad S = [\cos 2\theta],$$

$$\sigma_{active} = \zeta Q.$$

The utilization of this formulation is significant in characterizing the active forces produced by the network. The stress is separated into two components: active and passive. The passive



**Figure 3.10: Active surface models:** (A) Tissues or cell surfaces can be modeled as surface with stresses and torques along the thickness. (B) Internal and external forces act on a surface element. The kinematics of these surfaces, mathematical tools from differential geometry can be applied, using generalized coordinates ( $X$ ), metric tensor ( $g$ ), and curvature tensor ( $C$ ), where ( $dl$ ) is the length of the line element with tangential unit vector ( $v$ ). Adapted from (Salbreux and Jülicher, 2017)

stress arises from the viscoelasticity of the material and the bending, splaying, and twisting of the aligned elements. The active stress, on the other hand, is calculated by combining the strength of activity, represented by the parameter zeta, and the nematic order matrix. The sign of zeta determines the type of force dipole generated; a negative sign results in contraction of the system, while a positive sign leads to expansion along the nematic axis.

The active stress plays a crucial role in the motion of the system and can result in chaotic motion even in low Reynolds number systems, as evidenced in dense bacterial systems of *Bacillus subtilis* where jet flows and turbulent patterns have been observed, as well as in expanding monolayers where independent vortices have been recorded (Blanch-Mercader *et al.*, 2018, Wensink *et al.*, 2012). The nematic formulation have proven to be effective in capturing the physics of 2D confined systems and expanding systems (reviewed in (Saw *et al.*, 2018)).

In the context of 3D models, active surfaces are used to describe the actomyosin cortex near cell membranes or epithelium in embryos (Salbreux and Jülicher, 2017). This thin sheet of matter generates internal forces and torques that drive shape changes at the cellular or tissue level. The resulting three-dimensional structures can be conceptualized as curved, active two-dimensional surfaces. Forces and torques can be defined in terms of tension ( $t$ ) and moment ( $m$ ), and the model also considers the mirror and rotation symmetries of the surface elements (see fig 3.10).

Salbreux and Julicher's work has demonstrated that flat active membranes with up-down asymmetry exhibit stability dependent on active tension and active tension-curvature coupling term. This tension-curvature dependency has been observed in the pancreas of mice, where the morphology of epithelial tumors is determined by the interplay of cytoskeletal changes in transformed cells and the existing tubular geometry (Messel *et al.*, 2019). Specifically, small pancreatic ducts produced exophytic growth, whereas large ducts deformed endophytically,

consistent with theoretical predictions. Another example shows that curls of high curvature form spontaneously at the free edge of suspended epithelial monolayers, which originate from an enrichment of myosin in the basal domain that generates an active spontaneous curvature (Fouchard *et al.*, 2020). The extent of curling is controlled by the interplay between internal stresses in the monolayer.

While the molecular level behind epithelial morphogenesis, specifically the actin cytoskeleton, is well understood, there are still gaps in the theoretical and experimental framework that can bridge the gap between molecular dynamics and tissue-scale deformations. Vertex and continuum models have been developed to capture the physics of morphogenesis at the tissue scale, and phenomenological experiments provide insights into the constitutive relations of cytoskeletal components and tissues in specific conditions. However, combining vertex models and active surface mechanics could provide finer control over individual cell surfaces, enabling more precise bottom-up morphogenesis.

## Chapter 4

# Bottom up morphogenesis

### 4.1 Learn by building

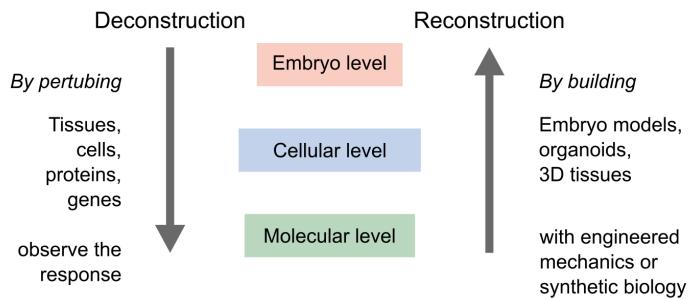
The mechanics and biology of epithelial tissues are complex, with mechano-chemical signaling and multiscale behavior all intertwined. The lens of active material has been instrumental in illuminating the role of molecular elements in undergoing shape changes during morphogenesis. Mechanistic understanding has been enhanced with new mathematical tools and advanced microscopy, enabling measurement of the forces involved in tissues.

The traditional and successful method for studying mechanics has been to deconstruct the system one component or parameter at a time. By manipulating genes or disrupting cellular processes, we can observe how mechanics change. This perturbative method allows for the alteration of biological systems at various levels, from molecular to tissue, (see fig 4.1) .

However, studying systems like organoids or embryos can only provide limited physical insights into the topological transitions of these structures, as experimental systems have limited physical control and ability to measure forces. An alternative approach is to learn by actively performing morphogenesis or reconstructing biological structures from their basic components.

For years, researchers have broken down biological systems into approachable parts - tissues, cells, proteins - in order to understand the behavior of each component. However, combining existing knowledge of these parts to recreate novel experimental systems could reveal the basic building blocks and effects of scale. This approach would complement top-down approaches in developmental biology. Synthetic biology, a perfect example of reconstruction, seeks to recreate life at various scales, from synthetic proteins to entire cells, in order to gain a deeper understanding of the indispensable components of life.

As active agents exist at every scale, emergent properties can appear at higher scales. Thus, it is essential to focus on higher scales or work with collectives of cells. This reminds me of the example of cars and traffic: Imagine you know the behavior of all individual car components,



**Figure 4.1: A conceptual representation of two approaches to understanding mechanics: reconstruction (bottom-up) and deconstruction (top-down).** In reality, they are not separate from each other. These methods inform each other, with past top-down research guiding new reconstruction, and new engineered cells or tissues furthering our understanding of the field in innovative directions.

but this information is not sufficient to understand the behavior of traffic flow. This requires a higher level of analysis.<sup>1</sup> Similarly, biological structures exhibit numerous collective behaviors, such as jamming, nematic order, instabilities, or self-organization (Trepat and Sahai, 2018).

Recreating structures from scratch also provides an opportunity to understand the role of physics at different scales. In the spirit of D’Arcy Thompson, we can explore the fundamental properties of matter in biological structures.<sup>2</sup> For instance, we can study the role of surface tension in guiding the shape of cellular aggregates or lumens. In this work, we focus on the mesoscale structures of epithelia ( $\sim 10 - 10^4 \mu\text{m}$ ).

We present our efforts to engineer an epithelial structure with a controlled microenvironment that is sensitive to self-organization and mechanical instabilities. The following sections will describe the ways of creating these structures from minimal ingredients.

## 4.2 How to build tissue structures?

Before embarking on the construction of a tissue structure, it is important to consider the desired form and function. Despite the diversity in the shapes and functions of tissues, certain elementary shapes can be seen in many cases, resulting from the interplay between physical forces and biochemical signaling. Examples of such shapes include spherical blastocysts, ellipsoidal embryos, or cylindrical vessels.

<sup>1</sup> Matthew Good’s commentary provides an insightful perspective on the complexity involved in building cells from interacting molecules. Meanwhile, Xavier Trepat argues that a bottom-up approach does not fully explain the emergent behavior of higher-level structures and emphasizes the need for constructing tissues at the mesoscale. Trepat uses the analogy of traffic jams to illustrate the importance of considering the collective behavior of cells in tissue engineering (Good and Trepat, 2018).

<sup>2</sup>. Thompson writes, ‘...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.’ (Thompson, 1979)

After considering the desired form and function of the structure, established cell lines are selected and synthetic structures are constructed using various techniques, such as geometry control and localized folding, as discussed in the [2.3](#) section. The resulting structures can be further studied to understand the interplay between physical forces and biochemical signaling, as well as their potential applications in various biological systems.

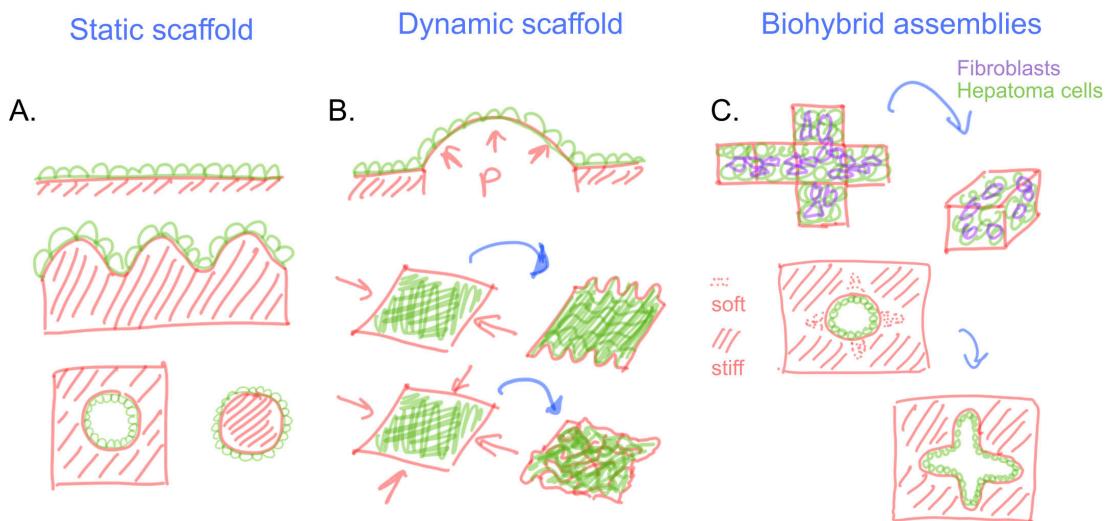
### 4.2.1 Controlling geometry and physical forces

From an engineering perspective, scaffolding is a commonly used approach for constructing synthetic epithelial structures. Scaffolds can be generated through 3D printing or microfabrication techniques, and cells can then be seeded onto the scaffold to attain the desired shape (*Torras et al., 2018*). This method allows for the creation of a well-controlled microenvironment for the cells in terms of geometry, stiffness, adhesion proteins, and cell culture media (see fig [4.2 A](#)) . Structures generated through this approach can be utilized to investigate tissue behavior in response to forces and curvature.

For instance, cells can be used to form a micro-vessel using a hydrogel with a cylindrical hole (*Dessalles et al., 2021*). The hydrogel and cells were housed in a microfluidic device that controlled pressure and flow in the vessel, and the authors were able to examine the role of hydrogel poroelastic properties in regulating the dynamics of the vessel. Another exciting study demonstrated the potential of epithelial tissues to form shape-programmable materials by using a collagen scaffold (*Mailand et al., 2022*).

Scaffolds can also be designed to dynamically change their shape (see fig [4.2 B](#)). For example, a cell monolayer on a flexible membrane can alter its curvature (*Blonski et al., 2021*), and a combination of stretching and unstretching a cell-laden hydrogel can produce distinctive folds and patterns (*Chan et al., 2018*). In some cutting-edge studies, researchers have utilized 4D bioprinting, where 3D printed objects undergo transformation over time (*Arif et al., 2022*). For instance, a flat hydrogel sheet containing endothelial cells and photo-crosslinking can be transformed into a tube (*Zhang et al., 2020*).

Additionally, the contractility of fibroblasts and hepatoma cells has been utilized to fold 2D structures into 3D shapes (*He et al., 2018*) (see fig [4.2 C](#)) . Microplates with an origami folding pattern are created, and the cells apply forces to generate a 3D structure. In other scenarios, cells are allowed to self-organize through the imposition of geometric constraints, which enhances the efficiency of organoid-like systems (*Gjorevski et al., 2016*). In the case of intestinal organoids, controlling the stiffness of the matrix in specific regions leads to growth and differentiation at softer areas, producing a highly reproducible structure (*Gjorevski et al., 2022*).

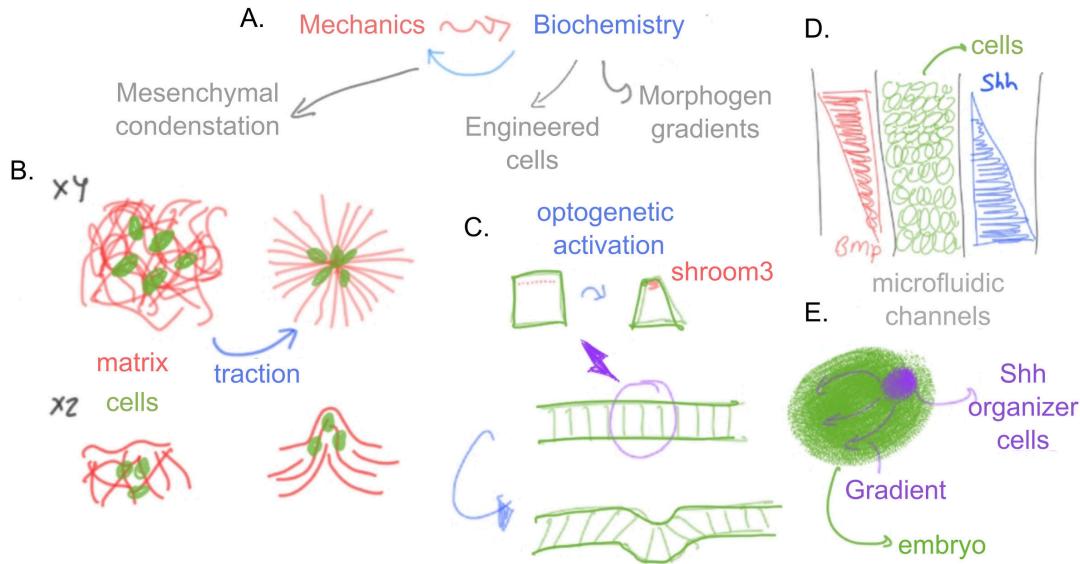


**Figure 4.2: Controlling geometry and physical forces:** The concept of scaffolding can be divided into two categories: static and dynamic scaffolds. (A) Static scaffolds are microfabricated structures that cells can adapt to and respond to geometrical cues, leading to the formation of a specific tissue organization (Brassard *et al.*, 2021). (B) In contrast, dynamic scaffolds consist of cell-laden matrices that are deformable, and their curvature can change dynamically due to external pressure or mechanical forces (Blonski *et al.*, 2021, Chan *et al.*, 2018). (C) Biohybrid assemblies can incorporate active contraction or pushing to create hybrid structures, such as origami folding triggered by fibroblast contraction (He *et al.*, 2018), or cells carving out an intestinal crypt-like geometry from a softer matrix (Gjorevski *et al.*, 2016).

#### 4.2.2 Manipulating biochemical signaling

Another approach to constructing biological structures involves controlling biochemical signaling to induce shape transformation. This approach utilizes natural processes in embryo morphogenesis, such as apical constriction in ventral furrow formation or cell jamming in the normal elongation of the zebrafish. Optogenetic tools, such as controlling Rho signaling, can be used to induce localized apical constriction with spatiotemporal control (Izquierdo *et al.*, 2018). This technique can also be applied to other proteins, such as Shroom3, to induce synthetic morphogenesis in neural organoids (Martínez-Ara *et al.*, 2022) (see fig 4.3 C).

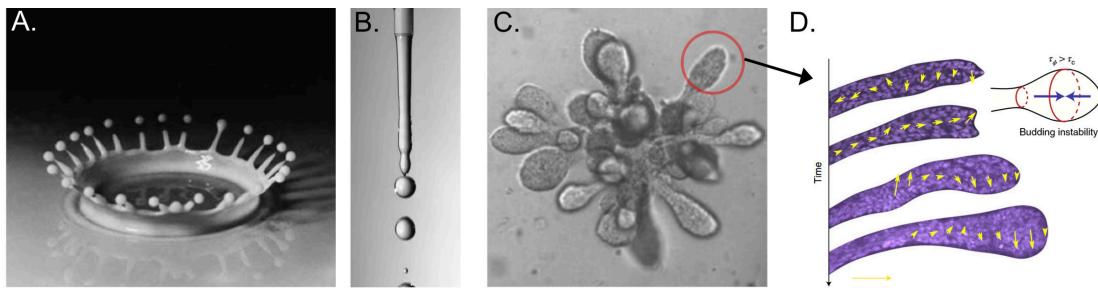
Epithelial-mesenchymal interaction is another crucial aspect of the tissue folding process. Hughes *et al.* demonstrated that cell clusters can remodel the matrix to create oriented stresses that lead to budding in tissues (Hughes *et al.*, 2018). By controlling the location and density of these cell clusters, it is possible to manipulate the curvature of the epithelia. Mesenchymal condensation serves as a folding template for the final tissue structure (Palmquist *et al.*, 2022, Shyer *et al.*, 2017) (see fig 4.3 B).



**Figure 4.3: Manipulating biochemical signaling:** Biochemical signaling and mechanics are interdependent in morphogenetic processes (A). The transport of signaling molecules can affect the cytoskeleton and mechanical properties of cells, while mechanical forces can also influence biochemical signaling. Microfluidics (D) is one method used to control biochemical signaling by providing opposing morphogen gradients through multiple channels (Demers *et al.*, 2016). Alternatively, cells can be genetically engineered to undergo apical constriction (C) or produce morphogen gradients (E) locally to form curved geometries (Cederquist *et al.*, 2019, Martínez-Ara *et al.*, 2022). Mesenchyme condensation (B) is another approach used to program curvature in developing tissues (Hughes *et al.*, 2018, Palmquist *et al.*, 2022).

The microenvironment plays a critical role in providing vital signals to tissues and can be manipulated to activate specific cellular functions. Microfluidic techniques can deliver appropriate morphogen gradients to the tissue with precise timing (Hofer and Lutolf, 2021). *In vivo*, multiple morphogens often act simultaneously. For instance, during neural tube development, there is an opposing gradient of sonic hedgehog (SHH) and bone morphogenic protein (BMP). With microfluidic devices, stable gradients can be generated, even in opposite directions (Demers *et al.*, 2016), thus mimicking symmetry-breaking events and directional neural tube patterning (see fig 4.3 D).

Moreover, genetic engineering of specific cells can be utilized to control signaling. Human pluripotent stem cells (hPSCs) can be programmed to express SHH (Cederquist *et al.*, 2019) (see fig 4.3 E). Mixing these cells with others could result in a polarized organoid and a patterned cerebral organoid.



**Figure 4.4: D’Arcy Thompson compares biological budding to splashes** (A) of fluids and Rayleigh-Plateau instability (Thompson, 1979) (B), where liquid splits up into smaller droplets. This mechanism could also be seen in organogenesis of mammary tissue (C, D) (Fernández *et al.*, 2021).

#### 4.2.3 Exploiting mechanical instabilities

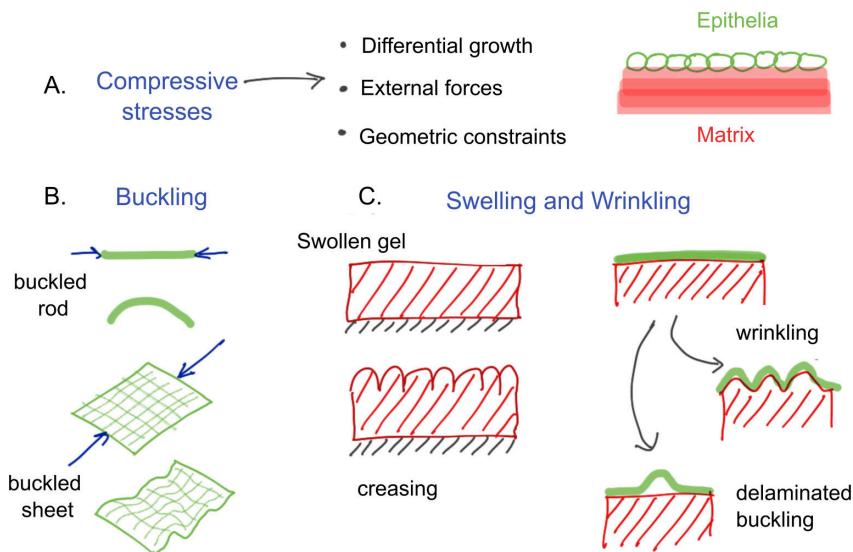
Morphogenesis, the process of shaping and formation of biological structures, often involves spontaneous pattern formation or symmetry-breaking events (Ishihara and Tanaka, 2018). These processes are often dictated by mechanical instabilities, which can lead to large deformations in soft matter systems. In material science, these instabilities are typically seen as problematic as they cause rapid breakage. However, in soft matter, large deformations can lead to interesting topological transformations, providing an opportunity for engineers to exploit these instabilities in the development of new actuators or soft robots (reviewed in (Pal *et al.*, 2021)).<sup>3</sup>

The significance of mechanical instabilities was foreseen by D’Arcy Thompson in his comparison of fluid splashes to hydrooids (see fig 4.4 A). He wrote that the shapes of a potter’s cup, glass blower’s bulb, and biological structures are simply glorified splashes formed slowly under conditions of restraint that enhance or reveal their mathematical symmetry (Thompson, 1979).<sup>4</sup> This conjecture has been confirmed through numerous quantitative studies on various systems, including ripples in leaves and wrinkles in the brain (Karzbrun *et al.*, 2018, Liang and Mahadevan, 2009).

There are various instabilities associated with solids and fluids. For example, the Rayleigh-Plateau instability explains why a fluid stream breaks into smaller packets, driven by the fluid’s tendency to minimize its surface area due to surface tension. The same instability can arise when fluid is surrounded by an elastic medium, instead of air, provided the surface

<sup>3</sup>“Mechanical instabilities have provided a unique approach to imbue “material intelligence” into soft machines without requiring the addition of rigid components. For example, binary actuators relying on mechanical instabilities can recreate logic modules and reproduce valving functionality using entirely soft elements.

<sup>4</sup>I cannot recommend enough the chapter “the forms of cell”. He states “Many forms are capable of realization under surface-tension, ... The subject is a very general one; it is, in its essence, more mathematical than physical; it is part of the mathematics of surfaces, and only comes into relation with surface-tension because this physical phenomenon illustrates and exemplifies, in a concrete way, the simple and symmetrical conditions with which the mathematical theory is capable of dealing.”



**Figure 4.5: Compressive stresses** occur frequently in many systems (A). We can consider epithelia and matrix as thin sheet supported by a compliant substrate. Thus, the tissue folding could be understood as buckling of sheets (B) or wrinkling or creasing of thin film supported by an hydrogel (C).

tensions can overcome the elastic stresses, leading to budding as observed in alveogenesis in human mammary tissue (Fernández *et al.*, 2021) (see fig 4.4 C-D). However, as tissues are active viscoelastic materials surrounded by viscoelastic medium, the timescales of these instabilities change, slowing down to hours instead of milliseconds in water droplets.

There are several types of mechanical instabilities associated with solids and fluids, including Rayleigh-Plateau instability, Kelvin-Helmholtz instability, Rayleigh-Taylor instability, viscous coiling and folding, and large-scale wrinkling and buckling (Gallaire and Brun, 2017, Kourouklis and Nelson, 2018). In this study, we aim to harness these instabilities to recreate epithelial structures.

Applying compressive stresses is one of the easiest ways to induce mechanical instabilities in solids. These stresses can occur in biological systems as a result of differential growth, swelling, or morphogen gradients and can lead to various forms of instabilities, including wrinkling, creasing, and buckling. Buckling occurs when a thin sheet is subjected to in-plane compressive stress, and if the stress is above a critical value, the sheet undergoes out-of-plane deformation instead of in-plane shrinkage (see fig 4.5 B). In contrast, wrinkling and creasing occur in similar compressive stresses, but the thin sheet is typically supported by a compliant substrate.

The creation of biological tissues *in vitro* has been a subject of great interest in the field of tissue engineering. To reproduce the characteristics of these tissues, researchers have turned to the use of hydrogels. These materials can be mechanically and chemically manipulated to simulate the behavior of biological matrices, which provide support for epithelial structures.

One of the ways in which hydrogels can be used to recreate the behavior of biological tissues is through the application of physical stress. For example, swelling of the hydrogel can cause it to undergo rapid large volumetric changes, producing crease-like patterns on the surface. If the hydrogel is constrained at the bottom, these creases can become permanent. Alternatively, if the hydrogel is supported by another flexible material, such as another hydrogel or an elastic substrate, the stresses produced during swelling will result in a wrinkling instability (see fig 4.5 A). These instabilities are important for understanding the formation of a variety of structures, including the gyrification of the brain cortex.

In a study by Tallinen et al., the gyrification of the brain cortex was replicated through the programming of materials to produce wrinkling (Tallinen *et al.*, 2016). The researchers created a synthetic brain with an inner core of an inert elastomer and an outer layer of a swellable elastomer. On swelling, the outer layer produced folds that closely matched the process of gyrification (see fig 4.6 A).

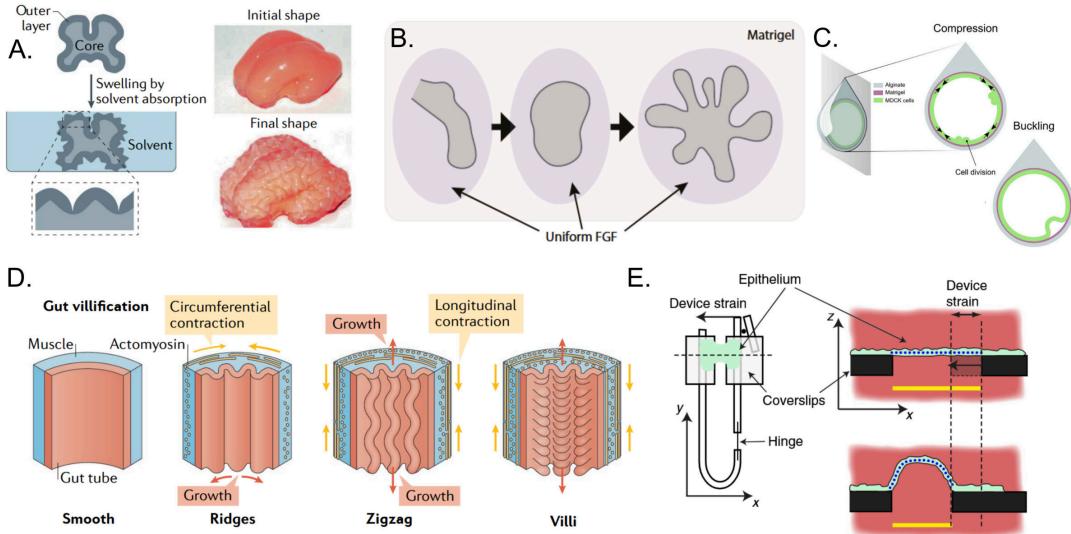
Similar mechanisms have been observed in other systems undergoing differential growth, such as the branching of lungs and formation of intestinal villi (Shyer *et al.*, 2013, Varner *et al.*, 2015) (see fig 4.6 B,D). These findings highlight the potential of hydrogels as a tool for understanding the physical mechanisms underlying tissue development. However, it is worth noting that the mechanisms described here are the subject of ongoing research and debate in the field of developmental biology.

The ability to recreate biological tissue growth conditions *in vitro* has been made possible through the use of hydrogels. Researchers have discovered that by mechanically and chemically controlling the hydrogel, they can generate desired mechanical instabilities (Dervaux and Amar, 2012). This can be accomplished through the swelling or pre-stretching of the gel, or by manually applying compressive stresses.

One way to simulate growth is through the direct stretching or compression of the gel. Chan et al. showed that the patterns produced can be controlled by modulating the shear modulus of the hydrogel with the epithelial layer and stretch (Chan *et al.*, 2018) (see fig 4.5 B). By pre-stretching the hydrogel before seeding cells, they were able to produce folded patterns with different wavelengths depending on the type of pre-stretching applied (uniaxial or biaxial).

Another type of instability in bilayers is delaminated buckling, which is often observed in thin film delamination in furniture. This can be induced through compressive stresses created during growth or collective tension. Recent studies have shown that growing epithelia confined in a sphere undergo delaminated buckling after reaching a critical growth-induced stress (Trushko *et al.*, 2020) (see fig 4.6 C), or through intercellular stresses (Oyama *et al.*, 2021) or by placing a biofilm on top of the epithelial monolayer (Cont *et al.*, 2020).

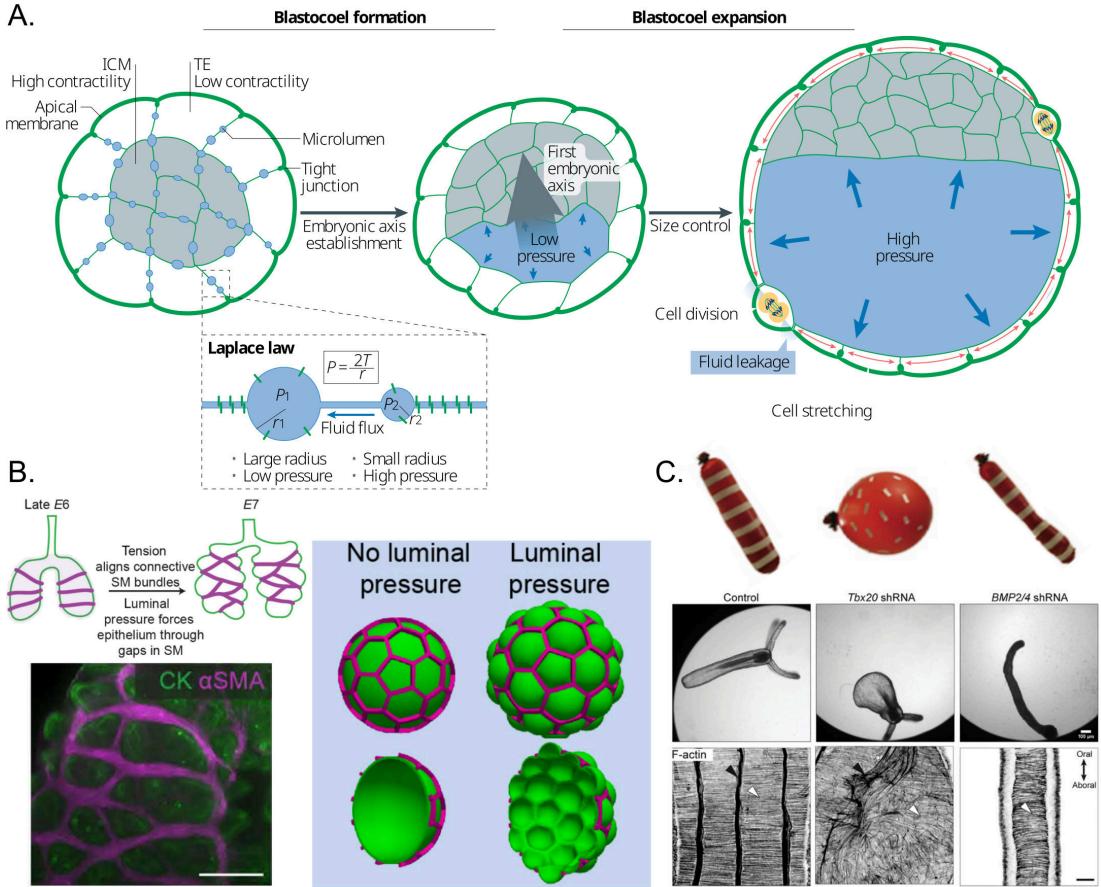
The formation of the ventral furrow in the drosophila embryo can also be considered as a buckling event. Although there are multiple explanations for this phenomenon, recent studies



**Figure 4.6: Examples of mechanical instabilities:** (A) Synthetic mini brains illustrate the wrinkling of the outer layer with swelling mimicking gyration (Tallinen *et al.*, 2016). (B, D) Other way around where inner layer of lung or intestinal epithelia develops folds when embedded into a hydrogel or muscle shell (Shyer *et al.*, 2013, Varner *et al.*, 2015). (C) It is also shown that simple epithelial tissues embedded into a shell would also buckle (Trushko *et al.*, 2020). (D) (Wyatt *et al.*, 2020) used matrix independent tissue with compression to illustrate that the epithelial tissue itself can undergo buckling. Panel A, D are adapted from (Collinet and Lecuit, 2021) and C from (Matejčić and Trepat, 2020)

have shown that the instability leading to the fold is caused by embryo-level forces (Fierling *et al.*, 2022, Guo *et al.*, 2022). Apart from instabilities, it is remarkable that the mechanical information can be encoded in the substrate. For instance, the tension produced by the cells in a pre-stretched membrane, on cutting would lead to curling (Tomba *et al.*, 2022), or through stretching a suspended epithelial layer would also do the same(Fouchard *et al.*, 2020).

It is noteworthy that there is currently only one established method for directly applying compressive stresses to suspended epithelial tissue. The Lab of Guillaume Charras has developed a technique using a cell-laden collagen gel sandwiched between two rods, where the gel is digested with collagenase to create a suspended monolayer (see fig 4.6 E). Through extensive experimentation, they have observed that the compression of more than 35% strain produces transient buckling events (Wyatt *et al.*, 2020). Importantly, the actin cytoskeleton plays a crucial role in buffering deformations in this system.



**Figure 4.7:** **Tissue hydraulics** plays an essential role in establishing (A) embryonic axis through lumen coarsening, and later the pressure regulates the size of the embryo. Laplace's law acts on the spherical cavities between cells to the whole blastocyst (Chan *et al.*, 2019, Collinet and Lecuit, 2021, Dumortier *et al.*, 2019). (B) Interestingly, if the inflated structure is surrounded by a mesh you see a stressball effect, where material inflates through the mesh. Similar phenomena is visible in growth and inflation of the lizard lungs. The smooth muscle constrains the deformation leading to stressball morphogenesis (Palmer *et al.*, 2021). (C) In cnidarians, the different orientation of F-actin leads to different shapes of the organism (Stokkermans *et al.*, 2022).

## 4.3 Tissue hydraulics

### 4.3.1 Hydraulic control of morphogenesis

In this thesis, we focus on the role of hydraulic pressure in morphogenesis. It has been well established in the field of developmental biology that fluid pressure plays a significant role in lumen expansion. For instance, in the mouse embryo, cell aggregates form small fluid cavities in intercellular junctions, which grow and coalesce into a large lumen, breaking the

symmetry of the embryo, due to the presence of an osmotic pressure gradient ((Dumortier *et al.*, 2019); reviewed by (Torres-Sánchez *et al.*, 2021), see fig 4.7 A). This process is powered by the pumping of ions and water by the cells, which generates pressure in the fluid-filled cavities, ultimately leading to the formation of spherical embryos. For any inflated spherical shell, the relationship between pressure ( $\Delta P$ ), curvature ( $R$ ), and surface tension ( $\sigma$ ) can be described by Laplace's law.

$$\sigma = \frac{\Delta PR}{2}.$$

The shape that is created under pressure depends on the material properties of the tissue. For example, a homogeneous material would create a uniform curvature, such as a spherical shape, while an anisotropic tissue with oriented cells would result in various shapes, such as cylinders or ellipsoids (Stokkermans *et al.*, 2021) (see fig 4.7 C). An interesting example of this phenomenon can be seen in the lobed epithelium of lizard lungs, which resembles the shape of a stress ball. Palmer *et al.* propose that the smooth muscle network functions as a mesh that constrains the epithelium, much like the outer layer of a stress ball (Palmer *et al.*, 2021) (see fig 4.7 B). Upon the application of pressure, the epithelium inflates in the regions between the gaps in the muscles.

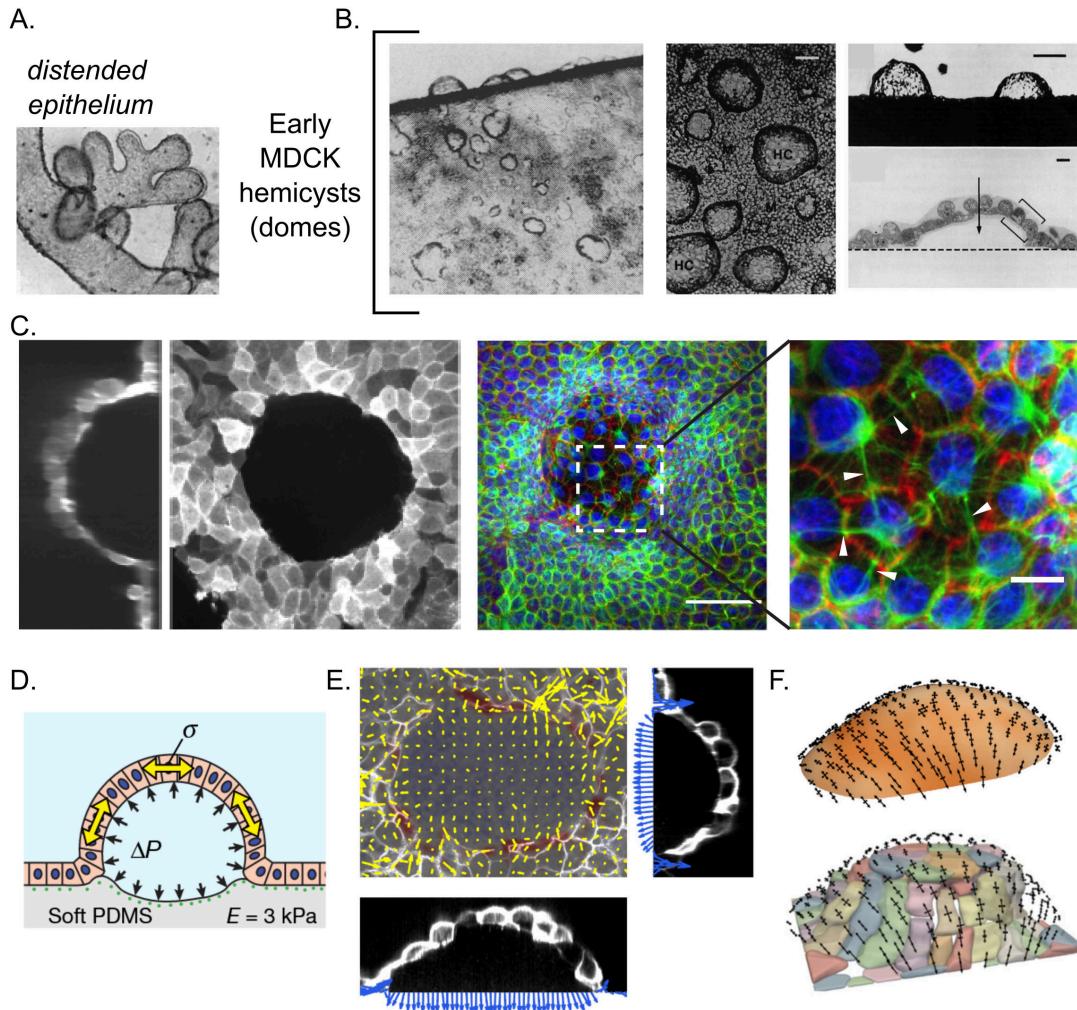
For embryos, an increase in pressure results in an increase in tension and stretching of the cells. Once a certain threshold is reached, the cell junctions may leak, causing a reduction in luminal pressure and shrinkage of the embryonic cavity. This system of pressure regulation through leakage acts as a mechanism for size regulation (Chan *et al.*, 2019). At the same time, it polarizes the embryo and promotes cell segregation and fate specification (see fig 4.7 A, reviewed by (Chan and Hiiragi, 2020)).

Similar coalescence and lumen coarsening have been observed in other systems (reviewed in (Schliffka and Maître, 2019)). The pressure can also be generated through secretion of the matrix, as seen in the case of the drosophila hindgut with mucins (Syed *et al.*, 2012), or through the secretion of hyaluronic acid in the formation of ear canals in zebrafish otic vesicles (Munjal *et al.*, 2021). Despite numerous *in vivo* experiments, there are very few systems in which epithelial tissue can be subjected to controlled shape and size *in vitro*.

### 4.3.2 Mechanics of domes

Many of the morphogenetic events are called doming because the shape vaguely resemble a spherical cap. For instance, doming of the retina in the eye or zebrafish embryo, or doming during duct formation of mammary or salivary glands. There are typically two mechanisms for these: first, an accumulation of the cells or matrix to create curvature; and second, trans-epithelial transport causing hydraulic pressure-driven shape change. The second kind is remarkable as they mimic various lumenized epithelia *in vivo*.

This is the most pertinent system to the thesis. I would briefly go into the historical



**Figure 4.8: Historical development of epithelial domes:**(A) Distended epithelium was observed in explant cultures in 1930-50s. (B) With MDCK cell line, spontaneously forming domes/hemicysts were characterized (Leighton *et al.*, 1969, Valentich *et al.*, 1979). (C,D) In our lab, shape and size of the domes were controlled with micropatterning adhesion protein (Latorre *et al.*, 2018). The pressure and tension was measured with Laplace's law and traction force microscopy. (E-F) For non-spherical domes, curved monolayer stress microscopy technique was implemented by segmenting the dome shape (Marín-Llauradó *et al.*, 2022).

developments in dome mechanics.

Fluid-filled dome formation in epithelial tissue culture has been recorded since 1933 (Cameron, 1953) (see fig 4.8 A). After several decades alongside the development of cell culture techniques, microscopy, and MDCK cell line <sup>5</sup>, in 1968, Leighton and colleagues observed that

<sup>5</sup>It is very important to acknowledge the contribution of Madin-Darby canine kidney (MDCK) cells to the field of mechanobiology and enhancing our understanding of tissues *in vitro*. Stewart H. Madin and Norman B. Darby,

the confluent MDCK cell monolayers formed hemispherical blisters (domes) (Leighton *et al.*, 1969) (see fig 4.8 B). They observed that these are different from renal tubules because the apical surface, with microvilli, was facing outwards. They saw that these fluid-filled structures are dynamically changing size and curvature. They would burst to deflate and leak fluid out in the medium (Valentich *et al.*, 1979). After sometime, they could heal and form the dome again. Later, other cell lines derived from mammalian and amphibian kidneys were often observed to form domes too (Dulbecco and Okada, 1980, Leighton, 1981, Lever, 1979)

Now the mechanism is clear as the epithelial cells perform critical barrier function alongside controlling the transepithelial flow of ions and water. It was shown that hindering sodium-potassium ion pumping reduces the likelihood of domes (Leighton *et al.*, 1969). Thus, on forming a confluent monolayer these cells perform their function of pumping ions from apical to basal direction (Valentich *et al.*, 1979). If the substrate is solid and impermeable the tissue accumulates enough pressure to delaminate and form a spherical structure.

Most domes observed have been spherical and circular in footprint, indicating uniform tension across the dome. This can be explained by considering the dome as a thin shell under pressure, similar to a bubble, and following Laplace's law. Early studies attempted to infer tension through geometry and pressure measurement (Tanner *et al.*, 1983), finding that the pressure was of the same order as physiological vessels (see fig 4.9 A-B).

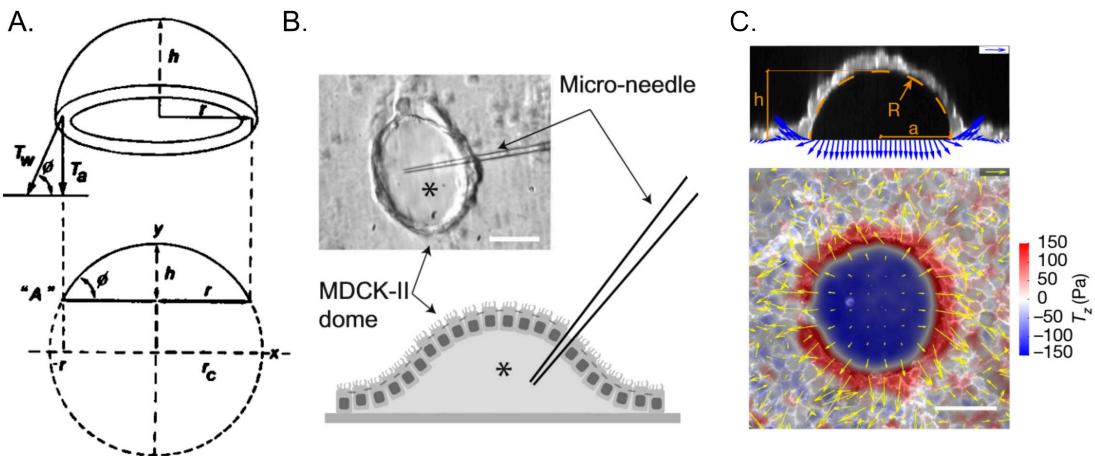
One study (Popowicz *et al.*, 1986) identified a "dome curve" when the frequency of domes was plotted against size, observing three classes of domes in terms of size. Smaller domes were observed to swell and increase in size. It was also suggested that there could be different subpopulations of MDCK cells. In the 1990s, many strains were characterized that formed different inflated structures, ranging from normal domes to tubules (Klebe *et al.*, 1995). One cell line, called super dome MDCK, formed larger domes.

Despite research into ion transport, hormone signaling, the role of tight junctions, and external shear stress, the understanding of the mechanics of domes and pressure has remained stagnant due to the lack of tools for measuring tension, pressure, and controlling the shape and size of these structures.

The work of Ernest Latorre in our laboratory has led to the development of a system for controlling the size of domes and studying the relationship between tension and pressure (Latorre *et al.*, 2018) (see fig 4.8 C-D). By utilizing protein patterning techniques, Latorre was able to create non-adhesive circular regions on soft PDMS gel, which, when seeded with MDCK cells, led to the formation of domes. The gel was embedded with beads to allow for the calculation of traction forces and pressures exerted by the monolayer (see fig 4.9 C). This system allowed for a deeper understanding of the rheology of tissue and the role of the cytoskeleton. He observed that stretching the actin cortex leads to dilution, and that tension reaches a stable

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Jr. isolated female cocker spaniel dog's kidney tubules cells in 1958. MDCK cells can self-organize in 2D and 3D; form monolayers and stratified layers; and undergo collective migrations. These cells are incredibly robust for experimentation.



**Figure 4.9: Methods for measuring pressure and tension:** (A) Earlier studies tried to estimate tension through geometry and thickness of the monolayer (Tanner *et al.*, 1983). (B) Later, pressure was measured by puncturing the dome with a micro-needle. However, the measurement of pressure is static, because the dome deflated after the puncturing (Choudhury *et al.*, 2022b). (C) Traction force microscopy technique provides a viable non-invasive solution for measuring pressure under to domes (Latorre *et al.*, 2018).

value regardless of strain. He also observed the surprising phenomenon of superelasticity, where cells are heterogeneously stretched in the dome when tension is uniform. To further understand the role of actin and keratin bundles in providing superelasticity, Latorre *et al.* developed a vertex model through which they could understand the instability triggered by actin dilution, and rescued by intermediate filaments.

Ariadna Marin-Llaurado extended Latorre's work by examining domes of varying sizes and shapes (see fig 4.8 E). This study found that different-sized spherical domes have similar tensions, and that pressure is compensated according to curvature. Marin-Llaurado couldn't rely on a simple formula for tension calculation, because the tension in non-spherical domes is non-uniform (Marín-Llauradó *et al.*, 2022). They used confocal microscopy to map dome curvature and calculated stresses computationally using a novel method called cMSM (curved Monolayer Stress Microscopy) (see fig 4.8 F). This method infers stresses just through geometry and pressure as in Young-Laplace relation. It does not need to make any assumptions related to material properties. The results showed that cells tended to align along the principal stress direction.

The mechanics of osmotic and hydraulic gradients are also crucial to understand. Chaudhary *et al.* demonstrated that kidney cells act like a mechanobiological pump (Choudhury *et al.*, 2022b). Using a two-layer microfluidic chip, the team was able to measure and apply pressure differences across an epithelial monolayer and observe that the tissue acted like a mechanical pump that stalls at high pressure. Remarkably, they discovered that diseased kidney cells

pump in a different direction than healthy ones. They were able to control both osmotic and hydraulic pressure. Another study by Ishida-Ishihara et al. investigated the connection between osmotic pressure and extracellular matrix swelling (Ishida-Ishihara *et al.*, 2020). The researchers found that osmotic gradients trigger Aquaporin transport channels, leading to dome formation through Matrigel swelling. However, these domes are gel-filled structures that differ from fluid-filled domes.

MDCK domes provide a model system for studying transport, cell fate, and tissue dynamics with a curvature. However, control over luminal pressure in these structures remains a challenge.



# Chapter 5

## Structure of the thesis

### 5.1 What is to be done?

Morphogenesis refers to the process of tissue deformation or growth, which results from the combination of both endogenous and exogenous mechanical forces (Collinet and Lecuit, 2021, Valet *et al.*, 2022). These forces may arise from the contractility of the epithelium and the surrounding matrix, as well as hydraulic pressure from the lumen (Chan and Hiiragi, 2020, Torres-Sánchez *et al.*, 2021). The various stresses act on different components of the tissue, such as cells and the extracellular matrix, which exhibit unique viscoelastic properties and remodeling time scales (Ambrosi *et al.*, 2019, Cavanaugh *et al.*, 2020, Kelkar *et al.*, 2020). However, comprehending how these stresses interact with viscoelastic properties to bring about particular morphogenetic events *in vivo* presents significant technical and conceptual challenges. These obstacles include disentangling the roles played by distinct components in a system, a lack of tools for quantitative measurements of stresses and mechanical properties, and an inability to apply controlled stresses over a wide range of amplitudes and rates.

In response to these challenges, bottom-up approaches have emerged as a complementary strategy for understanding the morphogenetic potential of individual components and building complex, functional tissues (Ingber, 2018, Trentesaux *et al.*, 2023). These approaches have been successful in engineering basic morphogenetic processes such as epithelial bending or buckling (Matejčić and Trepat, 2022). However, even though bottom-up approaches are proving to be successful, we still need tools that can measure and control the shape and stress of 3D epithelia simultaneously. Additionally, 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.

This thesis seeks to address these gaps in knowledge by investigating the mechanics of epithelial tissues. A comprehensive understanding of the principles that govern tissue form and function is essential for both advancing our understanding of fundamental physical rules

in biology and inspiring new engineering tools and design principles. To achieve this, we leverage cutting-edge technologies, such as 3D printing, microfluidics, and 3D cell cultures, to individually control morphogenetic driving factors.

Our approach provides a material science perspective for probing the intricate mechanisms involved in the generation of forces and shape changes at the cellular and tissue levels, and holds promise for discovering emergent phenomena and enabling the building of novel tissue forms and assemblies.

## 5.2 Objectives

### General aim of the thesis

This thesis aims to investigate the mechanics of epithelial layers under controlled pressure.

### Specific aims of the thesis

General aims are divided into specific goals:

1. Develop a novel technology for constructing three-dimensional epithelia using lumen pressure control.
2. Characterize the material response of the pressurized epithelial tissue.
3. Explore the mechanics of epithelial folds.

## 5.3 Thesis outline

Results are presented in Part 2 with four chapters that address the specific aims of the thesis and provide a understanding of the mechanics of epithelial layers subjected to controlled pressure.

- Chapter 6 details the construction of an experimental system designed to physically control epithelial monolayers. This chapter showcases the main result of the PhD, a novel microfluidic system that generates 3D epithelia with controlled pressure and shape. The chapter highlights the successful development of the microfluidic system, while also summarizing any failed or attempted methods used in constructing the device.
- Chapter 7 focuses on using the microfluidic device to understand epithelial mechanics. The chapter reports the results of rheological experiments and relates them to a computational framework that explains the observed phenomenology in terms of the viscoelasticity of the actomyosin cortex.
- Chapter 8 describes a buckling instability in pressurized epithelia. It is found that rapid deflation produces a buckling instability that leads to the formation of epithelial folds. Buckling occurs across different length scales to overcome compressive stresses, and folding patterns become more complex with increasing size. The chapter discusses the potential of guiding the folds by controlling the shape and size of the epithelia.
- Finally, in Chapter 9, the findings are summarized with a list of conclusions along with a brief discussion on future perspectives of this thesis.

In summary, the thesis presents a microfluidic-based technique to impose a controlled deformation on an epithelial monolayer while continuously monitoring its state of stress. This technique allows for investigation of the active viscoelasticity of epithelial layers over physiological time scales. The thesis also presents a 3D model of the epithelium, developed by Adam Ouzeri and Marino Arroyo, which explains the observed phenomena using the active viscoelastic properties of the actomyosin cortex. Furthermore, it is demonstrated that these viscoelastic properties, along with adhesion micropatterning, can be utilized to engineer epithelial wrinkles with predictable geometry. The results provide an understanding of the mechanics of epithelial layers subjected to controlled pressure and showcase the potential of the developed techniques to further explore the synthetic morphogenesis.

## **Part II**

# **Results**



# **Chapter 6**

## **A microfluidic device for generating 3D epithelia**

### **6.1 Introduction**

To generate three-dimensional epithelial structures in vitro from planar epithelial monolayers, we chose to utilize an existing system of epithelial domes (spontaneous domes) developed by Ernest Latorre and improved by Ariadna Marín-Llauradó (Latorre *et al.*, 2018, Marín-Llauradó *et al.*, 2022). This system involves seeding a Madin-Darby canine kidney (MDCK) cell monolayer on a substrate that is patterned with circular non-adhesive regions. The cells invade these regions and form a cohesive monolayer everywhere within 24 to 48 hours. Due to the active ion pumping mechanism of the MDCK cells in the apical-to-basal direction, the cells delaminate from impermeable substrates such as glass or soft PDMS gel and form spherical cap structures on the circular patterns, known as epithelial domes. Latorre and Marín-Llauradó demonstrated that they could form a variety of structures with controlled shape and size, ranging from spherical to tubular caps.

This system also enables the use of 3D traction force microscopy to measure pressure. The technique involves measuring the deformation of a soft PDMS gel embedded with beads to characterize the forces and pressures applied by the cells on the substrate. This method offers an innovative approach to measuring pressure compared to the previous technique of puncturing epithelial domes with a microneedle (Choudhury *et al.*, 2022b, Tanner *et al.*, 1983). It also allows for the characterization of the rheology of epithelia and the discovery of interesting material properties such as the superelasticity of cells during stretching (Latorre *et al.*, 2018).

However, the formation of epithelial domes is dependent on the ion pumping mechanism of the domes, making them spontaneous structures. Therefore, the timescales for the dome

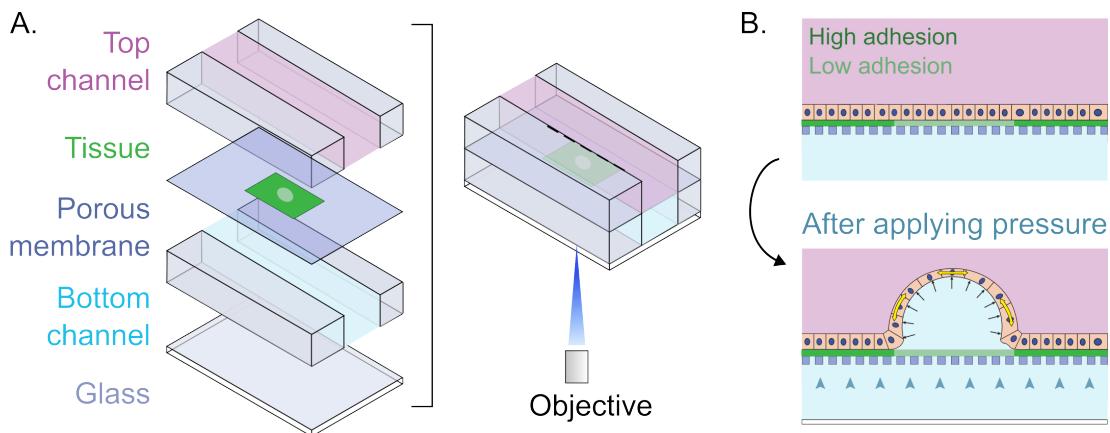
stretching are not controlled. This process can be marginally accelerated by a few hours through the use of drugs like Forskolin, which can activate transepithelial channels of  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  (Bourke *et al.*, 1987, Klebe *et al.*, 1995). However, to build and physically control the epithelial structure, pressure control is necessary. In this chapter, we will be discussing a microfluidic chip that can inflate an epithelial monolayer into a dome while also allowing us to measure and control the forces involved.

## 6.2 Monolayer Inflator

Drawing inspiration from the pioneering work on organ-on-chip microfluidic devices, we have deemed these platforms to be an ideal system for the precise manipulation of pressure, cell culture conditions, and the acquisition of high-resolution imaging data (Huh *et al.*, 2010, Nelson *et al.*, 2017). An illustrative example is the lungs-on-chip device, which comprises two distinct layers separated by a porous membrane. The top layer contains a channel for the epithelial cells, while the bottom layer has a channel for the endothelial cells. This device is assembled on a thin glass slide, which facilitates the collection of high-quality imaging data.

Therefore, we conceived the idea of a MONoLayer Inflator (MOLI) device, which utilizes a two-layer microfluidic channel with one side for epithelial monolayers and the other for the application of pressure (see fig 6.1). The epithelial monolayer side is micropatterned with a protein that contains non-adhesive or less-adhesive regions for dome formation. Our working hypothesis postulated that cells would adhere to the protein substrate uniformly, even in regions with lower adhesive properties. We anticipated that upon the application of pressure, cells would detach from the regions with weaker adhesion, leading to the formation of a dome-shaped structure.

We attempted to fabricate the devices by utilizing plastic stickers and photopolymerizable adhesive, but encountered difficulties such as fluid leakage and limited biocompatibility, rendering them unsuitable (Bartolo *et al.*, 2008, Sollier *et al.*, 2011). Consequently, we opted for the utilization of PDMS material to construct the microfluidic chip due to its facile handling and processing characteristics.



**Figure 6.1: Conceptual design of MOLI:** (A) Microfluidic device consist of a porous membrane sandwiched between two layers of schannels. (B) Upon application of pressure, cells from low adhesion will detach to form an epithelial dome.

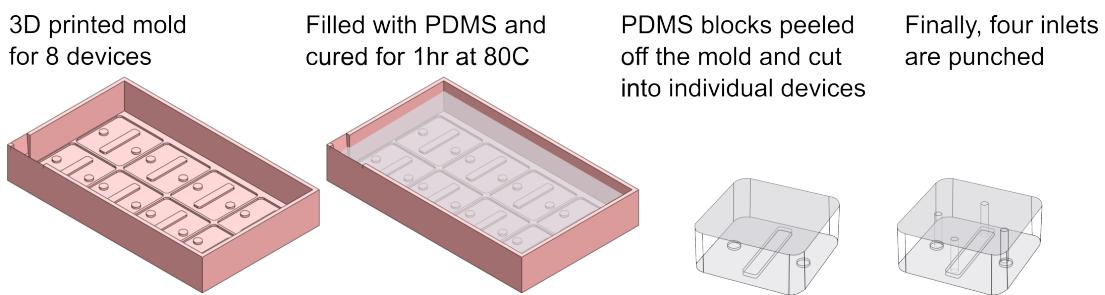
### 6.3 Fabrication of the device

The structure of the device consists of four layers: glass, bottom channel, porous membrane, and top channel. These layers are bonded together using ozone plasma activation.

For imaging epithelial structures with high-resolution confocal microscopy, the device must be mounted on a thin glass slide. We used #0 glass slides, which have a thickness range of 85–115  $\mu\text{m}$  and are designed for high-performance microscopy applications.

To ensure that the porous membrane is positioned as close as possible to the microscope objectives, whose working distance typically ranges between 200 $\mu\text{m}$  and 1000 $\mu\text{m}$ , it is crucial that the channel is sufficiently thin. To this end, we fabricated the bottom layer with a thickness of 100 $\mu\text{m}$ . This thickness provides adequate structural support for manual handling, while avoiding potential microfluidic issues arising from pressure loss and lower flow rates. To achieve the desired thickness, we utilized a spin coating method to fabricate a thin layer of polydimethylsiloxane (PDMS). Subsequently, the layer was precisely cut into the channel shape using a desktop cutting machine (Silhouette Cameo 4, Silhouette America).

The primary function of the porous membrane is to enable pressure application while preventing the migration of cells from the cell channel to the pressure channel. Initially, we used a membrane with pores of 10 $\mu\text{m}$  diameter based on literature. We attempted to create 10 $\mu\text{m}$  pores in a 100 $\mu\text{m}$  thin layer of PDMS using photolithography to facilitate manual handling. However, we encountered difficulty in fabricating 10 $\mu\text{m}$  pillars with a height of 100 $\mu\text{m}$  due to an excessively high aspect ratio, making the pillars too fragile and prone to breakage during fabrication (see appendix B). Therefore, we opted to employ plastic, polyethylene terephthalate (PET), membranes with 10 $\mu\text{m}$  pores.

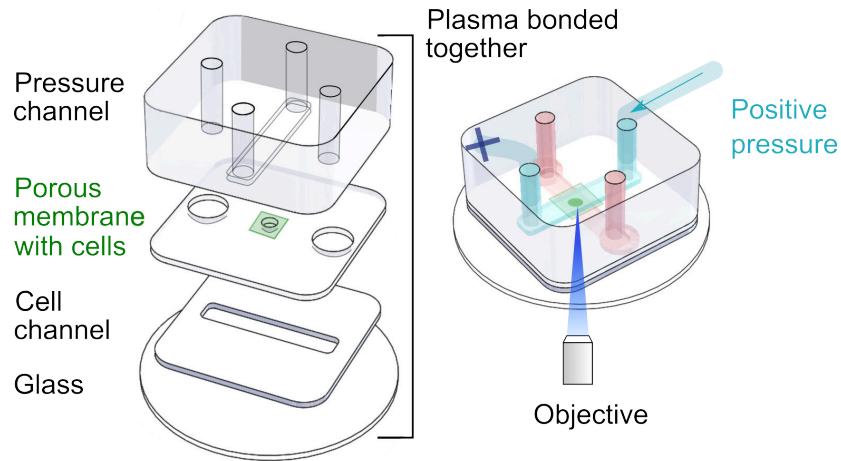


**Figure 6.2: 3D printed mold for the device** patterned to prepare eight devices at a time. The thickness of the PDMS block is controlled with volume of PDMS poured into the mold. After polymerization, the PDMS is cut into individual pieces and inlets are punched with a biopsy punch.

The thin ( $10\mu\text{m}$ ) plastic sheets were easily manageable, but we encountered difficulties with bonding and experienced leakages due to membrane wrinkling. To address these difficulties, we decided to modify the middle layer by using a PDMS layer with a hole attached to a small piece of membrane, instead of whole layer being a porous membrane. This approach allowed us to achieve stronger and leak-proof bonding by sandwiching a smaller area between the two PDMS layers. The middle PDMS thin layer was constructed with a  $1.2\text{mm}$  hole to expose the membrane to pressure, as this dimension is approximately the size of the field of view of a  $10\times$  objective.

The design of the top channel in the device involved a PDMS block with a  $5\text{mm}$  thickness and a  $1\text{mm}$  engraved channel. The decision to select the thickness of the top channel was based on the requirement for the block to be sufficiently thick to accommodate tubing for pressure application. To create the mold with the channel, a precision 3D printer (Solus DLP 3D Printer) was utilized. Additionally, four inlets with a diameter of  $1.5\text{mm}$  were punched into the block using a biopsy punch to facilitate two inlets for the pressure channel and two inlets for cell seeding purposes (see fig 6.2).

Ultimately, the integration of the layers was accomplished through a two-step bonding process utilizing an ozone plasma cleaner (refer to fig 6.3). First, we bonded the glass to the bottom channel and simultaneously bonded the middle layer to the top channel. Following this step, the two assembled layers were bonded together with the membrane sandwiched in the middle to create the final device.



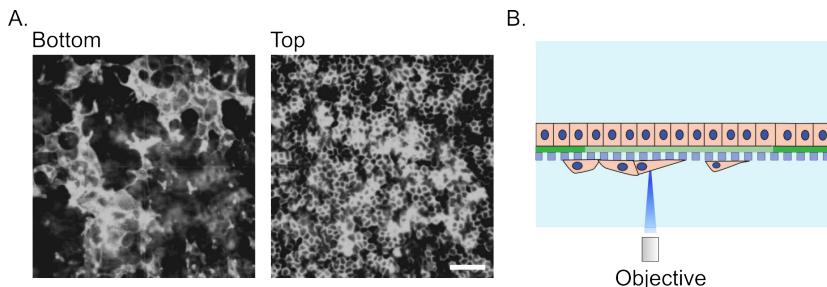
**Figure 6.3: Fabrication of MOLI:** Four layers assembled together with ozone plasma cleaning. Each channels has a inlet and outlet. Only the pressure channel is connected to the tubing; one side connects to the reservoir and other is sealed.

## 6.4 Protein patterning and "upside-down" cell culture

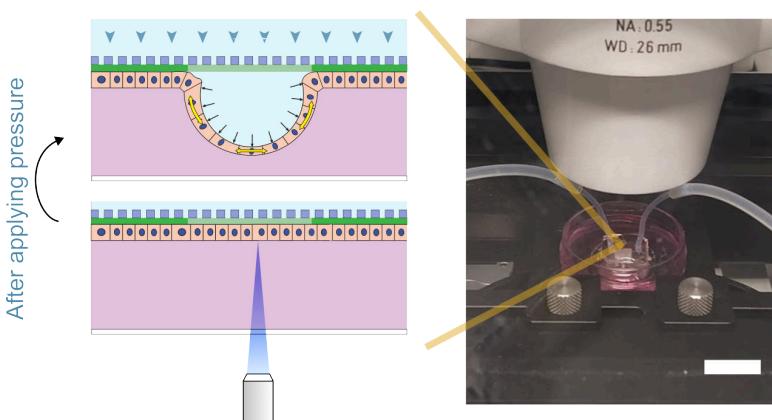
To overcome issues encountered in earlier prototypes, we opted to employ a glass-bottomed dish (35mm, #0 coverslip thickness, Cellvis) as the container for our experimental setup. This design choice was made to address concerns surrounding the potential for cell culture medium to spill over/under the device during pressure application, especially in the case of a leaky device.

In the context of the spontaneous dome system, the upper surface is accessible for various treatments and microcontact printing using a PDMS block. However, in our case, we have a completely sealed device, which necessitated the use of the photopatterning technique known as PRIMO. In brief, first the surface to be micropatterned is coated with poly-L-lysine (PLL) and then SVA-PEG chains. Upon illumination with UV light (375nm), PEG chains in selective regions could be cleaved and subsequently exposed to adhesion-promoting proteins. The PRIMO technique had been optimized previously for substrates made of glass and soft PDMS. We had to optimize the technique for use with a porous plastic membrane, which entailed increasing laser power to 1500mJ/mm<sup>2</sup> (for details see the Appendix A). We also optimized coating the devices with fibronectin, vitronectin and collagen. For the experiments featured in this thesis, we are using fibronectin mixed with fluorescent fibrinogen for finding the samples.

To ensure successful attachment of cells and formation of monolayer, a concentration of  $25\text{-}30 \times 10^6$  cells/mL was seeded for one hour, followed by rigorous flushing with fresh cell culture media to wash away any unattached cells. Early experiments revealed that while cells attached to the top side of the porous membrane, there were very few dome formations upon application of pressure. Additionally, imaging through the porous membrane was poor quality,



**Figure 6.4: Cells filtering through the membrane:** (A) Images of MDCK-CiBN CAAX GFP monolayer on the both sides of the membrane. Scale bar is  $80\mu m$ . (B) Schematic of imaging through the porous layer.

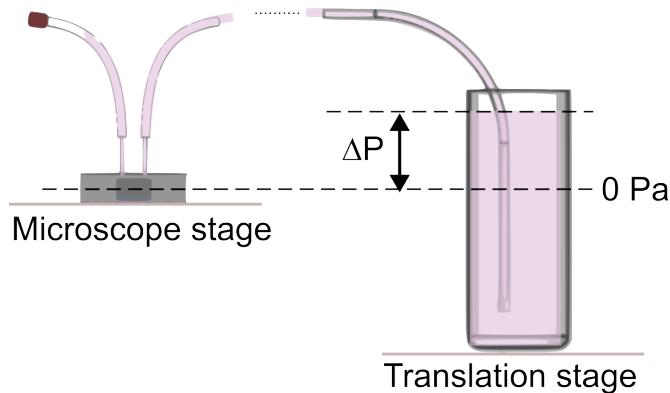


**Figure 6.5:**  
**Upside-down cell culture:** Illustration of upside-down cell culture and the experimental setup on the microscope stage.

as the cells were further away from the microscope objective, and some cells were filtering through the membrane from top to bottom (see fig 6.4).

To prevent cells from crossing the membrane, various plastic membranes with smaller pore sizes (ranging from 50 nm to 10  $\mu m$ ) were systematically tested. Considering the flow rates and cell filtration through the membrane, a 400nm pore size membrane was chosen. However, imaging the green channel (488nm) through these pores was impossible. Therefore, an "upside-down" cell culture approach was implemented, where the device was flipped immediately after seeding cells in the bottom channel to ensure attachment on the membrane instead of the glass (see fig 6.5). Thorough washing of the channel was necessary to prevent cell attachment to the glass, which would have obstructed the imaging of the domes.

Despite optimization efforts, achieving complete coverage of non-adhesive regions with a cell monolayer remained a significant challenge. To address this issue, the protein concentration in these regions was increased to facilitate cell attachment at the designated dome location. Upon application of pressure, cells from the lower adhesion region would detach, resulting in the formation of the dome structure.



**Figure 6.6:**  
**Hydrostatic pressure application:**

The device is positioned on a microscope stage and connected to a reservoir of media, which in turn is attached to a translation stage. By increasing the difference between the device and the air-liquid interface, we can measure and apply hydrostatic pressure.

## 6.5 Pressure control

For the application of external pressure, we selected hydrostatic pressure as the method of choice. Previous studies had reported a pressure requirement of approximately 100Pa, equivalent to 1cm of water column, for the formation of a dome. Initially, pipette tips were employed to apply pressure, but we observed that they were susceptible to bubble formation and leaks. Hence, we switched to using Polytetrafluoroethylene tubing, which was connected to a 50mL reservoir (Falcon tube) to mitigate these issues (see fig 6.6). By adjusting the height of the tube to match the air-liquid interface in the reservoir with the device, zero pressure was exerted on the cell monolayer. To apply pressure, we increased the height of the tube by 2cm, resulting in the application of 200Pa pressure to the monolayer, leading to the delamination of cells and the formation of domes.

Nonetheless, we exercised caution regarding the potential occurrence of bubble formation within the cell channel. To avoid this we subjected the media to a vacuum chamber for 30min prior to the experiment to eliminate any nascent bubbles that may have developed over time. During tubing insertion, however, the system posed a risk of reintroducing bubbles into the system. To counter this issue, we employed the two inlets for each channel to flush fresh media from the reservoir, ensuring that no bubbles were present.

To control the pressure, we used an automatic translation stage (Zaber High speed motorized linear stage) that could be programmed to lift the reservoir. We measured the pressure by tracking the height of the stage and the zero-pressure position. With this stage, we could apply pressure in the range of 0 → 1500Pa, and we could even apply negative pressure by setting it lower. For our experiments, we used the range of -200 → 1300Pa.

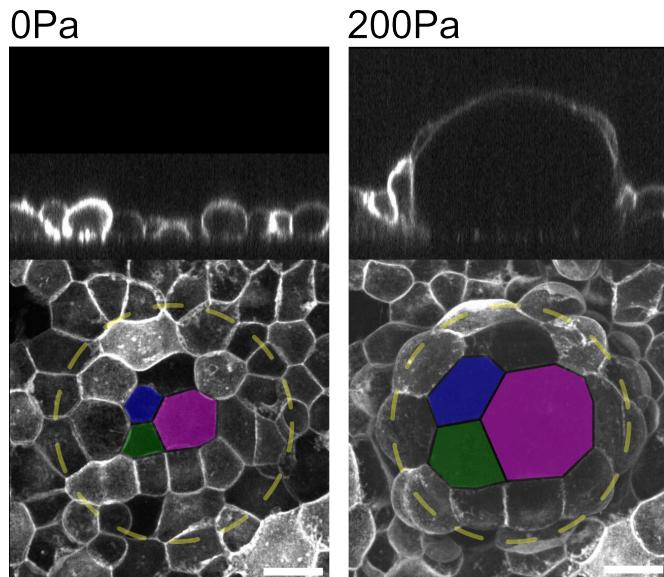
## 6.6 Imaging the epithelial domes

Following extensive optimization of protein patterning, cell culture conditions, and confocal microscopy techniques, we were able to generate domes in accordance with the intended pattern and exert precise control over the pressure required for their formation. To obtain images of the dome, we utilized a spinning disk confocal microscope with a 40x objective lens (NA 0.75), which allowed us to visualize the membrane (CIBN CAAX GFP) and adhesion protein (Fibrinogen) pattern in separate channels (488nm and 644nm, respectively). By incorporating a labeled adhesion protein, we were able to track the formation of the domes with greater ease and accuracy.

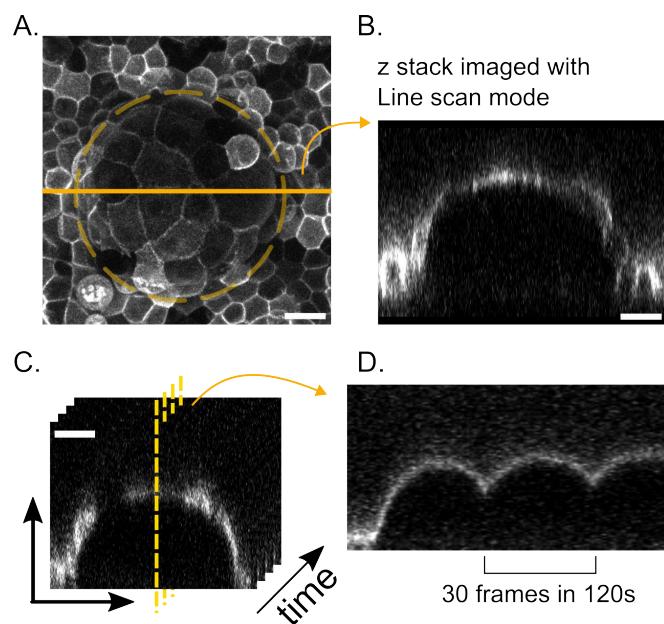
We initially focused on characterizing the mechanics of spherical domes at constant pressure to gain insights into epithelial behavior (see fig 6.7). Laplace's law was employed to calculate tension using pressure, cell shape, and tissue curvature data, which we could easily monitor. Unlike previous studies that did not control the pressure under the dome, our experimental system allowed us to inflate and deflate the domes in seconds. This forced us to monitor them by observing the base of the dome where the monolayer would intermittently come in and out of view.

Acquiring images of the dome stack in a confocal microscope required three minutes using a step size of 0.5 $\mu$ m (exposure 500ms) and a height of 100 $\mu$ m, which was slower than the rate at which we could deform the dome by changing the pressure (see fig 6.7). To investigate the rheology of the domes, it was necessary to monitor their dynamic response at faster pressure rates and shorter timescales while measuring dome strain and curvature. Since the dome possessed inherent symmetry, imaging the mid-section of the structure provided all the geometric information required.

Using the line scanning mode of a Zeiss Airy Scan Microscope, we imaged a single line of pixels across the midsection of the dome and acquired a confocal z-stack (1024 pixel, 4.1 $\mu$ s/pixel dwell time, and 1 $\mu$ m step size) along the height of the dome (refer to fig 1.8). This approach allowed us to obtain a cross-sectional view of the dome in a fraction of the time required for a normal stack. By enabling piezo stage movement, we imaged a 100 $\mu$ m tall dome in just 4 seconds and tracked its height evolution using a kymograph of the central part of the dome. It is important to note that this imaging method is primarily useful for tracking dome strain and curvature, and the cell images obtained are often of low quality.

**Figure 6.7:****Epithelial dome:**

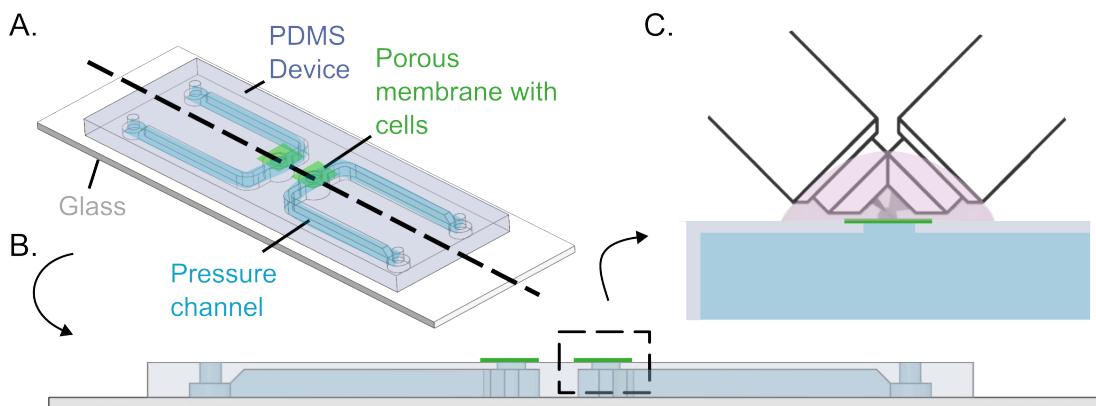
Representative confocal microscopy sections of domes at 0 Pa and 200 Pa. Images in the XY plane represent the dome's maximum projection, while images in the XZ plane represent a cross section at the center plane. Three cells are highlighted with color to show the stretching during the dome inflation. Scale bar is  $20\mu m$ .

**Figure 6.8:****Imaging the dome with Line scanning mode:**

(A) Confocal microscopy image of a dome's maximum projection. (B) Midsection of the same dome imaged with the line scan mode (LSM). (C-D) Timelapse of the dome in LSM and a kymograph showing dynamics of the domes when imaged at time-step of 4s. Scale bars are  $20\mu m$ .

## 6.7 Light-sheet MOLI

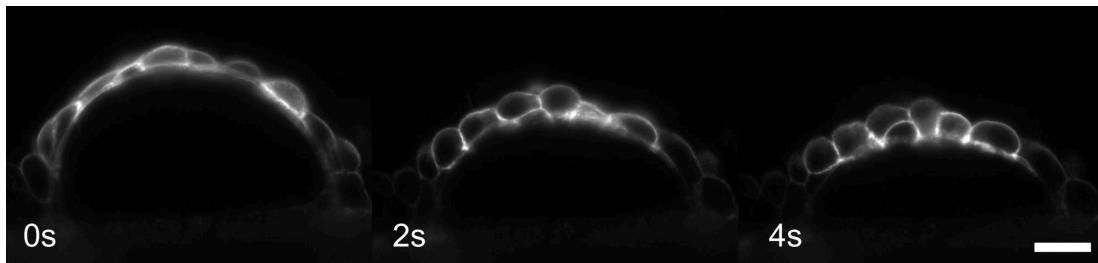
We utilized a Brucker QuVi SPIM light sheet microscope to capture rapid subcellular or cellular changes. The microscope was equipped with two immersion-upright 40x objectives (NA 0.8) at  $45^\circ$  to the horizontal plane. Drawing upon our proficiency in device fabrication, we devised a new setup that facilitated top imaging of cells and porous membranes (see fig 6.9).



**Figure 6.9: Light sheet MOLI:** (A) Isometric illustration of a single piece PDMS block engraved with two channels, so that we can have two devices in one. (B) Cross-section of the device. (C) The device is used with 40x immersion objectives coming at 45 deg angle. This limits to the field of view to  $332 \times 332 \mu\text{m}^2$ .

To simplify the fabrication process, we inverted the conventional MOLI device. This necessitated only a pressure channel and a middle layer with a hole and porous membrane. The device was manufactured sufficiently thick to plug in the tubing, and given the device and channels' size, we produced the mold using a standard 3D printer, Ultimaker 3D printer. We incorporated a ridge-like protrusion to manufacture the pressure channel and cell seeding hole in one go. The device was bonded to a microscope slide using unpolymerized PDMS, and we performed PRIMO patterning of the device by flipping it upside down. In this setup, seeding cells was easier, as the cell seeding part was exposed.

As expected, we were able to generate domes using the same system as before by applying pressure. The imaging technique we developed enabled us to acquire a full dome image in a mere 4 seconds, which involves using objective scan, where only one objective to scan the dome with 100 frames with step size of  $1\mu\text{m}$  at rate of 4ms. This allows us to observe fast-moving features that were indiscernible with other imaging techniques (see fig 6.10).



**Figure 6.10: Dome imaged with Light sheet MOLI:** Mid-section of a dome with membrane marker imaged every 2s. Showing the shape of individual cells undergoing changes during deflation. Scale bar is  $20\mu\text{m}$ .

## 6.8 Summary and Discussion

We have developed a microfluidic chip to generate 3D curved epithelia, utilizing a multilevel device consisting of two layers separated by a porous membrane. Seeding cells on the membrane in the bottom channel allowed for dome formation closer to the microscope objective, enabling high-quality confocal imaging. Hydrostatic pressure under the dome was controlled dynamically, allowing for monitoring of cells and tissue behavior. Additionally, we developed imaging strategies to capture dynamics of these 3D structures faster using line scanning mode of confocal microscope or light sheet microscope.

Using this device, we were able to form the domes and monitor cellular and tissue behavior. As demonstrated in previous studies, the most intriguing aspect of the system is that complex materials such as epithelial tissue, in order to maintain mechanical equilibrium, must adopt a spherical cap shape for a circular footprint. This uniform curvature and pressure imply uniform and isotropic tension, independent of tissue material properties (Latorre *et al.*, 2018, Marín-Llauradó *et al.*, 2022). The tissue tension can be easily measured by applying Laplace's law for spherical cap domes. However, in the case of non-spherical geometry, there would be anisotropic stresses that would require a computational model, such as curved monolayer stress microscopy, to solve an inverse problem to go from geometry to forces (Marín-Llauradó *et al.*, 2022).

The geometry of the domes is primarily controlled by the adhesion protein pattern, but delamination can still occur. In spontaneous domes, circular footprints were found to be the most common (Tanner *et al.*, 1983), while domes formed around sharp corners can blunt themselves through delamination (Latorre *et al.*, 2018). This must be taken into consideration when creating specific geometries.

Tissue tension and adhesion forces also interact with each other. In MDCK suspended monolayer, it is seen that cell-cell junctions are stronger than cell-substrate adhesion (Harris *et al.*, 2012), so if tension at the base of the dome exceeds the adhesion forces, it can lead to

detachment and delamination.

Furthermore, we have unintentionally developed a peeling system that enables us to observe tissue detachment from the substrate. If the dome retains its spherical shape, we can calculate the necessary forces for breaking cell-substrate adhesion and identify the contribution of focal adhesion molecular components.

This system provides a novel approach for testing material properties and probing mechanics at the tissue scale, allowing for simultaneous high-quality imaging and monitoring of cytoskeletal components and the nucleus. Additionally, we can create a 3D tissue with controlled lumen pressure, providing a well-controlled protocol that is suitable for replicating curvature-pressure-tension conditions in various cell lines, not just MDCK cells. However, our primary focus is on comprehending the mechanics of epithelial tissue under controlled pressure.

# **Chapter 7**

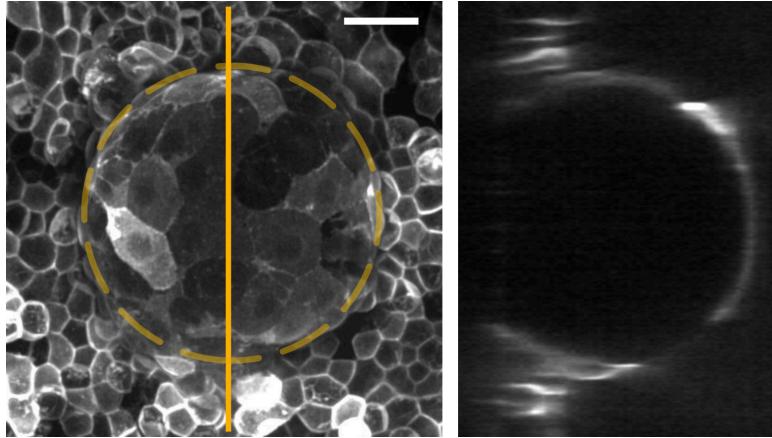
## **Dynamic material response of epithelial domes**

### **7.1 Introduction**

The main objective of this thesis is twofold: to develop a method for creating three-dimensional epithelia with controlled pressure and to investigate the tissue's material response under different tension regimes. Reviews from Torres-Sánchez et al. and Choudhury et al. demonstrate that pressure levels in different contexts can vary significantly across a wide range of timescales and magnitudes. (Choudhury *et al.*, 2022a, Torres-Sánchez *et al.*, 2021). For instance, the luminal pressure in blastocysts doubles during development, causing changes in cortical tension and strain (Chan *et al.*, 2019).

To investigate the relationship between cell strain, tension, and pressure, the MDCK dome system offers a suitable platform. Previous research by Latorre et al. observed a broad spectrum of pressure throughout dome evolution, with cells displaying various deformations, including active-superelastic behavior (Latorre *et al.*, 2018). However, the control in this system is limited to the footprint of the domes, with no capability for control of pressure and tension.

To address the first objective, we have developed the monolayer inflator (MOLI) device, enabling the creation of epithelial domes where cells can be stretched to over 100% of areal strain. This innovative MOLI system allows for better control of pressure and tension applied to the tissue, making it a valuable tool for our research. In this chapter, the MOLI system will be employed to subject tissues to various strain and tension regimes, enhancing our understanding of the epithelial tissue's material response under different conditions.



**Figure 7.1:**

**Spherical cap:**

An example of an epithelial dome at 200 Pa, which has increased its surface area almost four times the original footprint. The spherical shape of the dome can be seen in the cross-section. Scale bar is  $20\mu m$ .

## 7.2 Measurement of dome mechanics

To measure the kinematics of the domes, we focused on the midsection, assuming symmetry of spherical caps (see Figure 7.1). We measured the height ( $h$ ) and base radius ( $a$ ) of each dome, which allowed us to calculate the radius of curvature ( $R$ ) using trigonometry as

$$R = \frac{h^2 + a^2}{2h}. \quad (7.1)$$

Additionally, we measured the pressure ( $\Delta P$ ) to compute the tension ( $\sigma$ ) using Laplace's law

$$\sigma = \frac{\Delta PR}{2}. \quad (7.2)$$

For dome strain, we utilized the areal strain measure, which is based on the surface area, and compared the dome surface area ( $A$ ) to the area of the footprint ( $A_0$ ) by

$$\epsilon = \frac{A - A_0}{A_0} = \frac{\pi(h^2 + a^2) - \pi a^2}{\pi a^2} = \frac{h^2}{a^2}. \quad (7.3)$$

However, the line scanning modality of a Zeiss Airy Scan Microscope generated hundreds of images per experiment, making manual quantification of geometrical parameters impractical. To overcome this challenge, we generated kymographs of the top section of the domes, allowing us to extract geometric information more efficiently (as described in the previous chapter). Using these kymographs, we were able to measure the tension and strain of the domes with greater ease and speed. These measurement techniques enabled us to study the dynamics of epithelial domes at faster rates without the need for time-consuming 3D imaging.

### 7.3 Epithelial domes at constant pressure

To begin, we systematically inflated domes at varying pressures ranging from 0-400Pa. However, we found that domes could not form at pressures lower than 50-100Pa due to adhesion forces, and delamination occurred at higher pressures. Ultimately, we determined that 200 Pa allowed the domes to form without delaminating, falling within the previously reported pressure range (Choudhury *et al.*, 2022b, Marín-Llauradó *et al.*, 2022).

Our measurements revealed that when the domes were subjected to a constant pressure of 200Pa, they underwent a significant increase in areal strain during the first three to five minutes of pressure application. Following this initial increase, the areal strain reached a plateau and remained relatively constant for the next 5-10 minutes (see Figure 7.2 A). Notably, our findings also indicated considerable variability in dome-to-dome strain, with strains ranging from 50% to 300%. Nevertheless, the stabilization in strain across all the domes suggested that a steady state had been achieved by the epithelial tissue.

In analyzing the tension-strain relationship of these domes, we found that it exhibits a non-monotonic curve similar to the Nike "swish" symbol (see Figure 7.2 B). At low strains, the tension within the domes is exceptionally high, followed by a decline to a minimum value at an areal strain of one, where the dome adopts a hemispherical shape. The tension then increases once again, but the rate of increase is much slower than the rate at which tension decreases at lower strains. This non-monotonic tension-strain curve is atypical and represents an intriguing characteristic of the epithelial domes.

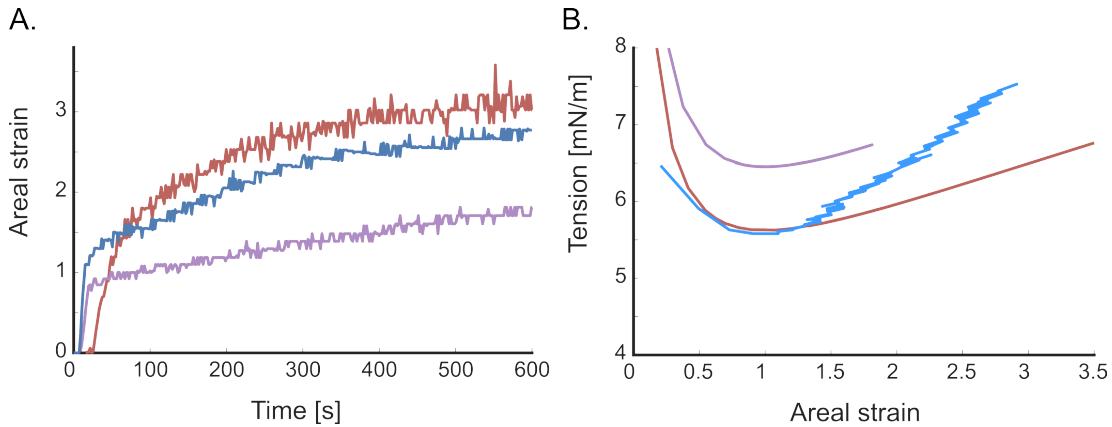
This tension-strain relationship of the domes can be explained by the geometric constraints imposed by the dome system and force balance, as governed by Laplace's law. The tension within the dome is intrinsically dependent on its radius of curvature, and the initial high value of the radius of curvature leads to a correspondingly high tension that subsequently diminishes to a minimum upon adopting a hemispherical shape (see Figure 7.3). Since the dome always maintains a spherical shape, the radius of curvature, areal strain, and tension are all interconnected. The expression of the curve can be derived by substituting areal strain ( $\epsilon$ ) in the expression of radius of curvature ( $R$ ) (Equation 7.1)

$$R = \frac{h^2/a^2 + 1}{2h/a^2} = a \frac{\epsilon + 1}{\sqrt{\epsilon}}. \quad (7.4)$$

Then, by substituting ( $R$ ) in Laplace's law (Equation 7.2), we obtain the relation for the tension in terms of strain.

$$\frac{\sigma}{a} = \frac{\Delta P}{4} \left( \frac{\epsilon + 1}{\sqrt{\epsilon}} \right). \quad (7.5)$$

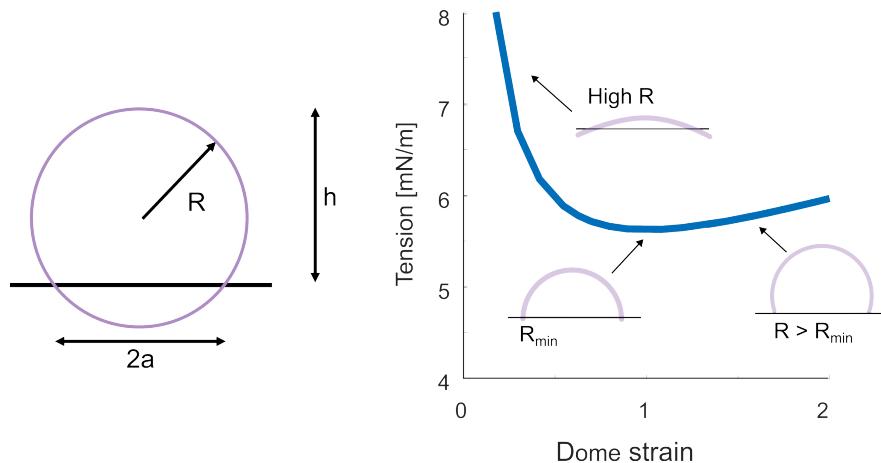
This relationship provides a specific link between tension and strain for a given pressure



**Figure 7.2: Epithelial domes at constant pressure:** Dynamic response of the representative domes at a constant pressure of 200 Pa: (A) Areal strain increases and reaches a steady state at around 5 minutes, and we can clearly see variability in the maximum strains. (B) The same domes produce a peculiar "NIKE swish" shaped tension and strain curve.

and base radius.

Therefore, it can be inferred that when a step pressure is applied, the dome undergoes a non-steady-state with out-of-equilibrium stresses while experiencing high tension related to high radius of curvature. The dome then rapidly inflates by following the tension-strain curve dictated by equation 7.5 to achieve a steady state. In this steady state, the external pressure is balanced with equilibrium tissue tension. It is evident that this Nike curve material response does not represent the quasi-static constitutive relation of the epithelial tissue. To determine the true constitutive relation, a different experimental strategy will be adopted in the following section.



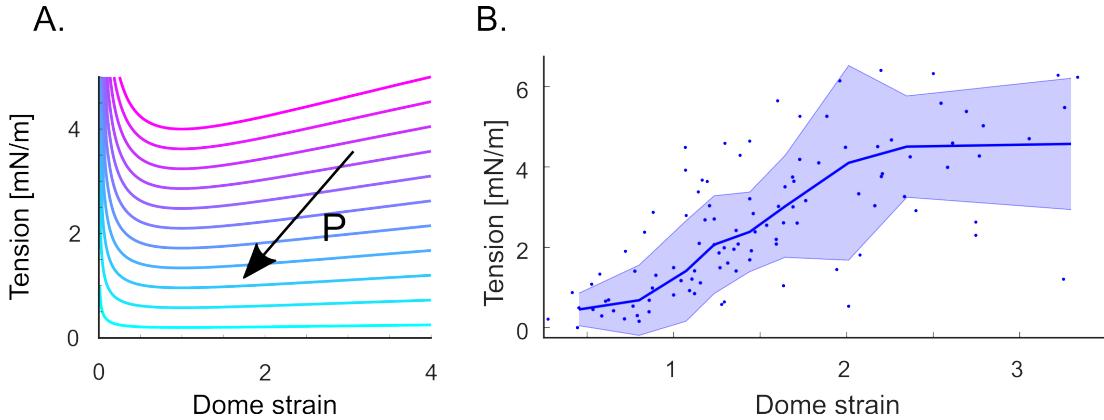
**Figure 7.3: Illustrative explanation for isobaric curve:** Tension and strain are related to each other through the geometric constraint of a spherical cap. Here, the base radius ( $a$ ) is constant, so the radius of curvature is almost infinite for domes with very small strains ( $<0.05$ ). As the strain increases, the radius of curvature decreases to a minimum corresponding to the base radius. Then it continues to increase again.

## 7.4 Constitutive relation of epithelia

The constitutive relation of a material describes the relationship between its deformation and the applied forces. In conventional experiments, quasi-static strain or tension is applied to determine this relation. However, our experiments could only control pressure. Increasing pressure slowly was not feasible for domes due to limited delamination at low pressures. To overcome this and obtain the tissue constitutive relation, we deflated a dome in steps, capturing steady-state tension and strain for different pressures. The locus of these steady states provides the constitutive relation.

Specifically, we applied a pressure of 200 Pa for 5 minutes, allowing the dome to reach a steady state. We then reduced the pressure in increments of 20 Pa, permitting the dome to reach steady state at each step (see Fig. 7.4). This process continued until the dome was completely deflated. Consequently, we captured the material response of the tissue at various pressures as the dome transitioned through different tension-strain states. It appeared to move between different Nike curves corresponding to pressure. By mapping all steady-state tension-strains of domes at different pressures, we determined a constitutive relation for the tissue.

The resulting constitutive relation showed an initial increase in tension with strain for lower strains. For larger strains, the tension plateaued, consistent with earlier studies on MDCK domes (see Fig. 7.4 B). It is important to note the significant variability in dome-to-dome tension, with recorded tensions around 4.5 mN/m aligning with the same order of magnitude as those in previous studies (Latorre *et al.*, 2018, Marín-Llauradó *et al.*, 2022).



**Figure 7.4: Constitutive Relation of Epithelia:** (A) We will set up experiments to probe the steady state at different pressures. We will start from the highest pressure, move along the isobaric line and achieve a steady state, and then move down to the next curve, and so on. (B) The constitutive relation between dome strain and tissue tension was experimentally obtained ( $n=12$ ). The line and shaded area represent the median and standard deviation, respectively, by binning 13 points in each bin.

To summarize, our experiments demonstrate that the tissue reaches a steady state under a specific static pressure, which enables us to determine its constitutive relation through deflation. In the next section, we will explore the dynamic material response of the tissue.

## 7.5 Dynamics of the epithelia domes

In this section, we investigated the dynamic material response of the domes by conducting cyclic stretching experiments. We subjected the domes to a triangular wave of pressure with a magnitude of 200 Pa at three distinct timescales, as depicted in Figure 7.5. The selected timescales of 20s, 266s, and 2000s were based on existing literature on tissue remodeling, particularly the work of Khalilgharibi et al. (2019) and Casares et al. (2015) (Casares *et al.*, 2015, Khalilgharibi *et al.*, 2019). These studies demonstrated that stress relaxation in tissues occur from tens of seconds to minute timescales due to F-actin remodeling and myosin-driven contractility. Additionally, in some cases, even faster deformation at timescales of a few seconds has been shown to impact cell remodeling (Andreu *et al.*, 2021). In our device, the fastest cycle that could be probed was limited to 20s because of the microscope's imaging speed.

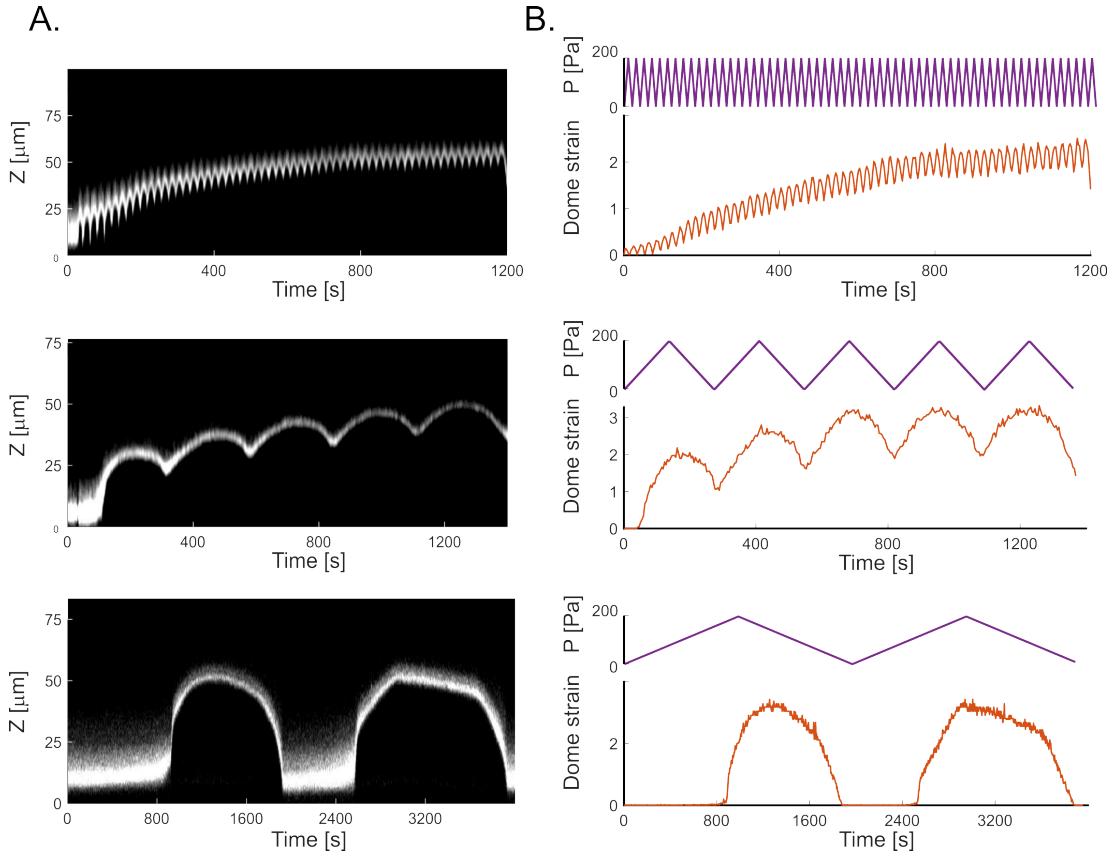
	Fast	Moderate	Slow
Time period (s)	20	266	2000
Rates (Pa/s)	20	1.5	0.2

For the fastest cycles, we observed that the maximum strain achieved by the domes in each cycle increased until they reached a steady state oscillation around 600 seconds. The experiment was conducted over 1200 seconds, equivalent to 60 cycles, during which we observed a cumulative buildup of strain over time. In the loading phase, the domes underwent stretching, while during the unloading phase, they experienced unstretching but failed to revert to zero strain after the initial cycles. In the concluding cycles, we noted that the dome oscillated between two distinct states of strain, resembling a limit cycle.

A similar response was observed for the moderate cycles, where the domes were stretched for five cycles of 266s each. The strain accumulated in the first cycle, with strains reaching higher values than those observed in the fast case. Moreover, after a few cycles, the dome appeared to reach a stable limit cycle.

In the slowest cycles, which lasted 4000 seconds, we observed that the domes did not form at lower pressures. As discussed earlier, the domes remained attached until a pressure of 100-150 Pa was attained, beyond which they underwent rapid inflation, leading to high strains of 200-350%. However, during cyclic stretching, we noted that strain accumulation did not occur, and there was no variation in the maximum strains attained during both cycles. The cells were able to stretch four times their original size and return to the original size at the end of each cycle.

These experiments clearly demonstrated the rate-dependent response of the domes, with faster rates resulting in strain accumulation and slower rates allowing for large, reversible deformations. This behavior resembles viscoelastic properties. In the next section, we will explore this viscoelastic behavior further to better understand the tissue's characteristics.



**Figure 7.5: Dynamic response of Epithelia:** (A) The XZ plane images and kymographs of domes subjected to cyclic pressure between 0 to 200 Pa with rates of 20, 1.5, and 0.2 Pa/s. The kymographs generated along the midsection of the domes indicated by yellow dotted lines. These indicate the evolution of height of the domes with respect to time. (B) The strain response of domes to cyclic pressure with different rates. Magenta represents pressure and red represents strain with respect to time. For A, B,  $n = 7$  domes for 20 Pa/s,  $n = 8$  for 1.5 Pa/s, and  $n = 7$  for 0.2 Pa/s.

## 7.6 Active gel tissue model

The role of actin cortex viscoelasticity in sustaining deformations over various timescales, from seconds to minutes, has been emphasized in the existing literature on epithelial mechanics (Clément *et al.*, 2017, Kelkar *et al.*, 2020, Khalilgharibi *et al.*, 2019). To further understand this behavior, Adam Ouzeri and Marino Arroyo developed a computational model that bridges active gel models of the actomyosin cortex with 3D vertex models at tissue scales (Ouzeri and Arroyo, 2023). Our collaboration necessitated close coordination, with Adam Ouzeri developing the model and me conducting experiments, facilitating the effective integration of the model with experimental data.

The model simplifies the complex cortex, comprising the actin filament network, hundreds

of actin-binding proteins, and myosin motors, into a thin layer of contractile gel. This layer possesses a thickness denoted as cortical thickness ( $\rho$ ).

The crosslinked actin filament network exhibits the behavior of an elastic network of semi-flexible filaments, whose material properties are represented by Lamé parameters ( $\mu$ ). Consequently, upon deformation, it can store elastic energy, although only at shorter timescales. At longer timescales, in response to stretching, the network dynamically reorganizes itself, releasing the stored elastic energy by relaxing stresses. This takes place through assembly/disassembly of the filaments or binding/unbinding of the crosslinkers. The dissipation of stresses is represented by a viscosity coefficient ( $\eta$ ).

However, the active component of the active gel model arises from myosin motor activity, generating contractile active tension in the network. This active tension ( $\gamma$ ) is hypothesized to be directly proportional to cortical thickness, with a proportionality constant denoted by coefficient of active tension ( $\xi$ ).

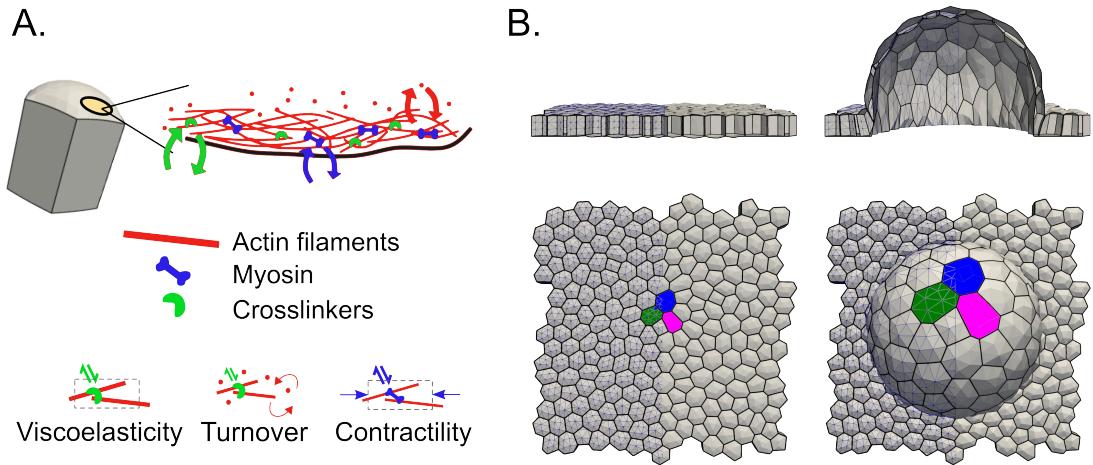
$$\gamma(\rho) = \xi\rho. \quad (7.6)$$

Several assumptions underlie this model. First, the cell volume is assumed to be conserved throughout the deformations. Second, cells cannot stretch indefinitely due to physiological constraints, such as reorganization of intermediate filaments, cell crowding, or compression of the nucleus. To limit strains, a strain stiffening mechanism is introduced, activated beyond high strains.

Regarding the actin network, it is assumed to be isotropic, whereas in reality, it also can be polar or nematic. Another assumption posits that the cortex undergoes constant turnover, maintaining a steady-state cortical thickness.

Based on the above assumptions and the cortex's physical attributes, the model represents each cell as an active gel surface. These active gel surfaces are then assembled to form a tissue, as depicted in Figure 7.6. The system's dynamics are formulated through a balance of diverse potentials, representing free energy, dissipation, active contractility, and external forces.

Due to this formulation, the system exhibits the behavior of an active hyperelastic material at shorter timescales, while at longer timescales, it behaves like an active viscoelastic material. This behavior can be explained through three timescales emerging from the theoretical framework: turnover timescale  $t_{to} = 1/k_d$ , viscoelastic timescale  $t_{ve} = \eta/\lambda$ , and viscoactive timescale  $t_{va} = \eta/\xi$ . The turnover timescale is driven by the cortex's polymerization rates ( $k_d$ ). The viscoelastic timescale is a ratio of the viscous remodeling coefficient to the Lamé parameters, representing elasticity. Lastly, the viscoactive timescale is the ratio of the viscous remodeling coefficient to the coefficient of active tension. All of these timescales are interconnected through the cortical thickness; thus, for simplicity, we can understand them as one remodeling timescale.



**Figure 7.6: Active gel tissue model:** (A) The cell is modeled as an active gel of cortex, which mainly comprises three aspects: viscoelasticity of the network, turnover dynamics, and active contractility. (B) These cells can be assembled into a tissue that can be used to perform in-silico experiments. An example of this is the digital dome being inflated, highlighting individual cells increasing their area.

Using this model, a virtual representation of the cell monolayer can be generated, including specific regions devoid of basal attachment to the substrate, which could be inflated into domes under pressure, similar to the experimental setup. These simulations will be referred to as "digital domes" in subsequent discussions (see Figure 7.6). By employing this model and comparing the results with the experimental data, we could effectively understand and investigate the biomechanical properties of the epithelial tissues, specifically the contribution of the actin cortex's viscoelasticity.

## 7.7 Active viscoelasticity of the epithelia

In this section, we interpret the experimental results within the context of the active viscoelastic model. The mathematical framework describes how cells, represented as an active gel, change shape. Specifically, the initial shape, known as the reference configuration ( $\Gamma_0$ ), is mapped to its current shape, called the deformed configuration ( $\Gamma$ ). This mapping enables the quantification of strains and deformations relative to the reference configuration using a metric tensor, which measures distances and angles within the material. When a material is deformed, the distances and angles within it change, and the metric tensor reflects these changes. In this case, each configuration possesses its own metric tensor.

For typical elastic materials, the reference configuration remains fixed in time, and deformations are measured relative to this fixed state. However, to account for the remodeling of the cortex through dynamic crosslinking, our model considers the reference configuration

as dynamic, necessitating a time-varying metric tensor ( $\mathbf{G}$ ). We can refer to this evolving reference configuration as the resting frame. By employing the dynamic metric tensor, we can calculate the resting area of a cell, which represents an imagined area where all stored elastic energy is dissipated. In terms of cell area, the relationship between the resting area ( $A_{rest}$ ) and the actual area ( $A_{actual}$ ) can be expressed as

$$A_{rest} = \int_{\Gamma_0} \sqrt{|\mathbf{G}|} dS_0, \text{ and } A_{actual} = \int_{\Gamma} dS. \quad (7.7)$$

Where  $dS_0$  and  $dS$  are infinitesimal area on the cortex in reference and deformed configuration respectively.

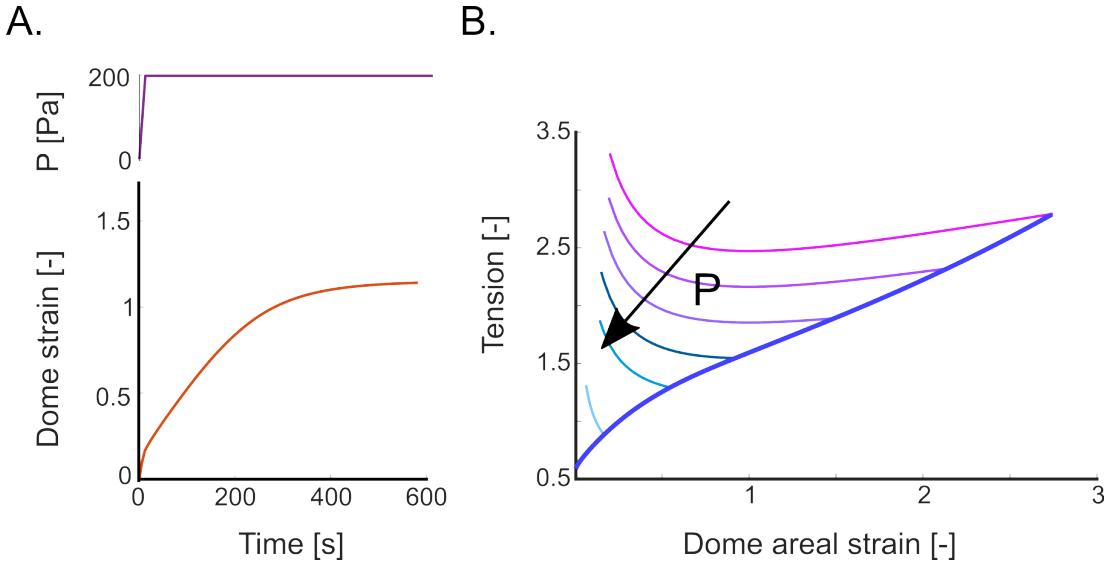
In the case of a polymer with dynamic crosslinks, the resting area of the material changes as the crosslinks break and reform in response to applied stress. The updated resting area can be used as a dynamic reference configuration for measuring subsequent deformations of the material. This concept of a resting area provides a useful way to think about the dynamic behavior of materials with internal structure and connectivity that change in response to applied stress.

Consider an example where the tissue is stretched instantaneously. The actual area changes instantly, but the resting area of the tissue stretches gradually and eventually catches up to the actual area due to the active viscoelasticity of the tissue. This process involves two types of stresses: viscoelastic stress and active tension. The total tissue tension is the sum of these two stresses. Upon stretching the tissue, viscoelastic stresses dissipate at viscoelastic timescales through remodeling, which allows the resting area to match the actual area, while active tension increases at turnover timescales. The effect of this remodeling on the tissue behavior is evident in experiments.

The simulations were conducted to mirror the experimental conditions. Upon subjecting the digital dome to constant pressure, consistent with our previous experimental observations, the digital dome reached a steady state while experiencing a reduction in cortical thickness as the cells stretched (refer to Figure 7.7). The remodeling of the cortex dissipates the viscoelastic stress and increases the active tension. At the steady state point, only the active tension remains balancing the externally applied pressure. The simulations show that the time to reach the steady state is mainly driven by the viscoelastic timescales. The tension-strain relationship of the digital domes also produced a non-monotonous “Nike” curve observed in experiments.

To derive the proper constitutive relation within the computational framework, the digital dome was subjected to quasi-static inflation. The resulting constitutive curve displayed characteristics similar to those observed experimentally, and additionally, exhibited stiffening at high strains, which can be attributed to a barrier mechanism (refer to Figure 7.7 A).

Furthermore, to assess the robustness of the constitutive relation obtained from our experi-

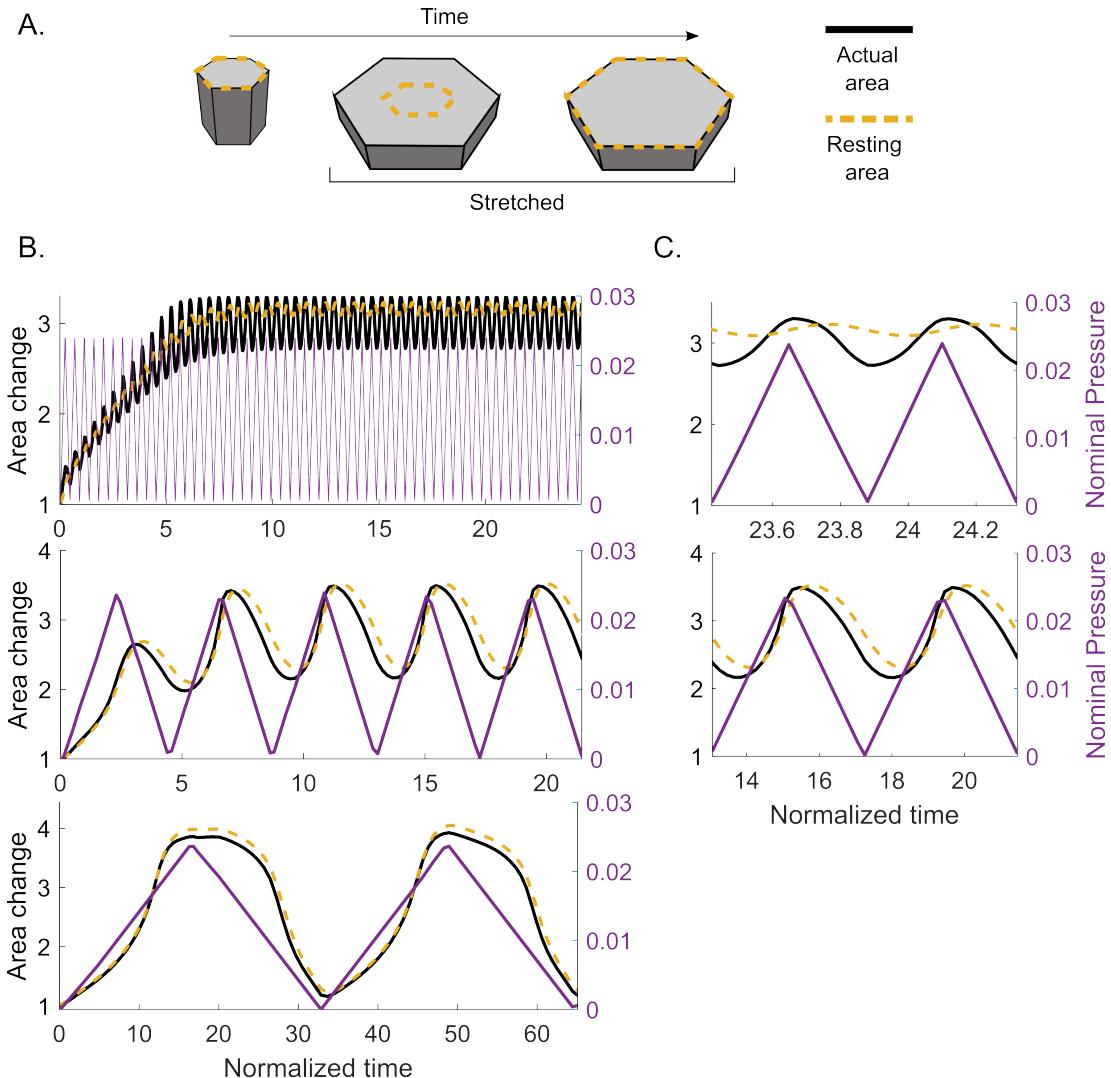


**Figure 7.7: Material response of the digital domes:** (A) When subjected to constant pressure, as in experiments, the digital dome inflated and reached a steady state. (B) These simulations also produced isobarics for different pressures, all leading to a steady state. Furthermore, subjecting it to a quasi-static increase in pressure produced a constitutive law (Navy blue curve) that can be mapped onto the locus of steady-state points.

ments, the digital dome was inflated to varying levels of pressure, generating tension-strain curves and steady-state points. The locus of steady-state points under different constant pressure conditions aligned with the quasi-statically obtained constitutive relation, as illustrated in Figure 7.7 B.

The effect of the remodeling timescale is especially noticeable in cyclic stretching experiments. When digital domes are subjected to cyclic pressure rates much slower than cortical dynamics, the deformations of the cells occur with the cortex being in a state close to steady state. The stored elastic energy is dissipated as the strain increases, and the cortical thickness does not change significantly. We observed that the resting area in the digital dome almost overlapped with the actual area (Figure 7.7 B). This slow rate of 0.2 Pa/s provides cells with sufficient time to remodel and dissipate viscoelastic stresses. Viscoelastic and turnover timescales in simulations are around 10-30s, which means that over a period of 2000s, the dome stretches to considerably large strains of 250-300% and returns to its original flat state.

In contrast, when cells in a dome are subjected to cyclic pressure at rates faster than cortical dynamics, they accumulate strains due to insufficient time to dissipate viscoelastic stress. In this case, the actual area changes more rapidly than the viscoelastic and turnover timescales permit. Consequently, along with the change in the actual area, the resting area also changes, but at a slower pace. During deflation, the resting area reduces but cannot decrease fully before



**Figure 7.8: Concept of resting area:** (A) Illustration of a resting and actual area of a cell in a monolayer during stretching. (B) Differences in results of resting and actual area when subjected to different rates of pressure. (C) Inset of the last two cycles.

the next inflation cycle begins, causing the tissue to continue stretching further. Eventually, a limit cycle is reached, wherein cells stably oscillate between two strains, and the resting area oscillates as well, but at a much smaller amplitude (Figure 7.7 B).

Interestingly, the simulations indicate that due to active viscoelasticity, there would be a lag between the peak of pressure and the peak of strain. This lag is clearly reflected in the comparison of the resting area and actual area, where the delay decreases with increasing pressure rates (Figure 7.7 C). The slowest pressure rate results in the least amount of delay,

while the fastest pressure rate results in the most delay. However, we can only experimentally observe this at moderate rates, as the experimental data from faster cycles is too noisy to observe the lag.

To sum up, the active gel model explains the material response of epithelial tissue depending on the rate at which pressure is applied. The concept of resting area enables us to understand that slower rates allow for cell remodeling and dissipation of viscoelastic stress, while faster rates result in strain accumulation due to insufficient time for dissipation. This active viscoelastic behavior is the outcome of timescales associated with cortical remodeling.

## 7.8 Summary and Discussion

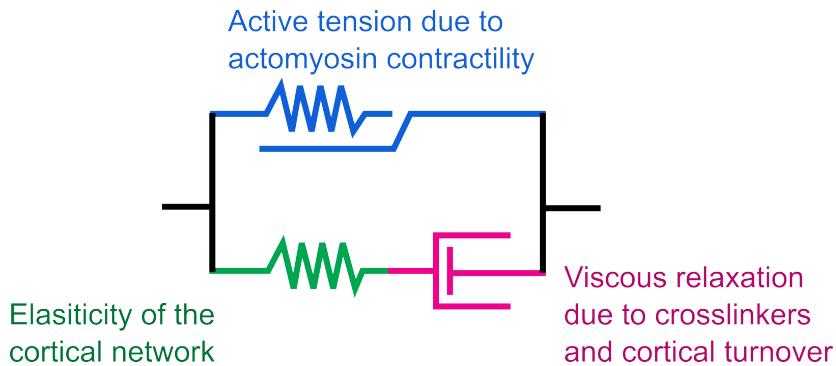
In this chapter, we investigated the mechanics of epithelial tissue by applying pressure at varying rates. Initially, we applied a constant pressure of 200Pa, which led to the dynamic inflation of domes and eventually reached a steady state in strain. Due to the spherical geometry of the tissue and Laplace's law, we observed a non-monotonous tension-strain curve in response to the constant pressure. We found that the true tension-strain curve exhibited increasing tension with respect to strains at lower values, but at higher strains, the tension appeared to be independent of the strains.

Furthermore, our measurements showed that the domes accumulated strain through the cycles when probed with fast-changing pressure and reached a steady-state in later cycles. However, when stretched slowly, the domes stretched to higher strains without accumulating strain at the end of the cycle. To understand the behavior of epithelial tissue, we used the computational framework developed by Adam Ouzeri and Marino Arroyo, which shows that the mechanical response of the domes to pressure is dependent on active viscoelasticity (Ouzeri and Arroyo, 2023). The digital dome studies highlighted the interplay of different timescales, which are the reflection of the interaction between cortical turnover, crosslinkers, and network reorganization, allowing for large deformations and rapid shape changes.

Our results can be interpreted using a multidimensional Maxwell model, which is a model that describes viscoelasticity. The classical Maxwell model consists of a spring and a dashpot, which represent the elastic and viscous elements, respectively. In our case, we can imagine a similar model with two branches: one branch includes a spring and a dashpot to represent the passive viscoelasticity, and a second branch includes an active spring to represent the active component (see fig 7.9). The active spring is always present, but if the system is stretched slowly, the dashpot would be driving the dominant mechanical response. Conversely, if stretched rapidly, the elastic spring deformation would dominate. By separating the passive and active components, we can better understand how each contributes to the overall viscoelastic behavior and associated timescales.

Previous research has approached the system in a similar manner, where epithelial tissue was modeled using viscoelastic models of springs and dashpots. One particularly interesting model was developed by Khalilgharibi et. al., which characterizes the response of a suspended monolayer to stretch and demonstrates that the dynamics are similar to that of a single cell, due to the role of the actomyosin cortex (Khalilgharibi *et al.*, 2019). They used a model with two springs in parallel, one of which can change its resting length dynamically. The model explains the relaxation of the monolayer, as the active contractility of the cortex changes the resting length of the active spring, which closely relates to our "resting area" concept.

Another study by (Clément *et al.*, 2017) found that viscoelastic dissipation could explain the shortening or elongation of cell junctions in *Drosophila* embryos. They demonstrated that the



**Figure 7.9: Representational viscoelasticity model:** The model can be understood using a spring and dashpot analogy with two branches: The first branch is an active spring representing the contractile forces applied by the actomyosin cortex. The second branch has two components, one for the elasticity of the network and the second for the viscous relaxation that occurs due to turnover of the network.

dissipation occurs at the minute timescale, which coincides with myosin pulses, and that actin turnover plays a key role in this dissipation. These pulses have a ratchet-like mechanical effect that drives junction shortening and causes tissue folding. This ratcheting effect is reminiscent of the cyclic stretching at faster rates, where cells stretch more and more every cycle. Similar to the authors of the study, we explain the strain accumulation by incomplete dissipation of viscoelastic stress due to deformation faster than the remodeling timescale.

In this chapter, we have connected the dynamic material response of epithelial tissue to active viscoelasticity using a computational model. Typically, rheological models are fitted to experiments. In our case, we employed a theoretical framework that bridges the physics of cortical actin dynamics to the time-dependent mechanics of tissues in terms of remodeling timescales. This allowed us to understand tissue phenomenology by characterizing the tissue response to different pressure regimes.

Although we did not use pharmacological treatments, the MOLI system is amenable to introducing drugs. Various components in the actin network enable cortical tension modulation through pharmacological interventions targeting specific molecular targets (Cartagena-Rivera *et al.*, 2016). For instance, Latrunculin depolymerizes the actin network, while Blebbistatin decreases cortical tension by inhibiting myosin activity. Conversely, Calyculin-A enhances contractility by accelerating Myosin II phosphorylation. In future experiments, these pharmacological interventions could be employed to identify the molecular pathways involved in tissue mechanical response.

Our experimental system focused on probing the response of suspended tissues at short timescales (minutes), which correspond to the timescale of acto-myosin network remodeling. We did not observe any cellular rearrangement, extrusion, or division at this timescale in our system, except for rare exceptions. Long-term experiments were not performed, as they were outside the scope of this study due to the suspected involvement of other cytoskeletal compo-

nents, such as intermediate filaments. Latorre et al. observed the activation of intermediate filaments in extremely stretched cells ( $>300\%$ ) and proposed that this caused re-stiffening, preventing the cells from stretching excessively (Latorre *et al.*, 2018). This observation motivated the strain-limiting mechanism imposed in our model.

Latorre et al. also demonstrated heterogeneity in cell stretching, along with active superelasticity. However, in our experiments, we did not observe any indication of superelasticity, as all cells were super-stretched simultaneously. This might be due to the relatively shorter timescales in our experiments compared to long-term quasi-static deformation of spontaneous domes.

A recently published study (Duque *et al.*, 2023) shows that strain stiffening is dependent on the strain and strain rates. The tissue stiffens at higher strains, but for higher strain rates, the stiffening is more pronounced at higher strains (15%) than lower strains (120%). They demonstrated that this response is due to the supracellular network of intermediate filaments. In our experiments, we could only control pressure and pressure rates. However, if needed, we can track the strains and quantify strain rates to explore the mechanics. When plotting tension-strain curves for different pressure rates, we noticed characteristics of stiffening at higher strains and higher rates. In the model, there is no rate-dependent strain stiffening. In the future, we could easily incorporate this aspect into the model.

By combining the model and experiments, we understand that the epithelium behaves as an active viscoelastic material. In the next chapter, we will endeavor to harness this knowledge of active viscoelasticity to generate transformations of domes into various structures.



# Chapter 8

## Epithelial Buckling: Transforming Domes into Folds

### 8.1 Introduction

Epithelial structures in biology exhibit a diverse range of shapes and sizes, including curved or folded forms. Understanding these structures can be complicated, particularly in the context of developmental biology. Interestingly, the etymology of the terms "development" and "complicated" provides insight into the importance of folding and unfolding processes. "Development" comes from "*desvelopemens*," meaning "unfolding," which describes the morphogenesis of an organism. In contrast, "complicated" comes from "*com-plicare*," meaning "folded together," which is fitting for describing the emergence of complex, folded structures in epithelial tissues.

By utilizing the MOLI tool, we can create 3D epithelial structures by inflating domes, thereby transforming a planar monolayer into a curved configuration. In this chapter, we will discuss how these structures can be made even more "complicated". By studying the mechanics of epithelial tissue, we have discovered that the dome can be deflated into folds by rapidly depressurizing it. We will explore the process of epithelial buckling and discuss how this knowledge has enabled us to transform domes into folded structures.

### 8.2 Rapid deflation produces a buckling instability

In the previous chapter, we observed gradual inflation of domes exposed to constant pressure, ultimately reaching a steady state, attributable to cytoskeletal remodeling. Areal strains exceeding 100%, more than twice the original area, were noted. Cyclic stretching resulted in the accumulation of strains in the tissue.

Employing a computational model, we investigated the mechanical effects of deflation on

the digital dome at the tissue and cell scales. Our analysis revealed that active viscoelastic dissipation through cortical remodeling enabled the dome to sustain high strains. Rapid stretching induced a discrepancy between actual and resting areas, suggesting that fast deflation may cause negative viscoelastic stress greater than active tensions, leading to buckling.

To explore the tissue's response to extreme rates of pressure, we exerted precise control over pressure, allowing no time for relaxation, resulting in buckling instability.

Our findings showed that digital domes gradually returned to a flat monolayer when deflated at a rate slower than the remodeling timescale. However, deflating the domes more quickly than the remodeling timescales resulted in buckling (see fig 8.1). Simulations revealed that the negative pressure was necessary for inducing buckling, which affected only rapidly deflating domes. This was due to negative stresses, as the pressure became negative while the curvature remained positive. In contrast, slowly deflating domes reached zero strain as the pressure approached zero.

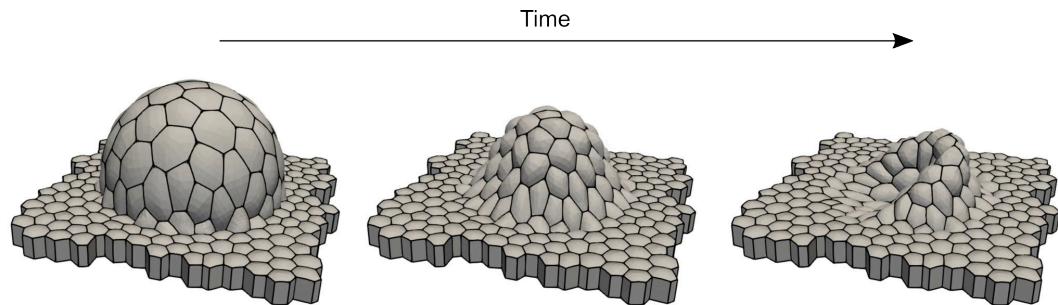
To systematically test our hypothesis regarding the factors affecting buckling events, we designed experiments with pressure profiles consisting of three stages (see fig 8.2). Firstly, we initiated a linear increase in pressure from 0 to 200 Pa over a period of 10 seconds. Secondly, we applied constant pressure for varying "hold times," which were chosen based on the timescales associated with actomyosin cytoskeletal remodeling. Finally, we decreased the pressure to -50Pa at varying "deflation rates."

It is important to note that we relied on qualitative characterization of buckling events, assuming that smooth and continuous curvature of the monolayer indicates the absence of buckling. We performed the experiments for all conditions and quantified the data by tracking the fraction of domes that underwent buckling.

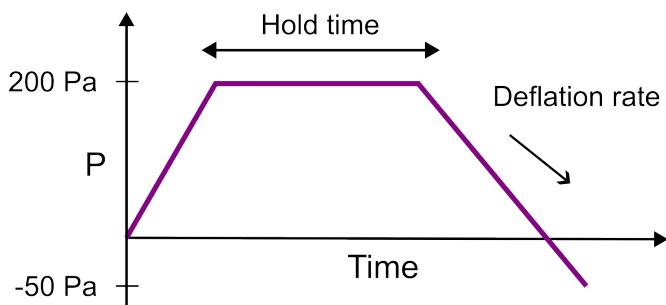
Our results showed that, at the fastest deflation rate of 200 Pa/s, buckling occurred for all hold times, ranging from 6s to 600s. This confirmed the hypothesis that rapid deflation would induce compressive stresses and cause buckling (see fig 8.3 A-B). On the other hand, slow deflation at a rate of 0.2 Pa/s rarely led to buckling, regardless of the hold time (see fig 8.3 C-D). This suggests that the tissue can effectively remodel its cytoskeleton and adapt to drastic changes in area to avoid buckling.

As seen in previous experiments, longer "hold times" allowed for more cytoskeletal remodeling, resulting in higher strains and a higher likelihood of buckling, even for slower deflation rates. Results plotted in a phase diagram illustrates the trend: as hold time increases, buckling becomes more likely at faster deflation rates and less likely at slower deflation rates with a shorter hold time (see fig 8.3 E).

The data obtained revealed that domes subjected to a 6-second hold time attained smaller strains compared to those with a 600-second hold time, consistent with the results from domes exposed to constant pressure (see Figure 8.3 F). The attainment of higher strains requires time for the dome to remodel and balance active tension with externally applied pressure.



**Figure 8.1: Digital dome undergoing buckling:** Here, a digital dome at a steady state is rapidly deflated to a negative pressure of  $-50\text{Pa}$ . As the cells don't have enough time to reduce their area, they collapse into a fold.

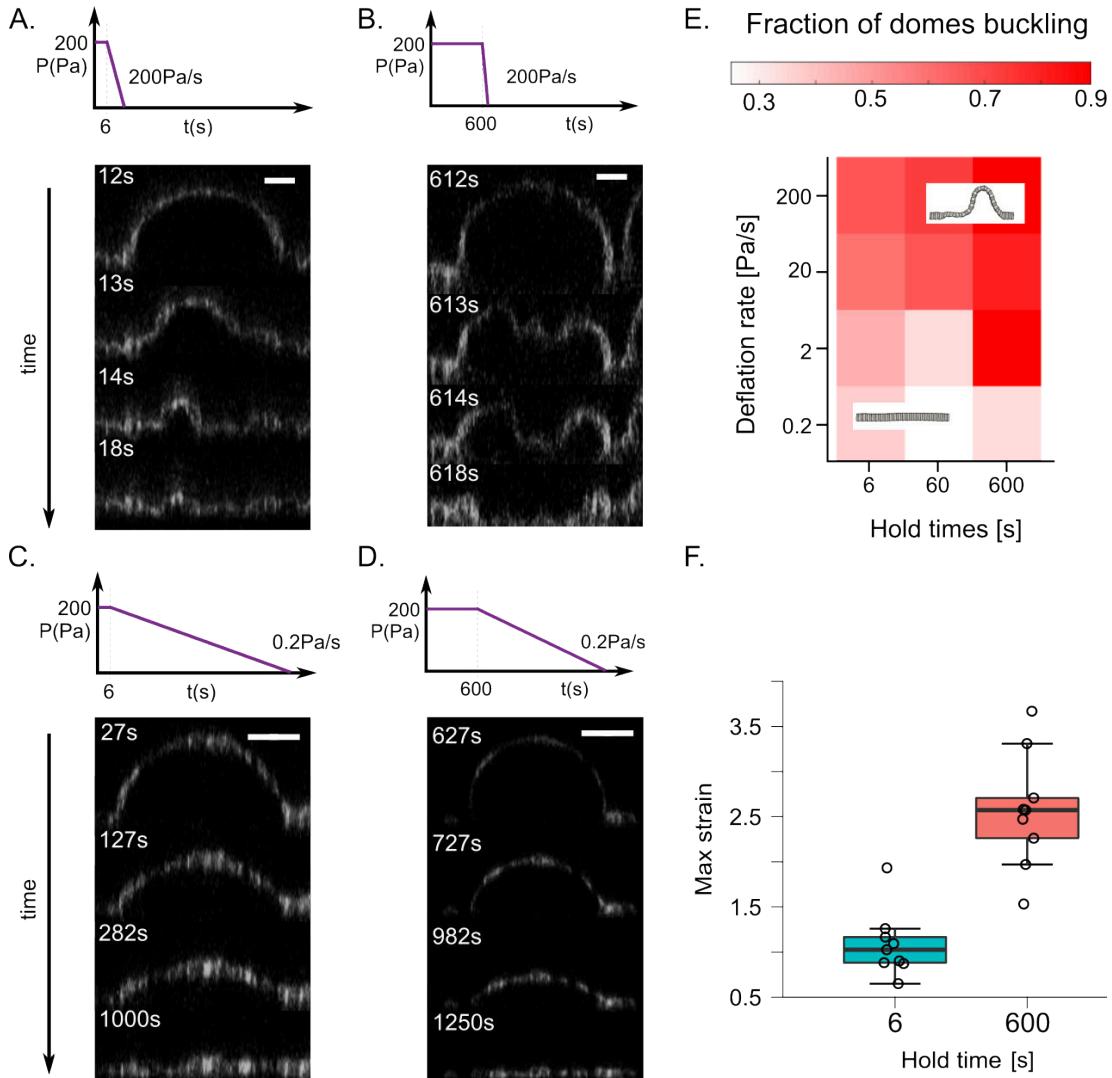


**Figure 8.2:  
Buckling protocol:**

The pressure is increased to  $200\text{Pa}$  for inflating the dome, and then the dome is given different amounts of time (hold time) to remodel before being deflated to  $-50\text{Pa}$  at different rates (deflation rate) to observe whether the dome buckles or not.

Despite the 6-second hold time, we observed buckling within the system. We intended to have a condition in which the dome undergoes rapid inflation and deflation before cytoskeletal remodeling could occur. We chose a 6-second hold time based on the imaging speeds we could not surpass. However, the rates of the inflation (10-second inflation plus 6-second hold) and deflation (1-second) process were too slow for the timescales of cytoskeletal remodeling (10-20 seconds). In conclusion, the experimental results suggest that deflation faster than the cytoskeletal remodeling timescale can cause buckling, even with a shorter hold time of 6 seconds.

It is important to note that we observed a wide variety of buckling patterns when examining midsections of the tissue through a line scan method. Some domes exhibited minor kinks in the folds, while others showed drastic buckling modes similar to those of plates.



**Figure 8.3: Buckling conditions:** (A-D) Representative montages of dome deflation for experiments and model at different deflation rates of 200 and 0.2 Pa/s after holding pressure constant of 200 Pa for 6 and 600 s. Scale bars are 20  $\mu$ m for XZ. (E) Diagram representing fraction of domes buckling for different deflation rates and hold time. Showing the optimum conditions for the buckling. (F) The maximum strain achieved is lower for 6s hold time compared to 600s conditions.

### 8.3 Multiscale buckling

The observation of tissue buckling was evident in the confocal line scan images. In order to gain a clearer understanding of the shape of the cells, we adapted a variation of MOLI for use with light sheet microscopy. The higher resolution 3D images of the dome revealed a variety of thicknesses of the cells, with thicker regions caused by the bulging of the nucleus and very

thin regions at the cell periphery (see fig 8.5 B).

To further investigate the phenomenon of buckling, we repeated the experiments described in the previous section, with rapid deflation and a long hold time. We discovered additional features beyond tissue-scale buckling, including kinks and crimps at cellular and subcellular scales. Upon closer inspection, we classified three levels of buckling:

1. Tissue
2. Cellular
3. Sub-cellular

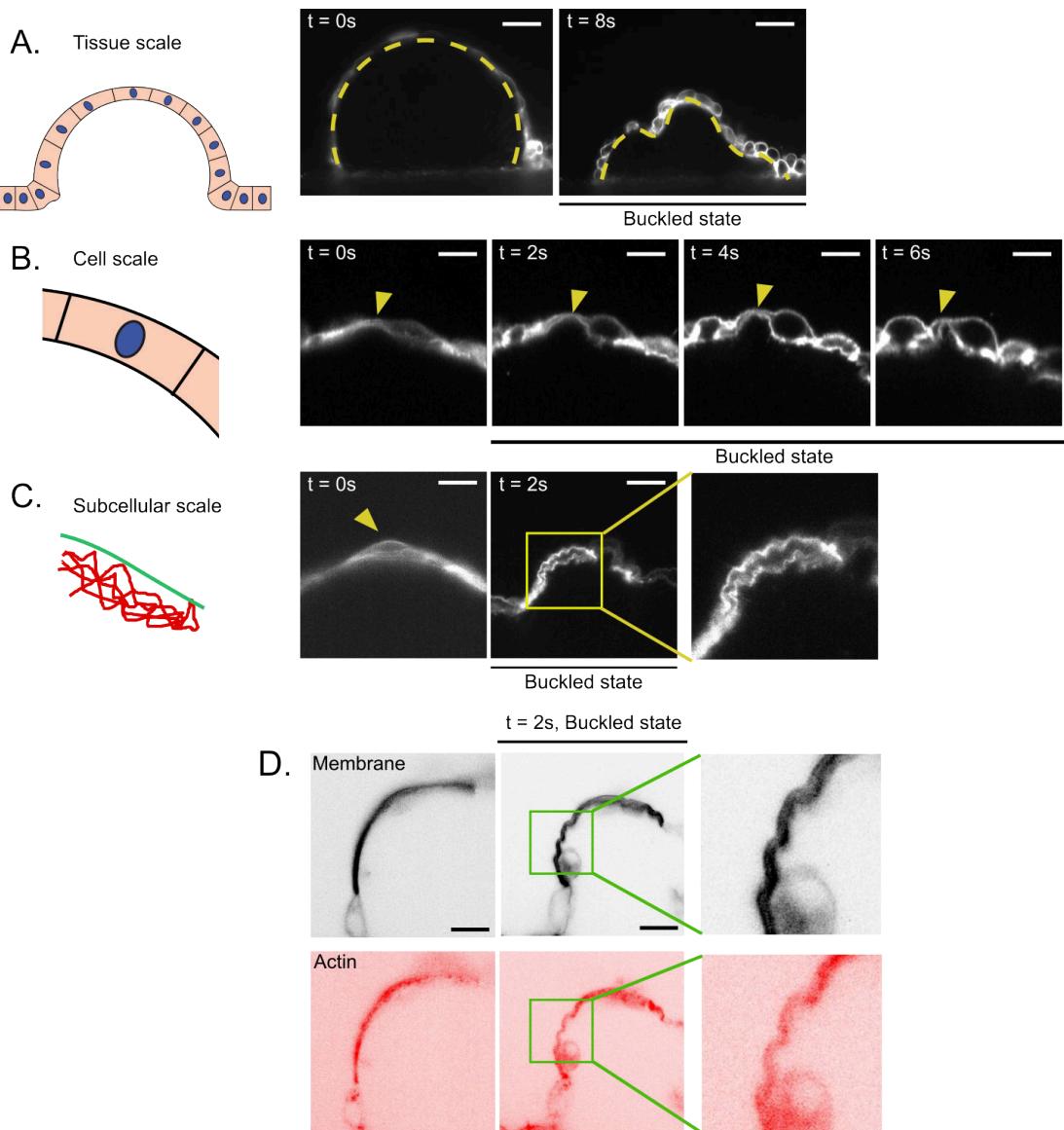
At the tissue scale, buckling was visible with cells collectively transitioning from a uniform curvature to a distorted shape (see fig 8.5 A). At this level, we observed cell deformation at a larger scale, including at the junctions between cells.

At shorter scales, we observed individual cells undergoing buckling (see fig 8.5 B). The length scale at which cell buckling occurred was much shorter than that observed at the tissue level. It appears as though the cell buckles as a single unit.

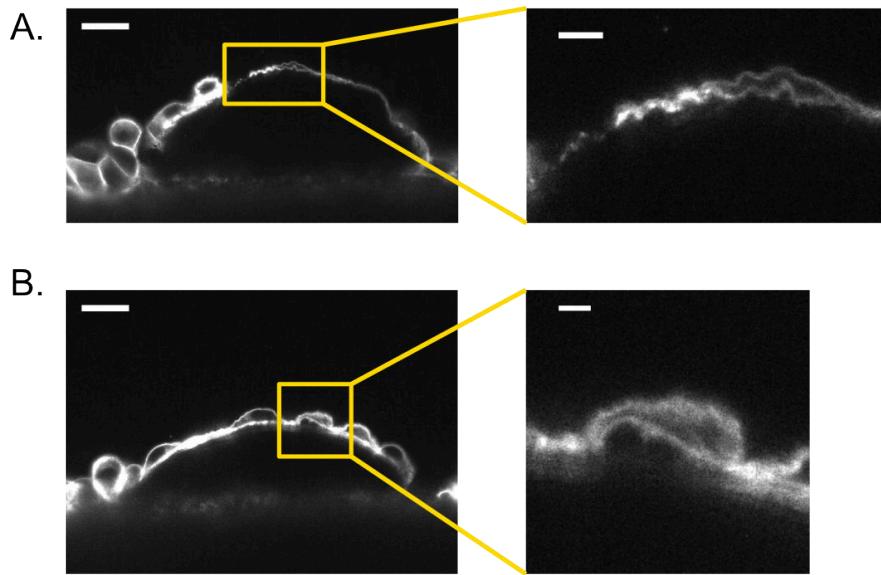
In some cases, buckling occurred at even shorter length scales, which we refer to as subcellular buckling, as the folds in the membrane were distinct from the cell level buckling. These folds occurred in the thinnest parts of the stretched cells, where the membrane buckled at much shorter wavelengths (see fig 8.5 C). Interestingly, these folds occurred on both the apical and basal sides of the cells.

In epithelial cells, the membrane is typically attached to the cortex through membrane-cortex attachment proteins such as ezrin, radixin, and moesin. It is reasonable to assume that the subcellular buckling observed in our experiments is due to actin cortex buckling. To confirm this, we imaged the actin cortex while the dome was undergoing buckling using SPY actin staining (see fig 8.5 D). Our results showed that the actin cortex followed the exact shape of the membrane during buckling.

We also observed that some domes did not appear to be buckling at the tissue scale, but were still exhibiting buckling at the cell or subcellular level (see fig 8.8). These categories are not strictly separated, as we observed multiple instances of tissue, cell, and subcellular level buckling (see fig 8.5 A).



**Figure 8.4: Multiscale buckling:** (A-C) Representative images of the domes undergoing buckling at different scales. Buckled and unbuckled states of the dome with zoom in section buckled component. Dotted yellow line represents uniform curvature in unbuckled state and non-uniform curvature in buckled state. Scale bar is  $20\mu m$ . (B) Evolution of a single cell in the dome during buckling. Highlighted by yellow arrow. (C) Some parts of cells undergo buckling producing short wavelength folds. (D) These folds are also present in the cortex too. Scale bar is  $5\mu m$  for (B-D).



**Figure 8.5: Cell level buckling:** In many instances where the tissue does not buckle, we observe individual cells buckling. Here is an example of cell buckling (A) and subcellular buckling (B).

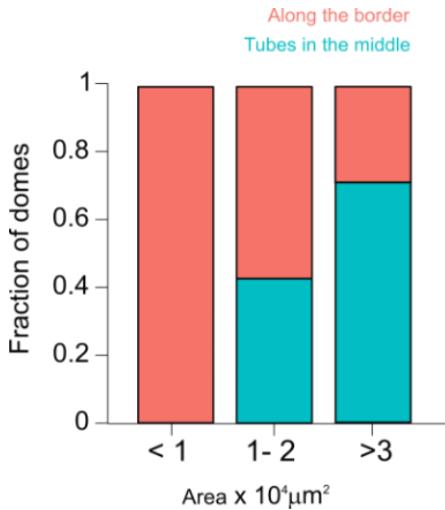
## 8.4 Generating epithelial folds

After optimizing the buckling conditions, we embarked on exploring epithelial folds. We have been imaging only the cross-section of the dome to capture the fast dynamics. We see the buckling in form of the squiggly lines. However, these buckling events are three-dimensional (see fig 8.3 B). During tissue buckling, a large area squeezed into original footprint area resulted in the formation of folds and wrinkles in the monolayer. Monitoring the base of the dome deflating, we observed that the tissue made contact with the substrate in certain regions first and others later. This led to the formation of folds in the regions where it made contact last (see fig 8.7 A-C).

To investigate if there was any pattern to these folds, we looked at spherical domes of various sizes. Broadly, we observed two types of folding patterns emerging (see fig 8.6):

1. Accumulation along the periphery
2. Folds in the middle

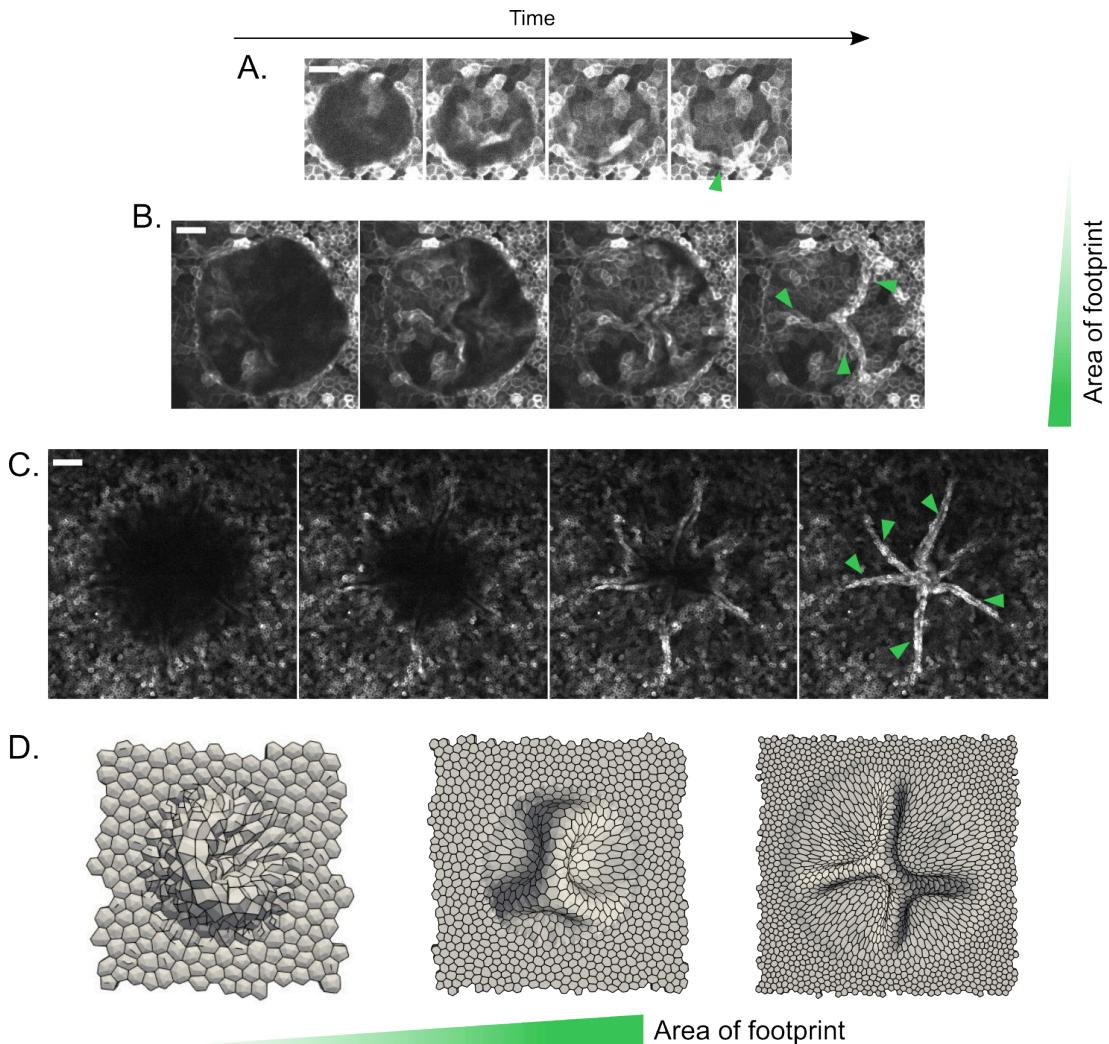
For domes with a footprint diameter smaller than  $\sim 110\mu m$ , we repeatedly observed that most of the buckling resulted in an accumulation around the periphery (see fig 8.7 A). The confocal timelapse from the base gave the impression of a donut-like structure, but three-dimensional imaging of the folds revealed a crescent-shaped fold like a croissant, taller on one side than the other. For larger domes with a footprint diameter greater than  $\sim 300\mu m$ , we observed more instances of domes forming a network of folds in the middle, with multiple folds

**Figure 8.6:****Buckling patterns:**

Buckling patterns observed in differently sized digital domes. The domes were grouped into three size categories and two categories based on location of folds (along the border and in the middle). We found that larger domes are more likely to buckle into a network of folds compared to smaller ones.

connecting each other by forming junctions (see fig 8.7 C). Finally, for domes of intermediate size, we observed a mixture of accumulation and folds, although the proportion of folds along the periphery decreased (see fig 8.6).

Interestingly, we observed the same folding patterns in our digital domes when performing the same deflation experiments (see fig 8.7 D). Larger digital domes produced more radial folds, and small digital domes formed an accumulation on the side. Intermediate-sized digital domes showed a mixture of both patterns.



**Figure 8.7: Buckling patterns in spherical domes of varied size:** Representative examples of digital domes undergoing buckling with time-lapse of their basal cross-section (A-C). In the first frame, the onset of buckling is visible where the dome makes contact in the middle. Subsequent frames show more of the fold coming into view, and when the dome completely deflates, a fold is formed (indicated by the green arrow). Panel (D) shows the final outcome of buckling for digital domes of different sizes. Scale bar is  $20\mu m$

## 8.5 Forming predictable folds

We were intrigued by how variations in the geometry of the domes might influence the pattern formation of folds. While we observed that spherical domes of different sizes produced varying and intricate buckling patterns, their axis-symmetric shape made it challenging to predict the patterns accurately. To overcome this limitation, we decided to generate large digital domes of

the same footprint area but with distinct geometries, including an ellipsoidal and a triangular shape. Remarkably, we found that the digital domes buckled into predictable patterns, with the ellipsoidal shape forming a line along the major axis and the triangular shape forming a Y junction fold.

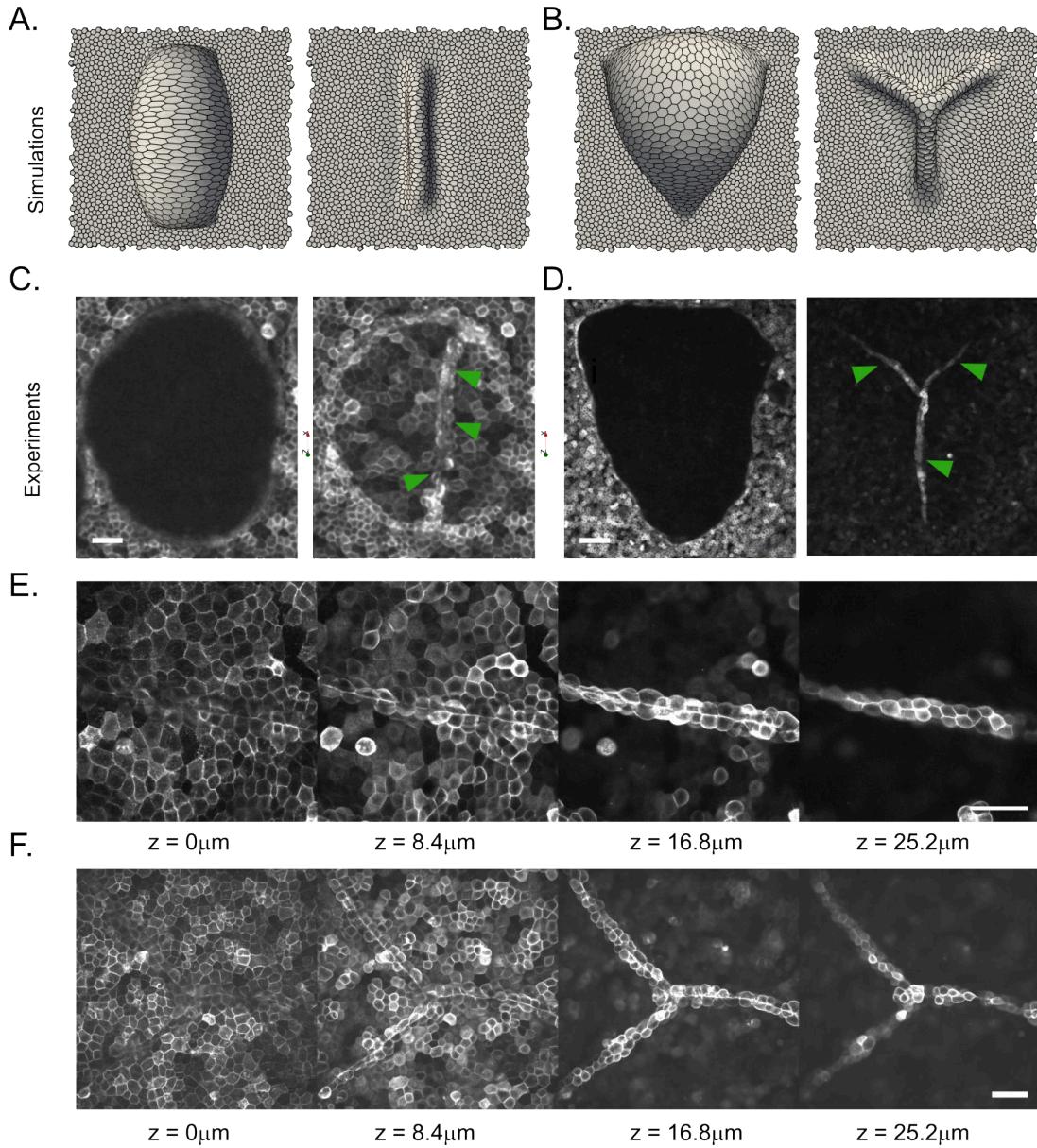
In order to explore the effects of geometry on the formation of folds in epithelial structures, we conducted experiments using MOLI with ellipsoidal domes of varying sizes. Our observations revealed that larger ellipsoidal domes would buckle into a fold along their major axis, just as digital domes, while smaller ellipsoidal domes produced a similar peripheral accumulation as spherical domes (see fig 8.8 B). This suggests that only larger domes, regardless of their shape, possess the capability to produce folds in the middle.

To further explore the possibility of creating more complex folds, we decided to buckle a dome with a triangular shape, anticipating that the vertices of the triangle could push the buckling along the medians of the triangle (see fig 8.8 C). As expected, the domes did buckle into forming a Y-shaped network of the fold (see fig 8.8 D). We also repeated the triangular and ellipsoidal shapes with digital domes and found similar patterns (see fig 8.8 A-B).

Furthermore, as expected, we found that the triangular domes buckled into a Y-shaped network of folds that resembled the pattern observed in digital domes (see fig 8.8 D). The timelapse of buckling indicated that the vertices of the triangular domes pushed the buckling along the medians of the triangle (see fig 8.8 C).

We observed that the folds exhibited variability in terms of stability, with some dissipating into the monolayer while others remained intact and even formed attachments with each other. These folded structures were stable for several hours and could be imaged for over 12 hours (see fig 8.8 E-F). Interestingly, when immediately inflated, these folds would unfurl themselves into a dome again.

Our results suggest that the MOLI system could provide a novel way of generating folds, with potential applications in tissue engineering. By controlling a few mechanical parameters such as geometry and pressure, it is possible to produce folds of predictable patterns.



**Figure 8.8: Controlling the patterns of fold:** A-B Simulations show digital models of ellipsoidal and triangular domes buckled into line and Y-shaped junctions. C-D Experimental results confirm the simulation findings. E-F Confocal z-stack images show the folds in the case of a line and Y-shaped junction. Scale bar is  $50\mu\text{m}$ .

## 8.6 Summary and Discussion

We utilized our device to generate dome from a flat monolayer then transform it into folds, alongside investigating the buckling response in relation to actin remodeling timescales. We discovered that buckling occurs at various scales, starting from the tissue level down to the actin cortex of individual cells, and is triggered when deflation occurs more rapidly than actin can remodel. We then explored the patterns of folding that emerge from different sized and shaped domes, and proposed a new method of creating controlled folds from planar monolayer. With the aid of computational models, we demonstrated the engineering potential of the dome system to produce structured folds by manipulating the geometry and pressure.

As mentioned earlier, mechanical instabilities are ubiquitous in biological systems, and the phenomenon of buckling has been observed in MDCK epithelial monolayers through various methods, such as growth in confinement or direct application of compression (Trushko *et al.*, 2020, Wyatt *et al.*, 2020). Wyatt et al. demonstrated that compressive stress greater than 35% strain can cause epithelial monolayers to buckle out of plane, and showed that active contractility can recover the out-of-plane deformation within tens of seconds.

Our study, on the other hand, is the first to offer visual insights into the minute details of the buckling process and its implications for tissue architecture at multiple scales.

From a mechanistic perspective, our result are a consequence of the hierarchical structure of the epithelial tissue, which comprises various components that sustain deformations and forces at different levels. Notably, the actin cytoskeleton plays a critical role in defining the shape of cells and tissues at multiple scales (Clarke and Martin, 2021). Additionally, a cell monolayer can be considered as an assembly of cells with their own surface tension and material properties, indicating a material with different length scales would buckle at different length scales. Therefore, if there is a local weakness in a cell or subcellular feature, it is expected to locally buckle. For instance, we only observed subcellular-level buckling in very thin cells while overall tissue does not buckle.

Furthermore, the short-wavelength folds resulting from subcellular buckling are intriguing to consider. It is worth noting that actin buckling is not a new phenomenon in the field, and there are minimal models of actin filaments with myosin motors on a lipid membrane demonstrating that myosin-induced contraction leads to actin filament buckling (Costa *et al.*, 2002, Murrell and Gardel, 2012, Wang and Qian, 2019). In membrane-actin droplets, researchers have reported multiple modes in the form of buckling and wrinkling, depending on the thickness (Kusters *et al.*, 2019). Interestingly, they found that thin shells undergo buckling and thin shells produce wrinkles in the membrane but not in actin, which could indicate different modes of buckling within a cell.

In the context of tissue-scale buckling, our results can be understood in terms of modes of buckling in thin shells. Our computational model suggests that the cortex behaves like a

hyperelastic material at short timescales, as in our case by the rate at which we are deflating these tissues. Similar results would be obtained if we repeated the experiment with elastic shells. The literature on thin shell buckling reveals similar aspects, such as the folds and patterns that emerge when different sized shells buckle (ref).

The slenderness, defined as the ratio of dome radius to thickness, intuitively guides the different modes of buckling. Thin shells with high slenderness would lead to a higher mode of buckling compared to thicker shells. However, our experiments go beyond understanding the system and show that we can program folds by minimally controlling two parameters: geometry and pressure. For non-spherical domes, anisotropic stresses are observed along the axes of the elliptical footprint for ellipsoidal domes (ref). These stresses can orient the folding in a particular direction to generate a programmed fold, such as Y junctions on the sides of a rectangular footprint.

In summary, this thesis presents a novel experimental system that allows us to inflate epithelial domes and deflate them into tubes. We demonstrate that the timescales of actomyosin cytoskeleton remodeling play a key role in this transformation. By controlling the geometry of the epithelia and the rate of deflation, we show that we can engineer epithelial folds of desired geometry.



# **Chapter 9**

# **Conclusions and Future Perspectives**

## **9.1 Conclusions**

The main conclusions of this study are as follows:

1. We have developed a microfluidics-based system for generating 3D epithelia using micropatterning technique PRIMO to create a non-adhesive region from which epithelial monolayers can detach and inflate into a dome.
2. Using the MOLI technique, we probed the mechanics of epithelial domes subjected to constant pressure and found that the dome reaches a steady state after five minutes of applying pressure. These experiments revealed a non-monotonic tension-strain response due to geometric constraints.
3. The constitutive response of the epithelial tissue showed that the domes exhibit an initial increase in tension with strain, tending to a tensional plateau at high strains, consistent with earlier studies demonstrating superelastic behavior in epithelia.
4. Dynamic material testing of domes with varying inflation/deflation rates demonstrated that the epithelium behaves as an active viscoelastic material.
5. We developed a complementary model to understand the different timescales involved in the tissue stretching process.
6. Rapid deflation to -50 Pa, faster than the remodeling timescales, leads to buckling instability.
7. Buckling occurs at multiple length scales, from the subcellular level to the tissue level, with differing characteristic lengths of the folds. The shortest folds occur at the membrane cortex level and the longest at the tissue scale.

8. Different sized spherical domes create different patterns. Smaller domes buckle into a fold along their periphery, while larger ones tend to create a network of folds in the center.
9. Folds can be programmed by controlling the shape of the dome. Elliptical domes produce a fold along the major axis, while triangular domes produce a Y-shaped network in the middle.

## 9.2 Future Perspectives

The experiments and theory presented in this thesis focus on the active viscoelasticity of tissues and the generation of folds using buckling instability. However, this experimental setup has implications for several projects within our research group.

For example, the current experiments only examined short timescales (<10-30 minutes) and focused solely on the actin cytoskeleton. Investigating the role of other cytoskeletal components, such as intermediate filaments, would be of great interest. Past studies have shown that intermediate filaments are critical in tissue re-stiffening. My colleague, Tom Golde, is currently using MOLI to study intermediate filament networks.

In addition, we are also utilizing the MOLI device to study a variety of different tissues, including stem-cell tissues, cancer tissue, and organoids. This could enable us to investigate the interplay between geometry, pressure, and cell fate. The inverted cell culture method we use also allows for high-resolution imaging. Two-channel system provides a conducive environment for maintaining complex culture conditions as well as allow for co-culture possibilities.

For the mechanobiology community, our setup is particularly appealing because, when the domes are stretched beyond 100%, the nucleus becomes compressed, which triggers various mechanotransduction pathways. We could easily examine the role of the nucleus and different mechanosensitive proteins when subjected to deformation. Additionally, we could use pharmacological treatments to alter tissue tension and understand the specific molecular pathways involved in maintaining tissue shape.

At the molecular scale, we could also investigate focal adhesions during delamination. Our experimental system delaminates tissue from the substrate, which presents an opportunity to study cell-substrate adhesion using protein patterning and live-cell imaging with focal adhesion markers. Furthermore, experiments conducted over longer timescales could allow us to explore the mechanics of cell-cell junctions and cellular rearrangements in response to prolonged stretching.

I must note the tissue hydraulic aspect, which has been largely overlooked in this thesis. Specifically, we have not extensively explored the trans-epithelial flow due to its negligible effect on our timescale. However, recent studies have demonstrated that the epithelial tissue can function as an active mechano-biological pump, generating its own pressure gradient over longer timescales. Thus, it would be worthwhile to investigate the role of fluid transport under controlled pressure in our microfluidic system.

Through my work with this system, I have identified numerous promising directions for future research. One particularly intriguing project, led by Thomas Wilson, involves the implementation of concepts such as shape changing, self-healing, and flexible epithelia in the creation of biohybrid devices. Our initial approach will involve the construction of a microflu-

idic chip, where the channels are composed of epithelial tissues that can be manipulated using optogenetic tools to open or close specific segments, akin to valves. This endeavor will enable us to generate novel synthetic epithelial tissue systems and develop a more comprehensive understanding of the underlying physical principles driving morphogenesis.

## **Part III**

# **Appendices**



## Appendix A

# Methods and Materials

### A.1 Fabrication of microfluidic devices

Polydimethylsiloxane (PDMS) gels (Sylgard PDMS kit, Dow Corning) were used to make the microfluidic devices. PDMS was synthesized by mixing the curing agent and elastomer in 1:9 weight ratio. This mixture was centrifuged for 2min at 900rpm to remove air bubbles. The unpolymerized PDMS was poured into a mold or spun to obtain the desired shape.

There are four parts to the device (figgg). First is the top block, a thick PDMS block with four inlets and one channel for the application of hydraulic pressure. The second is a  $200\mu m$  thin PDMS layer with a  $1.2mm$  diameter hole in the center with a  $400nm$  porous membrane (Polycarbonate filtration membrane  $0.4\mu m$ , Whatman membranes) attached to it. The third is another  $200\mu m$  thin PDMS layer with a channel for seeding the cells. Lastly, all these PDMS parts are attached to, the fourth part, a glass-bottomed 35mm dish ( $35mm$ , no.1.5# coverslip thickness, Cellvis).

The top block was made using replica molding in a 3D printed mold. This mold was 3D printed with vat polymerization and a digital light processing 3D printer (Solus DLP 3D Printer with SolusProto resin). The mold's surface was then silanized using Trichlorosilane (Trichloro(1H,1H,2H,2H-perfluoroctyl) silane, Merck) for preventing adhesion with unpolymerized PDMS. PDMS was poured into the mold and degassed for one hour. PDMS is cured with a hot plate at  $100^\circ C$  for  $30min$ . Once cured, PDMS is removed, cut into devices, and punched with  $1.5mm$ .  $200\mu m$  thin PDMS layers were made by spin coating  $4.5ml$  unpolymerized PDMS on a  $15cm$  dish at  $500rpm$  for  $1min$ . These dishes were incubated in an oven at  $80^\circ C$  to polymerize for  $12hr$ . These thin sheets were cut into the parts of devices using a Silhouette cutting machine (Silhouette Cameo 4, Silhouette America). The sheets were attached to a Silhouette cutting mat and then Silhouette software was fed with the pattern of the device layers. A sharp cutting tool in the machine cut the PDMS along the pattern. These cut PDMS were peeled off with help of 70% ethanol.

These devices are assembled with the aid of ozone plasma cleaner (PCD-002-CE, Harrick Plasma). Glass bottomed dishes and thin PDMS layers with cell channels were treated for  $1\text{min}$  under plasma. Then bonded together by placing the layers in contact for  $2\text{hr}$  at  $80^\circ\text{C}$ . Similarly, the top block and thin membrane with porous membrane were also bonded. These layers were later bonded together again using plasma cleaner.

## A.2 Patterning protein on the device

The devices were filled with 96% ethanol for removing air bubbles. Then, devices are treated with 5% v/v (3-aminopropyl) triethoxysilane (Merck) diluted in 96% ethanol for  $3\text{min}$  and rinse three times with 96% ethanol. Later the devices were filled with MilliQ water to remove ethanol traces. PRIMO (Alveole Lab) was used to pattern adhesion-promoting protein. For this setup, devices were incubated with PLL (Poly-L-lysine solution, Merck) for  $1\text{hr}$ , subsequently with SVA PEG ( $50\text{mg}/\text{ml}$  in  $8.24\text{pH}$  HEPES) for  $30\text{min}$ , and rinsed with HEPES. Before using PRIMO, devices were filled with a photoinitiator. Desired protein pattern was loaded into the PRIMO software (Leonardo, Alveole Lab).

PRIMO uses a microscope to shine the laser in the specific region according to the loaded pattern to cut PEG chains. After the PRIMO process, the samples were rinsed with phosphate-buffered saline (PBS, Merck). Then the samples were filled with fibronectin and fibrinogen ( $100\mu\text{g}/\text{ml}$  Fibronectin in 2% Far-red fibrinogen solution in 1X PBS) solution for  $5\text{min}$ . Then samples were rinsed again with 1X PBS. Fibrinogen labels the fibronectin with Far-red signal to image the coated protein pattern and allows for tracking the position of the domes. The PRIMOed samples can be stored at  $4^\circ\text{C}$  for two to three days before seeding cells.

## A.3 Cell culture in the device

To image cell shape and tissue structure Madin-Darby Canine Kidney (MDCK) cells expressing CIBN-GFP-CAAX were used for the experiments. CIBN-GFP-CAAX labels the plasma membrane. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco ThermoFisher) with 10% v/v fetal bovine serum (FBS, Gibco, ThermoFisher), L-glutamine (ThermoFisher),  $100\mu\text{g}/\text{ml}$  streptomycin and penicillin. Cells were incubated at  $37^\circ\text{C}$  with a 5%  $\text{CO}_2$  condition.

Before seeding cells in the device, it is filled with a cell culture medium. Cells are trypsinized and diluted at a concentration of  $25 - 30 \times 10^6\text{cells}/\text{ml}$ . The cell channel of the device is filled with  $30\mu\text{l}$  of cell solution and incubated for cell adhesion. After one hour of incubation, devices are rinsed with media to remove unattached cells. Devices were kept  $24\text{hr}$  in the incubation for the growth of a monolayer before the experiment. It is important to note that the inverted epifluorescence microscope is needed to see the cells. The bright-field microscope can not be

used for visualizing cells because of the porous membrane.

#### A.4 Staining actin with SPY-actin

To observe the dynamics of the cortex, we used SPY555-actin (Spirochrome), a bright dye optimized for quick labeling of F-actin in live cells with low background. To prepare the 1000x solution, we added  $50\mu l$  of anhydrous DMSO to the stock SPY555-actin. We then added  $1\mu l$  of the 1000x solution to  $999\mu l$  of cell culture medium. The resulting solution was introduced into the microfluidic chips and left in the incubator for 2 hours before imaging.

#### A.5 Fabrication method for the Light-Sheet MOLI device

The devices used with the light-sheet microscope consisted of a single PDMS block bonded to a glass microscope slide ( $76 \times 26mm$ , RS Components BPB016). The blocks were made using a 3D printed mold (Ultimaker 3 with Ultimaker PLA Printer Filament 1616). PDMS was mixed, centrifuged, degassed, and cured as described above for the normal devices. Once cured, the PDMS was removed, cut into individual devices and punched with a 1.5mm biopsy punch. The PDMS blocks were then attached glass slides using a thin layer of unpolymerized PDMS, that was coated onto the glass slides using a spatula. The devices were then kept on a hotplate at  $100^\circ C$  for  $30min$  to allow the PDMS bonding to fully cure. The  $400nm$  porous membranes were then attached to the devices. The edges of the membrane were carefully dipped into unpolymerized PDMS, before being placed flat on the top of the device. Particular care was taken to ensure the center of the membrane over the punched pressure-application hole remained free of PDMS. The devices were then kept at  $65^\circ C$  for an hour to allow the PDMS bonding to fully cure.

#### A.6 Device protein patterning and cell culture in Light-Sheet device

The light-sheet devices were protein patterned and cell cultured using the same methods and steps as outlined above for the normal devices, with the one minor addition of the use of a simple PDMS and glass cap for a few critical steps. The porous membrane for pressure application, and thus the site of protein patterning and cell seeding, for the light-sheet devices is exposed and on the top side of the devices. This mostly allowed for easy application of reagents as a droplet could be applied and aspirated directly.

However for the more sensitive steps in the procedure, a simple PDMS and glass device was used to create a temporary covered channel over the porous membrane to regulate the procedure and ensure the treatment of the devices was highly standardized. Specifically, the

cap was used for the application of photoinitiator during PRIMO, and for the application of cell solution during cell attachment. The caps were fabricated using  $2\text{cm} \times 2\text{cm}$  squares of a  $400\mu\text{m}$  thick PDMS layer, with a keyhole shape cut in from the side. Each PDMS piece was then stuck to a  $18\text{mm}$  diameter coverslip ( $18\text{mm}$ , 1# Cover glasses circular, Marienfeld 0111580) using the surface tension of the liquid. The experimental apparatus and measurements for the light-sheet devices were the same as the normal devices as outlined above.

### A.7 Application and measurement of the pressure

The pressure is applied via hydrostatic forces similar to the previous studies (Choudhury *et al.*, 2022b, Palmer *et al.*, 2021). The two channels in the chip were separated by the porous membrane. Cells are on the bottom side of the membrane. The pressure in the channel (top side of the membrane) is used to inflate the structures on the top. This channel has one inlet and one outlet for removing bubbles. The inlet is connected to a  $35\text{ml}$  reservoir of cell culture medium (in a  $50\text{ml}$  falcon tube) by tubing (PTFE Tubing 1/16inch OD for Microfluidics, Darwin microfluidics) and the outlet is connected to a shutoff valve (Microfluidic Sample Injection / Shut-off Valve, Darwin microfluidics). Once bubbles are removed, closing the valve would apply the pressure on the basal side of the cells according to the difference between the height of the fluid level. All tubings are connected to the chip with a steel insert (Stainless steel 90deg Bent PDMS Couplers, Darwin microfluidics). We are able to find zero by matching the height of the device to the liquid and air interface in the reservoir. This is confirmed with the experiments, where on applying pressure domes form but on slow reduction in pressure to zero causes domes to deflate.

### A.8 Confocal Microscopy

For timelapse imaging of domes at a larger time interval ( $> 1$  min), an inverted Nikon microscope with a spinning disk confocal unit (CSU-W1, Yokogawa) was used with Nikon 40x, 20x, and 10x air lenses. For shorter time intervals ( $< 10$  s), a Zeiss LSM880 inverted confocal microscope was used with laser scanning mode. Fast imaging was enabled by imaging a single line in the middle of the dome.

### A.9 Light-sheet microscopy

The imaging of the light-sheet devices was done with a dual-illumination inverted Selective Plane Illumination Microscope (diSPIM) (QuVi SPIM, Luxendo, Brucker) with Nikon 40x immersion lenses (Nikon CFI Apo 40x W 0.8 NA NIR water immersion objective). For the

buckling experiments, only single objective illumination and detection was used for the fast imaging of  $2\text{s}/\text{frame}$ .

## A.10 Quantification of the dome areal strain and tension

As mentioned earlier, the domes were imaged in 3D with confocal microscopy. We used ImageJ to manually section the dome in the middle in the YZ plane, XZ plane is a plane parallel to the monolayer, with Reslice function along the Z axis. This section was used to calculate the height ( $h$ ), radius of curvature ( $R$ ), and base radius ( $a$ ). Strain ( $\epsilon$ ) and tension ( $\sigma$ ) were calculated as,

$$\epsilon = \frac{h^2}{a^2}, \quad \text{and} \quad \sigma = \frac{\Delta PR}{2}.$$

The raw data was extracted in ImageJ and then MATLAB was used to compute and plot the strain and tension.

## A.11 Analysis of the kymographs

For cyclic pressure or buckling experiments, the domes were imaged at low resolution and high noise levels to capture fast dynamics. The previous method of manually quantifying each time point is not feasible. Thus, we used the ImageJ function of the Reslice function along the time axis. We resliced it along the Y-time axis in the middle of the dome, such that we get a kymograph of height as a function of time. Also, we performed the reslicing along the XT axis at the plane of the monolayer, such that we get the kymograph of the base radius with respect to time. These kymographs were in form of images save manually with ImageJ.

A custom-built MATLAB code was used to digitize the kymographs, where maximum intensity along each time was considered as the current dome height position. The first 30s of the experiment pressure is zero, so the unstretched monolayer position is determined from those time points. Dome height is calculated with the difference between the current position and the initial position. Base radius is calculated similarly by subtracting two sides. The radius of curvature is calculated using the relation between the base and height of the dome as

$$R = (h^2 + a^2)/2h.$$

## A.12 Qualitative analysis of the buckling event

Whether domes are buckling or not was determined manually checking every frame during the deflation. If dome maintains the smooth circular geometry in XZ plane during the deflation, we mark the dome as “not buckling”. However, if the dome has a visual discontinuity in the curvature or a kink it is then considered to be “buckling”.

Imaging the fast events in XY plane was done in an ad hoc manner. To capture the folds, the dome as imaged closer to the apical surface of the monolayer. The type of fold was determined by carefully observing the way which monolayer makes contact with the imaging plane. If there is one point of contact in the center and spreads outwards, it is considered as accumulation along the periphery. In case where there are multiple points of contact and they all join in the middle, it is considered as a network of folds.

## **Appendix B**

# **MOLI Protocol**

### **B.1 Device Fabrication**

#### **B.1.1 Main Block Fabrication**

1. Place the empty 3D printed mold on hot plate at 100°C for 1 hour.
2. Mix around 20 ml of PDMS (Sylgard kit) at a 1:9 ratio.
3. Pour PDMS into a 50ml centrifuge tube, and weight out another of equal mass of water.
4. Centrifuge for 3 mins at 900rpm.
5. Fill the mold with 12ml of PDMS.
6. Place the mold with PDMS into a desiccator for 1 hour or until there are no bubbles remaining.
7. Then place the mold on a hot plate at 100°C for 30 mins, until it is polymerised and solid to touch with tweezers.
8. Carefully cut the PDMS block out from the mold and place it into a dish with the pattern side up.
9. Repeat Steps 5 to 8 to create more blocks and then with any remaining PDMS, fill the mold with a small amount and set it on the hot plate 100°C, so as to keep the mold clean.
10. Punch four holes in the devices with the 1.5mm biopsy punch.
11. Cut out the individual devices with the scalpel.
12. Store devices with the pattern facing up in a dish.

#### **B.1.2 Membrane Fabrication**

1. Meanwhile, prepare around 10ml of PDMS at a ratio of 2:8. Centrifuge as in Steps 3 and 4 in the previous section.

2. Using a syringe, measure 4ml of PDMS into a large petri dish.
3. Use the nitrogen gun to remove bubbles and evenly spread the PDMS across the dish, whilst not letting it touch the sides.
4. Leave the dish on a level surface at room temperature for at least 2 hours to allow the PDMS to coat evenly.
5. Place in the oven at 80°C for an hour (or 60°C for 2 hours).
6. Follow section 1.2 to cut the membranes.

#### B.1.2.1 Silhouette Cutting Method

7. Cut the biggest rectangle possible in the PDMS membrane with scalpel.
8. Use a bit of 90% ethanol to peel off the membrane from the dish.
9. Place the membrane on the silhouette cutting mat. Make sure to have membrane completely dry before placing it.
10. Press the membrane against the mat to get rid of all the bubbles and making adhesion with the mat more secure.
11. Load the cutting mat in the Silhouette machine.
12. Open cutting pattern in the silhouette software.
13. Select the cut settings:
  - *Material = Vinyl*
  - *Depth = 10*
  - *Speed = 5*
14. Make sure the PDMS is at a correct location corresponding to the drawings.
15. Press ‘Send to silhouette machine’.
16. During the cutting process, be aware of pieces of PDMS delaminating and getting stuck in the machine.
17. After the successful cutting, unload the cutting mat.
18. Remove all the small PDMS and holes before then peeling off the actual layers and storing them in a dish.

## B.2 Plasma Bonding

### B.2.1 First Bonding

1. Items needed in the clean room:

- PDMS blocks and membranes
  - Glass bottom dishes
  - Scalpel
  - Tweezers
  - Cutting mat
2. Have a hot plate next to the plasma cleaner at 100°C with cloth on top.
  3. Plasma clean a large dish with the top blocks and the middle layers. Use the high setting for 30 seconds.
  4. Combine the block and membrane (so as to create an enclosed channel with a central hole). Make sure to line up holes and apply a lot of pressure to the PDMS.
  5. Flip over the blocks (membrane side up) in the dish, and place on the hot plate.
  6. Place the bottom channel layers into the lids of the glass bottom dishes, with two membranes per lid.
  7. Plasma clean the layers and glass bottom dishes the same as Step 4.
  8. Holding each dish at arms length, spray it with ethanol so that it gets very lightly coated, and then attach a membrane to the centre of the dish. Apply some pressure and ensure that there are no bubbles but do not apply too much force as the glass bottoms of the dishes are fragile.
  9. Put the lids onto the dishes and place them onto the hot plate.
  10. Once plasma area is cleaned, place the samples into the oven at 80°C. Leave for a minimum of 2 hours.

### B.2.2 Second Bonding

1. Cut any protruding parts of the membrane from the bonded PDMS blocks, and then cut the corners off to create an octagonal shape.
2. Cut 400nm porous membrane disks into small pieces of around 4mm × 4mm (9 pieces per sheet). Store these between layers of a cloth in a large petri dish.
3. Mix around 20ml of PDMS and put small amount (<2ml) into a petri dish.
4. On the underside of the glass bottom dishes, mark the edges of the bonded channel with a pen.
5. Return to clean room with devices, membranes and unpolymerised PDMS.
6. Have a hot plate at 100°C.
7. Hold a piece of paper with tweezers, slightly dip one edge of the paper into the wet PDMS and remove excess by placing it on a cloth.

8. Gently, paint the wet PDMS onto the middle layer around the hole with paper. Be cautious of using too much PDMS might result in blockage on the hole.
9. Place the porous membranes over the central hole of the PDMS block and press down the edges gently.
10. Similar to the first bonding, place the blocks into the glass bottom dish lids with two per lid.
11. Plasma clean the blocks and glass bottom dishes, following the same specifications as Step 4 in Section 2.1.
12. Bond the blocks and dishes by placing the blocks porous membrane side up on your finger and align the channel using the pen marks as a guide. Ensure that the holes are lined up and the two enclosed channels are perpendicular. Press the PDMS block on the cloth to ensure attachment.
13. Put lids onto the dishes and place them onto the hot plate.
14. Once plasma area is cleaned, return with the samples and place them into the oven at 80°C.
15. After 30 mins, use a 20ml syringe to pour PDMS around the base of the devices for a better seal, covering the remainder of the glass base.
16. Return the devices to the oven and leave for a minimum of 2 hours.

## B.3 PRIMO

### B.3.1 PRIMO Preparation

1. Wash the devices with ethanol. Pipette 80µL of 70% ethanol thought each channel, ensuring that it only emerges from the opposite hole, and that there are no bubbles in the channels. Aspirate off the excess.
2. Repeat this with MilliQ water or PBS.
3. Pour a few drops of MilliQ water around the edge of the device to reduce evaporation.
4. Fill each channels with 80µL of PLL.
5. Leave at room temperature for 1 hour.
6. After 50 mins, create SVAPEG solution (will require 80µL per device so scale accordingly):
  - Accurately measure around 20mg of SVAPEG powder.
  - Add 20× the amount of HEPES buffer solution to the powder (400µL for 20mg of powder).
  - Mix well, ensuring all powder is included. Do this immediately before using.

- Using a desiccator, store the SVAPEG powder under argon, sealed with para-film and in a vacuum bag.
7. Pipette 40 $\mu$ L of HEPES buffer solution into each channel and aspirate the excess.
  8. Soon after mixing the SVAPEG solution, fill each channel with 40 $\mu$ L of it.
  9. Leave for 30 mins at room temperature or overnight at 4°C, with enough MilliQ water around them to prevent them becoming dry.

### B.3.2 PRIMO Protocol

1. Prepare the fibronectin solution (will require 40 $\mu$ L per device):
  - Combine 20 $\mu$ L of fibrinogen [FGN] with 980 $\mu$ L of PBS.
  - Filter the solution with 0.22  $\mu$ m filter.
  - Take 600  $\mu$ L of the mixture and add 60  $\mu$ L of fibronectin [FN]. Discard leftovers.
  - Store in the ice.
2. Add 40  $\mu$ L of photo-inhibitor to the lower channel of the devices. Do this in batches of three or four.
3. Preform PRIMO:
  - Use the PRIMO enabled microscope.
  - Focus on porous membrane, which will be when it appears darkest.
  - Find boundary of the 1.2mm hole and match 1200 $\mu$ m circle.
  - Select ‘Scan’ and then ‘Lock’.
  - Select ‘ $\mu$ Pattern’ and load template.
  - Select *Lines = 4* and *Dose = 500*.
  - Set Spacing to the negative of the ‘*Find Size Height*’ ( $-1223\mu$ m), but adjust to get the best pattern match.
  - Turn off both ‘Eye’ Icons on the left side.
  - Select ‘Forward’ in lower-right corner of screen and wait for the process to complete, which should be around 17 mins.
4. After PRIMO is complete, pipette 80 $\mu$ L of PBS into each channel and aspirate excess.
5. Then pipette 40 $\mu$ L of FN solution (from Step 1) into the lower channel. Do this in batches of three or four.
6. After exactly 5 mins (important to be accurate), wash all channels with PBS twice.
7. Place a few drops of MiliQ water around the devices and store in fridge at 4°C. Note that cells must be seeded with 2 days of PRIMO completion.

## B.4 Cell Seeding

1. Warm media and trypsin in the cell culture bath.
2. Treat all devices with UV for 15 mins using the fume hood ('Disinfect' → 'UV'). Ensure all lids are opened.
3. Detach cells from the flask using 3ml trypsin, as normally done in cell passing.
4. While cells are in the oven detaching, get two 15ml vials and fill one with media.
5. Aspirate the top of the devices and fill all channels with media (from vial). Aspirate the excess.
6. After removing cells from the oven, mix with 7ml of media.
7. Using a Neubauer Chamber, count the number of cells in the sample.
8. Meanwhile, fill the other 15ml vial with the rest of the cell culture and centrifuge for 3.5 mins at 1000rpm.
9. Aspirate away the media from the vial, leaving only the cells at the bottom.
10. Add new media, such that the sample is diluted to 25-30 million cells per ml.
11. Pipette 35mL of culture into the lower channel of each device. Always mix the cells in the vial with reverse pipetting between samples and quickly aspirate off the excess. Put a few drop of media on top and around each device.
12. Flip tray over and place in the incubator.
13. After 1 hour, flip the devices back round and aspirate any media off the device tops.
14. Quickly pipette 200 $\mu$ L of media into the device lower channels to remove unattached cells, while aspirating off the excess media.
15. Put a few drops of fresh media on top of the devices to slow evaporation.
16. Using a microscope, check the devices. Use the CY5 channel to check for a pattern, and the GFP channel for a uniform layer of cells.
17. Rinse devices again with media and place a large droplet on top of the devices such that when the lids are replaced, a 'column' of media is formed.
18. Place in the incubator again (the correct way up) and leave overnight.

## B.5 Experiment

1. Warm up two reservoirs of CO<sub>2</sub> media (one of around 35ml and one of around 10ml) for around 30 mins.
2. Then place the reservoirs in a desiccator for 30 mins (with lids unscrewed).

3. Clean tubing with PBS.
4. Connect the translation to both power and the computer. Place the 35ml of media in the stage holder.
5. Connect vacuum and open to 200psi. Tape tubes into place around the microscope.
6. Open stage control software *ZABER*, and move stage to where the level of the media is the same as that of the devices.
7. Move condenser as high as possible and connect pressure stage tubing to one of the upper-channel device outlets. Use the vacuum to ensure the full tube is filled with media before connecting, as it is important that there are no bubbles in the tubing or device.
8. Connect the vacuum T-device to the other upper-channel device outlet.
9. Slowly lift the media reservoir out of the stage until domes start to appear in patterned areas. Return reservoir to stage.
10. Using stage software, re-form domes and identify the best one(s) for imaging.
11. Perform the experiment of choice.
12. After experiment, disconnect everything and clean tubing with ethanol and PBS.



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