

Technische Universität München
TUM School of Natural Sciences

Microfluidic Engineering of Multicellular Assemblies

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Vollständiger Abdruck der von der TUM School of Natural Sciences der Technischen Universität München zur Erlangung einer

Doktorin der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitz: Prof. Dr. Karen Alim

Prüfende der Dissertation: 1. Prof. Dr. Andreas Bausch

2. Prof. Dr. Joachim Rädler

Die Dissertation wurde am 29.01.2025 bei der Technischen Universität München eingereicht und durch die TUM School of Natural Sciences am 25.04.2025 angenommen.

Kurzfassung

Organoidkultur repliziert die morphologische Komplexität von nativem Gewebe äußerst präzise und bildet die verschiedenen Entwicklungsstadien der Organogenese nach, während derer sich multizelluläre Systeme zu räumlichen Mustern aus unterschiedlichen Zelltypen selbst organisieren [1]. Das Verständnis der physikalischen Mechanismen, die der Selbstorganisation von multizellulären Systemen zugrunde liegen, ist entscheidend, um biologische Prozesse für Anwendungen wie beispielsweise das Tissue Engineering gezielt steuern zu können. Diese Arbeit hat das Ziel, (i) die physikalischen Prinzipien der Ausrichtung von Endothelzellen zu entschlüsseln und (ii) ein mikrofluidisches System zu entwickeln, um die Skalierbarkeit und Reproduzierbarkeit der Organoidkultur zu verbessern.

Der erste Teil dieser Dissertation befasst sich mit der Untersuchung der kollektiven Ausrichtungsdynamik von humanen Aortenendothelzellen (HAOEC) mithilfe eines mikrofluidischen Systems. In der Aorta richten sich HAOECs in Fließrichtung des Blutes aus und etablieren dabei eine weitreichende Ordnung, die an Verzweigungsstellen durch lokale Bereiche von Unordnung unterbrochen wird, welche $-1/2$ topologischen Defekten entsprechen [2]. Vor kurzem wurden topologische Defekte als Katalysatoren für verschiedene biologische Funktionen identifiziert, beispielsweise für Apoptose [3], Zellansammlung [4] und Zellprotrusion [5]. Die Rolle topologischer Defekte bei der Vermittlung von Übergängen zwischen lokalen und globalen Ordnungszuständen in biologischen Geweben ist jedoch bisher weitgehend unerforscht. Dieses fehlende Wissen über Zwischenzustände zwischen ungeordneten und geordneten Zellorganisationen führt zu heterogenen Beobachtungen, wie sie auch bei der Ausrichtung von Endothelzellen festgestellt wurden [6]–[9]. Der Einsatz von großflächiger, zeitaufgelöster Mikroskopie zeigte, dass HAOECs unter Fluss einem dreiphasigen Ausrichtungsprozess folgen, der eine Zwischenphase von FehlAusrichtung beinhaltet. Diese Phase ist von der Nukleation topologischer Defekte begleitet, die durch String-Anregungen stabilisiert werden. Physikalische Modellierungen zeigten, dass diese String-Anregungen einen neuartigen Mechanismus für die langreichweitige Übertragung mechanischer Kräfte in multizellulären Systemen darstellen könnten. Obwohl 2D-Modelle ideal sind, um die physikalischen Prinzipien zu untersuchen, die der Selbstorganisation zugrunde liegen, müssen diese Konzepte in komplexeren, dreidimensionalen zellulären Systemen verifiziert werden. Die Untersuchung von 3D-vaskularisierten Netzwerken und kollektiven Zelldynamiken während der Angiogenese stellt einen ersten Schritt dar, um diese Erkenntnisse auf die Selbstorganisation von 3D-zellulären Strukturen, wie beispielsweise Organoiden, anzuwenden.

Der zweite Teil dieser Dissertation beschäftigt sich mit der Entwicklung eines Systems für die automatisierte Aussaat von Organoiden, um die Skalierbarkeit von 3D-Zellkulturanwendungen zu verbessern. Obwohl die Organoidkultur großes Potenzial birgt, um komplexe biologische Systeme *in vitro* zu erforschen, ist sie weiterhin mit bedeutenden Einschränkungen konfrontiert, wie zum Beispiel Zellheterogenität, mangelhafte Reproduzierbarkeit, hohe Kosten und Probleme bei der Skalierbarkeit [10]–[13]. Mikrofluidik auf Tröpfchenbasis bietet eine Hochdurchsatzlösung, indem sie Zellen in Hydrogeltröpfchen einschließt und so eine kontrollierte Organoidbildung ermöglicht. Vereinzelte murine Bauchspeicheldrüsenkrebszellen wurden in Kollagentröpfchen eingebettet und entwickelten sich zu verzweigten Organoidstrukturen, die die in herkömmlichen 3D Kulturmethode und sogar *in vivo* beobachteten Strukturen präzise nachbildeten. Die präzise Steuerung des Temperaturgradienten im mikrofluidischen Gerät gewährleistet eine einheitliche Kollagenpolymerisation. Die Tröpfchengröße beeinflusst die Morphologie der Organoide, und in Tröpfchen kultivierte Organoide zeigten eine beschleunigte Reifung im Vergleich zu in größeren Gelen gezüchteten Organoiden. Das Verständnis möglicher Verschiebungen von Entwicklungsphasen aufgrund veränderter Kulturbedingungen ist von entscheidender Bedeutung, um die korrekte Übertragung von Erkenntnissen aus diesem aufstrebenden Feld der 3D-Zellkultur zu gewährleisten.

Die präsentierten Erkenntnisse zu den physikalischen Mechanismen der Zellausrichtung bilden die Grundlage für die Konstruktion realistischerer und funktionalerer vaskulärer Netzwerke, die sich durch kollektive Zelldynamik ausbilden. Darüber hinaus bietet die Mikrofluidik auf Tröpfchenbasis eine robuste Plattform, um die Skalierbarkeit und Reproduzierbarkeit der Organoidkultur zu verbessern und sicherzustellen, dass zuverlässigere und zugänglichere 3D-Zellkultursysteme realisiert werden können.

Abstract

Organoid culture closely replicates the morphological complexity of native tissue and precisely mimics distinct developmental stages observed during organogenesis, during which multicellular assemblies self-organize into spatial patterns of distinct cell types [1]. Understanding the physical mechanisms underlying the self-organization of multicellular systems is crucial to guiding biological processes in applications such as tissue engineering. This thesis will (i) uncover the physical principles in endothelial cell alignment and (ii) develop a microfluidic pipeline to enhance the scalability and reproducibility of organoid culture.

The first part of this dissertation focuses on studying the collective alignment dynamics of human aortic endothelial cells (HAOEC) using a microfluidic setup. In the aorta, HAOECs align in the direction of the blood flow, establishing long-range order, which is disrupted at bifurcation points by local areas of misalignment that correspond to $-1/2$ topological defects [2]. Topological defects recently have been identified as catalysts for many biological functions, such as cell apoptosis [3], accumulation [4], and protrusion [5]. However, the role of topological defects in mediating transitions between local and global order in biological tissues remains largely unexplored. This lack of knowledge about intermediary states between disordered and ordered states of cell organization produced heterogeneous observations, as seen in endothelial cell alignment [6]–[9]. Using large-scale, time-resolved imaging revealed that HAOECs under flow follow a three-stage alignment process that includes an intermediate stage of dealignment. This intermediate stage is accompanied by the nucleation of topological defects which are stabilized by string excitations. Physical modeling demonstrated that these string excitations could constitute a novel mechanism for long-range force transmission in multicellular assemblies. While 2D models are ideal for understanding the physical principles underlying self-organization, these concepts must be verified using more complex 3D cellular systems. Studying 3D vascularized networks and collective cell dynamics during angiogenesis represents a first step toward applying these insights to the self-organization of 3D cellular assemblies, such as organoids.

The second part of this dissertation focuses on establishing a pipeline for automated organoid seeding to enhance the scalability of 3D cell culture applications. While organoid culture holds great potential for studying complex biological systems in vitro, it still encounters significant limitations, including cell heterogeneity, poor reproducibility, high costs, and scalability issues [10]–[13]. Droplet-based microfluidics provides a high-throughput

solution by encapsulating cells within hydrogel droplets, allowing controlled organoid formation. Singularized murine pancreatic ductal adenocarcinoma cells were incorporated into the collagen droplets and developed into branched organoid structures, recapturing faithfully the structures found in bulk assays and even in vivo. The tight control of the temperature gradient within the microfluidic device ensures uniform collagen polymerization. Droplet size influenced organoid morphology, and droplet-grown organoids displayed accelerated maturation compared to bulk-grown organoids. Acknowledging that altered culture conditions might shift developmental timescales is crucial to ensure the correct translation of findings from this emerging field of 3D cell culture.

The presented insights into the physical mechanism of cell alignment set the basis for engineering more realistic and functional vascular networks, which form by collective cell dynamics. In addition, droplet-based microfluidics provide a robust platform to enhance the scalability and reproducibility of organoid culture, ensuring the realization of more reliable and accessible 3D cell culture systems.

List of Publications

This dissertation is partially based on the author's following works which have been submitted for publication in peer-review journals.

Topological Excitations govern Ordering Kinetics in Endothelial Cell Layers

Iris E. Ruider, Kristian Thijssen, Daphné Raphaëlle Vannier, Valentina Paloschi, Alfredo Sciortino, Amin Doostmohammadi, and Andreas R. Bausch
Nature physics (under revision)

Accelerated maturation of branched organoids confined in collagen droplets

Iris E. Ruider, Anna Pastucha, Marion K. Raich, Wentao Xu, Yan Liu, Maximilian Reichert, David Weitz, and Andreas R. Bausch
Lab on a Chip (under revision)

Previous Publications:

Automatic image annotation for fluorescent cell nuclei segmentation

Fabian Englbrecht, **Iris E. Ruider** and Andreas R. Bausch
PLOS ONE, 16, 4, e0250093 (2021).

In Vivo Photocontrol of Microtubule Dynamics and Integrity, Migration and Mitosis, by the Potent GFP-Imaging-Compatible Photoswitchable Reagents SBTubA4P and SBTub2M

Li Gao, Joyce C. M. Meiring, Adam Varady, **Iris E. Ruider**, Constanze Heise, Maximilian Wranik, Cecilia D. Velasco, Jennifer A. Taylor, Beatrice Terni, Tobias Weinert, Jörg Standfuss, Clemens C. Cabernard, Artur Llobet, Michel O. Steinmetz, Andreas R. Bausch, Martin Distel, Julia Thorn-Seshold, Anna Akhmanova, and Oliver Thorn-Seshold
Journal of the American Chemical Society, 144, 12, 5614–5628 (2022).

A MEC-2/stomatin condensate liquid-to-solid phase transition controls neuronal mechanotransduction during touch sensing

Neus Sanfeliu-Cerdán, Frederic Català-Castro, Borja Mateos, Carla Garcia-Cabau, Maria Ribera, **Iris E. Ruider**, Montserrat Porta-de-la-Riva, Adrià Canals-Calderón, Stefan Wieser, Xavier Salvatella, and Michael Krieg
Nature Cell Biol, 25, 1590–1599 (2023).

Patents

The methods presented in dissertation have contributed to the following patent applications.

Organoids in Extracellular Matrix Droplets

Iris Elke Ruider, Jean Carlos Serrano Flores, Andreas R. Bausch, David A. Weitz, and Perry Ellis

U.S. Provisional Application No. 63/432,700, filed December 14, 2022.

Organoids in Extracellular Matrix Droplets

David A. Weitz, **Iris Elke Ruider**, Jean Carlos Serrano Flores, Andreas R. Bausch, and Perry Ellis

Application No. PCT/US2023/083972, filed December 14, 2023.

In preparation:

Human iPSC-derived epiblastoid, gastruloid and chamber-like heart organoid model systems

Andreas Bausch, Alessandra Moretti, Daphne Perle Rahaelle Vannier, **Iris Elke Ruider**, Ann-Caroline Heiler

Invention report submitted to TUM ForTe.

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Preface

Tissue engineering aims to create functional replacements for damaged tissues caused by injury, age, or disease. Organoids are particularly promising as they closely resemble in vivo tissues and organs, and can replicate the functions of these tissues [14], [15]. Their benefit extends beyond regenerative medicine, as they present an ideal model system for pharmaceutical research. Organoid growth and development heavily rely on the cell's potential for self-organization [1]. Understanding the physical principles underlying self-organization is essential to pave the way toward more reproducible and realistic organoid models. Therefore, this thesis focuses on (i) understanding the key concepts governing cell alignment dynamics of endothelial monolayers exposed to flow using a microfluidic setup and (ii) developing a pipeline for automated organoid seeding to enhance the scalability and reproducibility of 3D cell culture systems.

This thesis presents three distinct microfluidic applications designed to guide collective cell behavior in 2D and 3D and to improve the scalability of organoid formation. Therefore, the first chapter provides an overview of relevant concepts of hydrodynamics required to enhance the understanding of the subsequent chapters.

Chapter two introduces an air pressure-driven pump system used to control the magnitude of the shear stress applied to endothelial cells. Endothelial cells can sense and align in the direction of the flow. Large-scale time-resolved imaging showed that the global alignment dynamics of endothelial cells are disrupted by an intermediary phase of temporal dealignment during which the nucleation of topological defects was observed. The findings presented in this chapter demonstrate that the interaction of the intrinsic activity within the endothelial monolayer and the external superjacent flow drives the non-monotonous alignment dynamics of the endothelial cells. Using concepts from active nematic theory and physical modeling showed that topological defects arise from active nematic stresses within the cellular monolayer. These defects interact via string excitations, which may represent a novel mechanism for long-range force transmission in biological tissues.

The third chapter introduces a microfluidic chip for angiogenesis, representing a more complex model system to study collective endothelial cell alignment in 3D. Typically, angiogenesis in microfluidic systems is achieved using a complex cocktail of growth factors combined with fibroblast co-culture. However, such models often are based on the diffusion

of growth factors as angiogenic triggers, with limited integration of interstitial flow. The microfluidic chip presented in this chapter constitutes a minimalistic model for angiogenesis relying solely on interstitial flow and a gradient of vascular endothelial growth factor A (VEGFA) to promote sprout invasion, thereby also providing a suitable platform for component screening.

The fourth chapter establishes an automated organoid seeding platform using droplet-based microfluidics to improve the scalability and reproducibility of 3D cell culture systems. Pancreatic ductal adenocarcinoma cells (PDAC) were embedded into collagen droplets of different sizes to determine how these altered mechanical parameters affect organoid growth. Eventually, the comparison of droplet-grown organoids to bulk-grown organoids shows that organoids mature faster in droplet-based culture conditions. Recognizing these shifts in developmental timescales is crucial to ensure an accurate translation of experimental results from this emerging field of 3D organoid culture.

This thesis concludes with a summary of the presented findings and a discussion of their potential impact.

The fifth chapter features detailed information on the protocols and materials used throughout this thesis.

Chapter 1

Basics of fluid mechanics

Introduction

Fluid mechanics describes and quantifies the behavior and motion of liquids and gases under static and dynamic conditions. While fluid dynamics comes from physics, it has found broad applications in engineering, medicine, and biology. Hydrodynamics plays a key role in many pathological conditions, influencing both the initiation and progression of disease. For example, in atherosclerosis, the accumulation of lipids, smooth muscle cells, and cell debris obstructs blood vessels, and specific blood flow patterns, such as low-shear stress regions, have been correlated with the onset of plaque formation [16]. Similarly, fluid flow also plays a crucial role in cancer progression, where interstitial flow promotes vascularization of cancerous tissue, resulting in enhanced tumor growth and the potential for metastasis [17]. This chapter briefly introduces key concepts of hydrodynamics to enhance the understanding of the subsequent chapters, building on the principles presented hereinafter.

1.1 The ideal fluid

An apparent difference between a solid and a liquid is that a solid has a constant shape and volume. In contrast, while maintaining constant volume, a liquid adapts its shape to its container. Although this definition only holds true because of the earth's gravitational field, it conveys in an easily accessible manner that solids and liquids have very distinct physical properties [18]. A solid exposed to a shear force experiences elastic shear stress, resulting in its deformation. On the contrary, a shear force applied to a liquid gives rise to a velocity gradient, which results from the momentum transport between the liquid's molecules. The viscosity is a measure of the rate at which the momentum is transferred between adjacent layers of the moving fluid. In an ideal fluid, the momentum transport between the constituent molecules is assumed to be zero. Therefore, the viscosity of an ideal fluid, η , is zero. Besides zero viscosity, an ideal fluid is also characterized by its

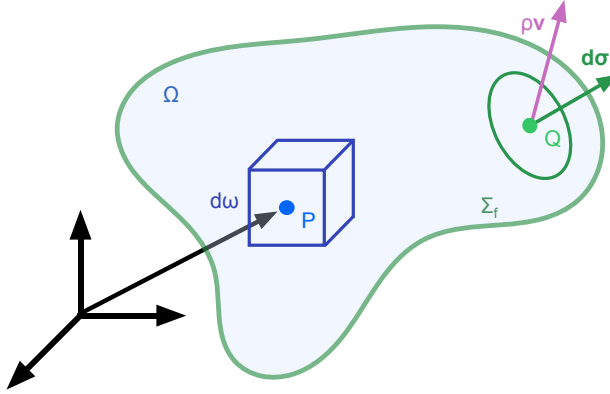


Figure 1.1: Conservation of mass in hydrodynamics. The variation of mass for a fluid body of volume Ω is described by the flux $\rho \mathbf{v}$ flowing across its surface Σ . For every point $P \in \Omega$, it is possible to define a volume element $d\omega$. Similarly, for every point $Q \in \Sigma$, it is possible to define a surface element $d\sigma$ oriented orthogonal to the surface in Q . The illustration has been adapted from [20].

incompressibility $\rho = \text{const}$, where ρ denotes the fluid density [19]. While some fluids, known as superfluids, such as helium below 2.17 K, are characterized by zero viscosity, most fluids have non-zero viscosity [20]. The viscosity is a measure of how much a liquid resists flow. Fluids with high viscosity are, for example, honey or pitch, whereas water or ethanol are considered fluids with lower viscosity [18]. The pitch drop experiment is one of the longest-running experiments in the world. It impressively demonstrates how viscosity impacts fluid flow: it takes seven to thirteen years for one single drop to form [21].

1.1.1 Continuity equation

To describe the behavior of a liquid, it is much more convenient to focus on its macroscopic properties, such as pressure p , density ρ , or fluid velocity $\mathbf{v}$, instead of keeping track of the motion of each single molecule contained within the liquid. Those parameters are commonly used to describe liquids with spatial resolution. For example, the quantity $\mathbf{v}(\mathbf{x}_0, t_0)$ denotes the velocity of the fluid at position $\mathbf{x}_0$ and time t_0 and not the velocity of a single molecule at this point in space and time. Therefore, the time derivative of any quantity takes the form of the material derivative. For the velocity, the material derivative takes the following expression:

$$\frac{d\mathbf{v}}{dt} = \frac{\partial \mathbf{v}}{\partial t} + \mathbf{v} \nabla \mathbf{v}. \quad (1.1)$$

Fluid mechanics, as well as many other disciplines in physics, is governed by the conservation of mass. The mass of the fluid contained within a volume Ω is defined as $m(t) = \int_{\Omega} \rho(\mathbf{x}, t) d\omega$ (Figure 1.1). The temporal variance of the fluid mass within this volume is given by

$$\frac{dm(t)}{dt} = \int_{\Omega} \frac{\partial \rho(\mathbf{x}, t)}{\partial t} d\omega. \quad (1.2)$$

Assuming that the mass cannot be created nor destroyed within the volume, the temporal variance of the mass can also be expressed by the fluid flow crossing the volume's surface Σ :

$$\frac{dm(t)}{dt} = - \int_{\Sigma} \rho \mathbf{v} \cdot d\sigma. \quad (1.3)$$

Setting those two equations equal and applying the divergence theorem yields

$$\frac{\partial \rho}{t} + \nabla (\rho \mathbf{v}) = 0, \quad (1.4)$$

which describes the continuity equation [20], [22].

1.1.2 Euler equation

An ideal fluid characterized by a stationary flow is defined by its local temporal derivatives being equal to zero, such as $\frac{\partial \mathbf{v}}{\partial t} = 0$. Therefore, each molecule within the fluid can be easily tracked, and classical mechanics apply. A small volume Ω contained within that fluid of mass m and surface Σ is subjected to volumetric and surface forces, $\mathbf{F}_V$ and $\mathbf{F}_S$. Using Newton's second law the equation of motion reads

$$\mathbf{F}_V + \mathbf{F}_S = m \frac{d\mathbf{v}}{dt}. \quad (1.5)$$

The volumetric force is caused by an external field such as gravity or an electric or magnetic field and is proportional to the fluids' volume. Considering the earth's gravitational field, $\mathbf{g}$, the volumetric force is given by

$$\mathbf{F}_v = \int_{\Omega} \rho \mathbf{g} d\omega. \quad (1.6)$$

For an ideal fluid, the viscosity is zero. Therefore, the surface forces acting on the fluid are described by

$$\mathbf{F}_S = - \int_{\Sigma} p d\sigma = - \int_{\Omega} \nabla p d\omega. \quad (1.7)$$

Using the divergence theorem, equation 1.5 can be rewritten as

$$\rho \mathbf{g} - \nabla p = \rho \frac{d\mathbf{v}}{dt}, \quad (1.8)$$

yielding Euler's equation [20], [22].

Flow in a fluid channel with pressure gradient: In a channel with a pressure gradient where the pressure at point $x_1 = 0$ is p_1 and p_2 at a distance L from point 1, with $p_1 > p_2$, the incompressible fluid flows from higher to lower pressure (Figure 1.2). We can try to describe the fluid flow using Euler's equation. The pressure varies along x but is constant along y and over time, $p(x, y, t) = p(x)$. Similarly, the velocity can be written as $\mathbf{v}(x, y, t) = (v_x(x, t), 0)$. The incompressibility of the fluid implies $\nabla \cdot \mathbf{v} = \partial_x v_x(x, t) = 0$, where ∂_x denotes $\partial_x = \partial/\partial x$. The influence of the gravitational force on the fluid flow can be neglected, and thus, the Euler equation simplifies to

$$-\partial_x p(x) = \rho \partial_t v_x(x, t). \quad (1.9)$$

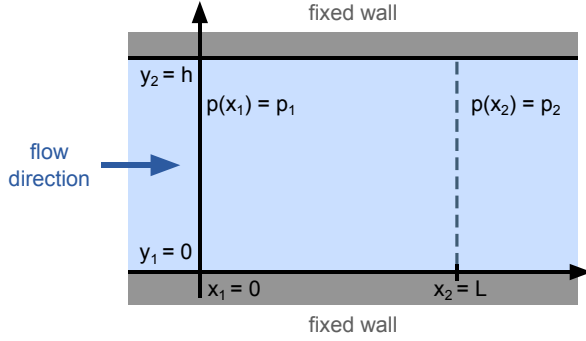


Figure 1.2: Fluid flowing through a channel. The pressure gradient applied along the x-axis gives rise to a fluid flow through the channel. The illustration has been adapted from [22].

Together with the incompressibility condition, equation 1.9 yields $\partial_x^2 p(x) = 0$. Using the boundary conditions, the pressure within the pipe can be expressed by $p(x) = \frac{p_2 - p_1}{L}x + p_1$. Therefore, we can solve equation 1.9 and obtain an expression of the fluid velocity in the channel as a function over time

$$v(x, t) = -\frac{p_2 - p_1}{L\rho}t + \text{const}. \quad (1.10)$$

The above expression implies that the velocity within the fluid channel would continue to increase over time. Needless to say, this is an unphysical behavior and does not correspond to how fluids behave in the real world. Euler's equation is derived for an ideal fluid assuming zero viscosity, which explains why it fails to accurately describe the fluid motion. Therefore, to obtain a correct description of the dynamics of viscous fluids, it is essential to incorporate viscosity into the equations of motion [22]. This is achieved by the Navier-Stokes equation, which will be introduced in the next section.

1.2 The Navier-Stokes equation

The Navier-Stokes equation is derived, similar to Euler's equation, from Newton's second law applied to a fluid of volume Ω and surface Σ exposed to volumetric and surface forces, equation 1.5. The volumetric forces $\mathbf{F}_V$ are described by equation 1.6. While the stress tensor for an ideal fluid can be significantly simplified because of zero viscosity, for a viscous fluid, the stress tensor is given by $\sigma_{ik} = -p\delta_{ik} + 2\eta\dot{\epsilon}_{ik} + \eta^*\dot{\epsilon}_{ll}\delta_{ik}$, where η and η^* represent the first and second coefficients of viscosity. The second term in the equation describes the contributions to the stress tensor related to pure shear stress, and the third term describes the contribution of the stresses associated with a uniform expansion or compression of the fluid. Developing the stress tensor similarly to equation 1.7 yields the Navier-Stokes equations

$$\rho\mathbf{g} - \nabla p + (\eta + \eta^*)(\nabla(\nabla \cdot \mathbf{v})) + \eta\nabla^2 \mathbf{v} = \rho \frac{d\mathbf{v}}{dt}. \quad (1.11)$$

The expression $\nabla^2 \mathbf{v}$ denotes the Laplacian of the velocity [20], [22]. The Navier-Stokes expression is very complex, and often, it is very challenging to provide an exact analytical

solution. For an incompressible fluid, it is possible to significantly simplify the Navier-Stokes equation to

$$\rho \mathbf{g} - \nabla p + \eta \nabla^2 \mathbf{v} = \rho \frac{d\mathbf{v}}{dt}. \quad (1.12)$$

Dimensionless Navier-Stokes equation: To obtain the dimensionless Navier-Stokes equations, we consider an incompressible fluid flow of characteristic velocity, V , and characteristic length, L . This could, for example, be a fluid flowing with velocity V around a sphere of length L . The characteristic timescale is then given by $T = L/V$. The velocity, space, and time can now be described by the dimensionless quantities

$$\mathbf{v}' = \frac{\mathbf{v}}{V}, \quad x' = \frac{x}{L}, \quad \text{and} \quad t' = \frac{t}{T}. \quad (1.13)$$

Substituting those expressions into the Navier-Stokes equation and applying $p' = p/(\rho V^2)$ yields the dimensionless Navier-Stokes equation

$$\frac{d\mathbf{v}'}{dt'} = -\nabla' p' + \frac{\eta}{\rho L U} \nabla'^2 \mathbf{v}'. \quad (1.14)$$

The Reynolds number is defined by

$$\text{Re} = \rho L U / \eta. \quad (1.15)$$

Therefore, two distinct systems that are characterized by the same Reynolds number are considered to be similar as they can be described by the same dimensionless Navier-Stokes equation. Furthermore, the Reynolds number also provides a measure of the ratio between viscous and inertial forces within the system. If the Reynolds number is too big, the viscous forces dominate, and the system is considered turbulent, which typically occurs for Reynolds numbers above $\text{Re} > 2000$ [20], [22].

Flow between two plates: The Navier-Stokes equation adequately integrates internal friction within the fluid and will allow us to deduce a correct expression for the fluid motion generated by a pressure gradient as discussed above. We consider a stationary, viscous, and incompressible fluid exposed to a pressure gradient. At $x_1 = 0$, the pressure is p_1 , and at a distance L , the pressure drops to p_2 , with $p_1 > p_2$ (Figure 1.2). The velocity of the fluid is described by $\mathbf{v}(x, y, t) = (v_x(x, y), 0)$. The incompressibility condition $\nabla \cdot \mathbf{v} = \partial_x v_x = 0$ implies that $v_x(x, y) = v_x(y)$. The boundary conditions impose that the velocity at the plates must be zero, $v_x(y = 0) = v_x(y = h) = 0$. Because of the incompressibility and the stationary flow, the Navier-Stokes equation can be written as

$$\partial_x p(x) = \eta \partial_y^2 v_x(y). \quad (1.16)$$

Therefore, the pressure and velocity of the fluid can be rewritten as

$$p(x) = p_1 - \frac{p_1 - p_2}{L} x \quad \text{and} \quad v_x(y) = \frac{p_1 - p_2}{2\eta L} y(y - h). \quad (1.17)$$

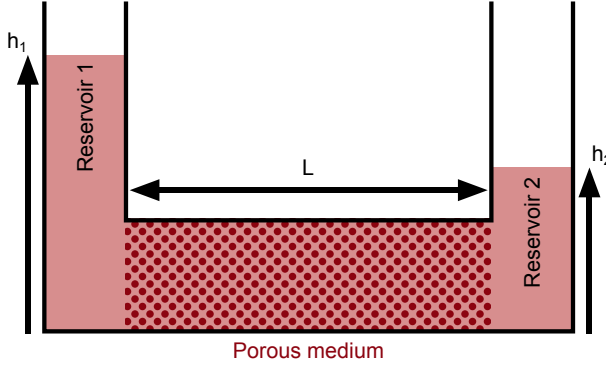


Figure 1.3: Fluid flow through a porous medium. Darcy's law describes the fluid flow through a porous medium. Two fluid reservoirs are unevenly filled, creating a pressure gradient. The pressure gradient drives a fluid flow through a chamber of length L and cross-section A filled with porous medium. The illustration has been adapted from [23].

The fluid velocity within the two parallel plates is described by a parabolic function where the fluid velocity is zero along the plates and peaks at $y = h/2$. By using the definition of the stress tensor introduced in this section, we can rewrite the stress tensor as $\sigma_{xy} = \eta \partial v_x / \partial y$. Therefore, velocity gradients give rise to shear stresses within a flowing fluid.

Darcy's law: Darcy's law is a special form of the Navier-Stokes equation that describes fluid flow through a porous medium. It was first postulated by Henry Darcy [23], [24]. We consider a rectangular channel filled with a porous medium connected to two reservoirs of identical dimensions. Naturally, when the reservoirs are filled at the same height, there is no fluid flow from one reservoir to the other. However, when a reservoir is filled to a height h_1 and the other reservoir is filled to a height h_2 , such that $\Delta z_0 = h_1 - h_2 > 0$, there is a hydrostatic pressure gradient that gives rise to a fluid flow (Figure 1.3). The fluid flow from reservoir 1 to 2 is given by

$$v_x = \frac{K \rho g \Delta z_0}{\eta L}. \quad (1.18)$$

The parameter K denotes the intrinsic permeability of the porous medium, and L is the length of the channel filled with the porous medium [23]. Equation 1.18 is an adequate solution to describe the flow of the fluid velocity provided that the cross-section of the reservoirs A_r is infinitely large compared to the cross-section of the connecting chamber facing the flow A . However, if this is not the case, the reservoir will drain, reducing the hydrostatic pressure. Thereby, the flow velocity between the two reservoirs will decrease over time [25], [26]. This is described by

$$v_x(t) = \frac{K \rho g \Delta z_0}{\eta L} e^{-\frac{\rho g A K}{\eta A_r L} t}. \quad (1.19)$$

Chapter 2

Controlling collective endothelial cell alignment dynamics

Introduction

Topological defects are essential to mediate transitions between disordered and ordered states [27]. In condensed matter physics, the importance of topological defects for such transitions is commonly recognized and has contributed to several advances ranging from superfluidity and melting [28]–[30] to superconductors [31], [32]. In biology, recent studies demonstrated the relevance of topological defects for a manifold of biological functions in multicellular assemblies. Notably, nematic defects act as key regulators of collective cell behavior, thereby controlling processes such as cell death [3], cell differentiation [5], and fruiting body formation in bacterial colonies [33]. However, these studies only focused on the local impact of topological defects on cell behavior, neglecting to investigate how topological defects drive global cell dynamics. Time-resolved, large-scale imaging showed that human aortic endothelial (HAOEC) cells undergo a transition from an initially disordered state to a highly aligned state driven by the formation of topological defects. This chapter starts by introducing the functional role of endothelial cells (EC) and basic concepts of active nematic theory. This provides the groundwork for understanding the shear stress-driven ordering of endothelial monolayers. Together with physical modeling, the experimental observations demonstrate how topological defects orchestrate global alignment and dynamic organization in endothelial cell monolayers.

2.1 Biological function of endothelial cells

Physiological function of endothelial cells: Endothelial cells (ECs) were already described as early as the 1850s by the pathologist Rudolf Virchow [37]–[39]. Endothelial cells line the inner wall of blood vessels and attach to an underlying basement membrane composed of several different extracellular matrix proteins such as collagen type-4, laminin, and fibronectin (Figure 2.1 a). As part of the vasculature, endothelial cells control the

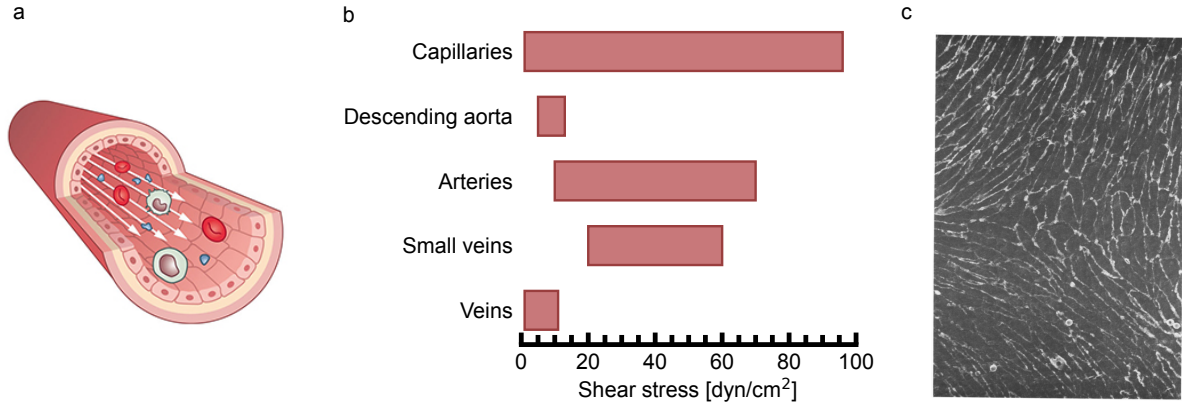


Figure 2.1: Endothelial cells line the blood vessels in the human body. (a) Endothelial cells are in direct contact with the circulating blood and, thus, are exposed to shear stress. Image taken from [34]. (b) Endothelial cells are exposed to distinct levels of shear stress depending on the type of blood vessels. Values obtained from [35] and [36]. (c) Rabbit aortic endothelial cells stained with silver nitrate. The endothelial cells form topological defects at bifurcation points. The image was obtained from a scanning electron microscope at $630\times$ magnification. Image taken from [2].

transport of ions, molecules, and cells from the inside of the blood vessels to the surrounding tissue and vice versa by carrying out a barrier function [40]. Thus, they are essential for regulating metabolic activity, wound healing, immune response, and disease progression [41]. The shape and orientation of endothelial cells are not uniform throughout the vasculature. In areas of laminar flow, as in the aortic tree, endothelial cells adopt a rod-like shape with a longer major axis and a shorter minor axis [38], [42]. The major axis is parallel to the blood flow axis, as Virchow first observed more than 160 years ago [37], [38]. At bifurcation points where the flow pattern is more turbulent, the endothelial cell morphology is more rounded, and a clear alignment pattern is not evident (Figure 2.1 c) [2], [42]. Endothelial cells are directly exposed to blood flow. Therefore, they experience shear stress. The magnitude of this shear stress varies based on blood flow velocity and the blood vessel's diameter. In human arteries, endothelial cells typically sense shear stress ranging from 10 to 60 dyn/cm² (Figure 2.1 b) [35]. Flaherty et al. provided one of the first experimental evidence demonstrating the ability of endothelial cells to sense and respond to shear stress in vivo. In their study, the authors surgically removed a patch of rabbit aorta and reimplanted it with a rotation of 90°. After reinsertion, the aortic endothelial cells were aligned orthogonally with the blood flow. However, ten days after reimplantation, the endothelial cells on this patch of rabbit aorta reoriented themselves so that they pointed again parallel to the blood flow axis [43]. Demonstrating through in vivo studies alone that endothelial cells possess the intrinsic ability to sense and respond to shear stress proved especially challenging [44], [45]. Thus, in the 1980s, researchers focused on investigating the response of endothelial cells to shear stress using in vitro studies, which allowed for a more rigorous control of experimental parameters. Those studies showed that en-

endothelial cells cultured in vitro under static conditions exhibit a more rounded morphology [44]. Furthermore, those studies confirmed that similar to in vivo studies, endothelial cells elongate and orient themselves parallel to the flow axis upon exposure to shear stress [44], [46], [47]. On top of that, in vitro studies demonstrated that endothelial cells' response to shear stress involves the establishment of stress fibers pointing in the direction of the flow, as well as an increase in their proliferation rate [48]–[50]. The magnitude of the response of endothelial cells was reported to correlate with the magnitude of the shear stress [7], [47].

Lack of a theoretical concept to describe endothelial cell alignment: A comparison of the reported experimental results of those studies, carried out by Reinitz et al., reveals a high variability in the observed results. Distinct experimental conditions and measuring those values at various time points during the alignment process can partly explain the discrepancy in the published experimental data [39]. The lack of a theoretical model that successfully captures the underlying mechanisms can explain the seemingly ad-hoc choice of time points during such a dynamic process as cell alignment. Another limitation common to all of these studies is the low number of cells evaluated to determine the response of the cellular monolayer ranging from a few dozen to a few hundred cells. Measuring such a small number of cells captures only the localized response instead of capturing the global behavior of the endothelial cell monolayer. These concerns regarding statistics continue to be relevant for studies published up to the 2010s. In 2015, Reinitz et al. presented a study in which they significantly increased the number of analyzed cells to between 3000 and 6000 cells, representing a 500 % increase compared to previous studies. By enhancing the number of analyzed cells, Reinitz et al. successfully documented an initial dealignment of endothelial cells followed by a consecutive alignment in the flow direction for human umbilical vein endothelial cells [39]. In the same year, Ohta et al. introduced the global order parameter S , frequently used in liquid crystal theory, to quantify endothelial cell alignment. Given that Ohta et al. used a small number of endothelial cells, as low as 40 cells per measured condition, the observations obtained from this study reflect just the local response of endothelial cells to shear stress [51]. In line with the concepts of the two previously presented studies, this chapter focuses on quantifying the response of endothelial cells on a global scale using concepts from active nematic theory. The analysis of 12,000 to 40,000 endothelial cells in one field of view spanning several millimeters shows that the alignment of human aortic endothelial cells follows a three-stage process disrupted by a temporal dealignment. Active nematic theory provides a better understanding of these cellular dynamics during the alignment process, and physical modeling can reproduce the experimental observations.

2.2 Active nematic systems in biology

Liquid crystals as a mesophase: The concepts from liquid crystal theory can describe the alignment dynamics of human aortic endothelial cells under flow. The liquid crystalline phase is a state with characteristics of both liquid and solid matter. Therefore, it is often

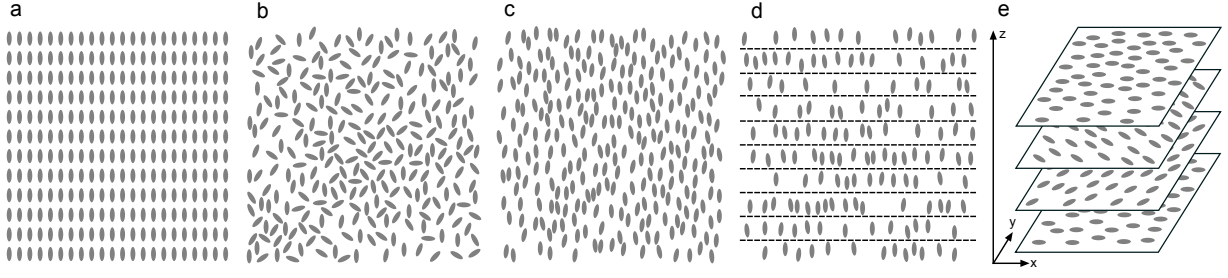


Figure 2.2: Liquid crystals constitute a mesophase between liquid and solid. (a) A solid possesses both orientational and positional order. (b) There is no orientational or positional order in a liquid. (c) The nematic phase is one type of liquid crystal. The constituent particles lack positional order but orient along a preferred orientation axis. (d) Smectics are another type of liquid crystal. Besides the orientational order imposed on the particles, the particles are additionally organized in layers in smectics. (e) In a cholesteric crystal, another type of liquid crystal, the particles are organized in planes, and the director rotates in between the planes.

referred to as mesophase. Typically, the molecules in a solid material adhere to a high orientational and positional order (Figure 2.2 a). On the contrary, in a liquid material, the molecules do not possess a defined orientation, nor does their position follow a certain pattern (Figure 2.2 b). The liquid crystalline state, sharing characteristics of both liquid and solid matter, is characterized by a high orientational order of its components and, simultaneously, the lack of a positional order. In liquid crystals, the particles can move freely as in a liquid while maintaining a specific preferred orientation [52], [53]. Liquid crystals are typically composed of rod-like particles and are classified into three different phases: (i) the nematic phase (Figure 2.2 c), (ii) the smectic phase (Figure 2.2 d), and (iii) the cholesteric phase (Figure 2.2 e). In the nematic phase, the constituting particles orient along a preferred orientation axis, and their motion is unrestricted. In the smectic phase, the molecules still point along a preferred orientation. Additionally, they self-organize into layers within which these particles can move freely. Molecules that possess a chirality can form a cholesteric phase. Here, on top of an organization into layers, the orientation of molecules contained in those layers varies periodically [52], [54].

The order parameter: The nematic phase constitutes the simplest phase of all liquid crystals. The nematic director $\hat{\mathbf{n}}_0$ describes the preferred orientation throughout the material. The nematic director field $\mathbf{n}(\mathbf{r})$ provides the average orientation of the particles in a specific region of the material and is given by:

$$\mathbf{n}(\mathbf{r}) = \begin{pmatrix} \cos\theta(\mathbf{r}) \\ \sin\theta(\mathbf{r}) \end{pmatrix}, \quad (2.1)$$

where $\theta(\mathbf{r})$ is the angle between the average orientation and the preferred orientation $\mathbf{n}_0$. The nematic director describes the orientation but cannot provide any information on the

magnitude of the order in the system because of the head-tail symmetry $\mathbf{n} = -\mathbf{n}$. To obtain a measure for the magnitude of the order, we compute the structure tensor

$$\mathbf{Q} = (2\mathbf{n} \otimes \mathbf{n} - \mathbf{I}) = \begin{pmatrix} 2\cos^2\theta - 1 & 2\cos\theta\sin\theta \\ 2\cos\theta\sin\theta & 2\sin^2\theta - 1 \end{pmatrix}. \quad (2.2)$$

Here, $\mathbf{I}$ is the identity matrix, and q is the magnitude of the order, also referred to as the order parameter. We note that the structure tensor is symmetric and traceless. Moreover, the eigenvector with the biggest eigenvalue corresponds to the nematic director of the system $\mathbf{n}_0$. Its associated eigenvalue is the global order parameter $q = \langle (2\mathbf{n} \otimes \mathbf{n}_0 - \mathbf{I}) \rangle = 2 \langle \cos^2(\theta_{ij}) \rangle - 1$ [52], [55], [56]. The global order parameter in 2D is defined in the interval $q \in [-1, 1]$. For $q = 1$, the system is perfectly aligned along the nematic director $\mathbf{n}_0$. The order parameter assumes zero, $q = 0$, in the case of an isotropic system. When the system is aligned orthogonal to the preferred orientation axis $\mathbf{n}_0$, the order parameter equals $q = -1$ [52].

The deformation free energy: Most nematic systems will not perfectly align their particles with the preferred orientational axis but will display deformations within the nematic director field. Those deformations can, for example, be introduced by boundary conditions or by interaction with external electric or magnetic fields. The free energy $\mathcal{F}_d$ is the energy required to impose certain deformation on a perfectly aligned system. The definition of the free energy must (i) be minimal for a perfectly aligned system $\mathcal{F}_d = 0$, (ii) respect the head-to-tail symmetry of the nematic director field $\mathcal{F}_d(-\mathbf{n}) = \mathcal{F}_d(\mathbf{n})$, and (iii) energy contributions from the boundary surface are neglected. Those implications entail that the free energy depends on the gradient tensor $(\nabla\mathbf{n})^2$. For the sake of simplicity, the development of the terms constituting the gradient tensor is omitted, as it is explained in much detail by Gennes and Prost in *The Physics of Liquid Crystals* [56]. The distortion free energy is given by

$$\mathcal{F}_d = \frac{1}{2} \left(K_1 (\nabla \cdot \mathbf{n})^2 + K_2 (\mathbf{n} \cdot \nabla \times \mathbf{n})^2 + K_3 (\mathbf{n} \times \nabla \times \mathbf{n})^2 \right). \quad (2.3)$$

Here, K_1 , K_2 , and K_3 are constants associated with the three deformations that can be introduced into a nematic liquid: splay, twist, and bend (Figure 2.3 a and b). As the deformation free energy is a measure of energy per volume and the nematic director is, per definition, unit-free, the units of the constants K_i are dyn or J/cm . For the sake of simplicity, the three constants are often set equal $K_1 = K_2 = K_3$ [52], [53], [56]. In that case, the deformation free energy simplifies to

$$\mathcal{F}_d = \frac{1}{2} \left((\nabla \cdot \mathbf{n})^2 + (\nabla \times \mathbf{n})^2 \right). \quad (2.4)$$

When the nematic phase is confined to the XY plane, this equation is further simplified to

$$\mathcal{F}_d = \frac{1}{2} K (\nabla\theta)^2 \quad (2.5)$$

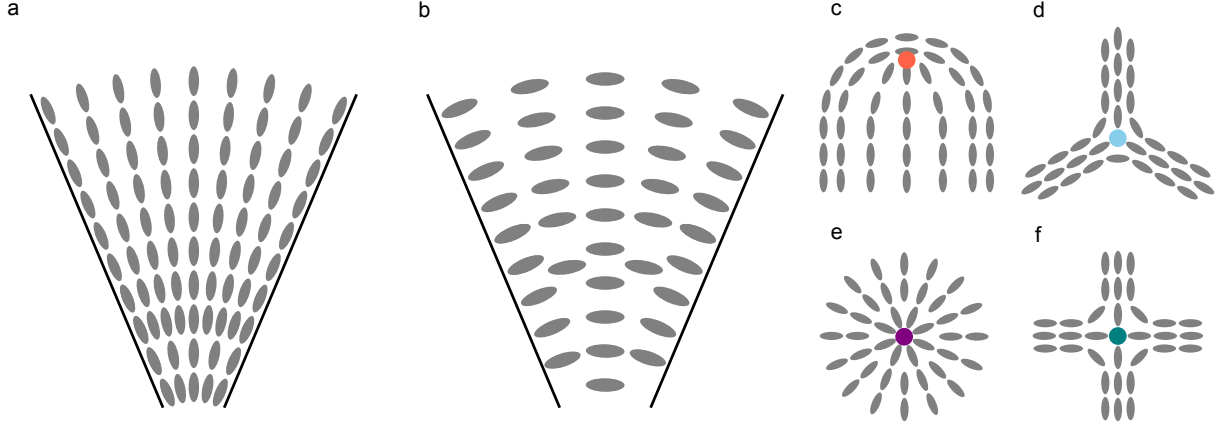


Figure 2.3: Deformations in nematics. (a) Splay deformation in 2D. (b) Bend deformation in 2D. (c) A red dot indicates the core of the $+1/2$ defect. (d) A blue dot indicates the core of the $-1/2$ defect. (e) A purple dot indicates the core of the $+1$ defect. (f) A green dot indicates the core of the -1 defect.

because the second term in equation 2.4 vanishes. The angle the nematic director $\mathbf{n}(\mathbf{r})$ forms with the x-axis is denoted as $\theta(\mathbf{r})$ [56]–[58].

Topological defects: In 2D nematic systems, the only possible deformations are bend and splay. Sufficiently high deformations in the nematic director field can entail the breakdown of local order, and point singularities arise. In the center of such point singularities, the orientation is not well defined as domains of different orientations meet. Those singular points in the orientation field are referred to as nematic defects (Figure 2.3 c, d, e, and f). Because those nematic defects cannot be resolved by a simple local rearrangement of misoriented particles, they are also called topological defects [54], [59]. We can compute the distortion free energy for nematic defects using equation 2.5. For stable nematic defects, the equilibrium condition needs to be satisfied

$$\nabla^2 \theta = 0. \quad (2.6)$$

To solve this equation, we introduce the angle $\phi(\mathbf{r}) = \arctan(y/x)$ defined as the angle formed by the vector $\mathbf{r}$ with the x-axis. Equation 2.6 yields a solution of the form

$$\theta(\phi) = m\phi + \theta_0, \quad (2.7)$$

where $m, \theta_0 \in \mathbb{R}$ are constants. The nematic director field $\mathbf{n}$ needs to be well-defined throughout the whole material. This implies

$$\oint_{\theta} d\theta = 2\pi m = k\pi, \quad (2.8)$$

with $k \in \mathbb{Z}$. Because of the head-tail symmetry and the continuity of the nematic director field, the angle θ can only change by π or any multiple of it. The constant m describes

how much the orientation of the particles changes after a full clockwise turn around the singularity and can take only well-defined values $m = \pm 1/2, \pm 1, \pm 3/2, \dots$. The different types of nematic defects are distinguished using the winding number m , often referred to as the charge of the defect (Figure 2.3 c, d, e, and f) [56], [58], [59]. We can now compute the energy of nematic defects. The energy per unit length for a system of dimensions $\rho_{\max}$ and a disclination of size a is defined as

$$\mathcal{F} = \frac{1}{2}K \int_a^{\rho_{\max}} 2\pi \frac{m^2}{\rho} d\rho = \pi K m^2 \ln \left(\frac{\rho_{\max}}{a} \right) + \mathcal{F}_c. \quad (2.9)$$

The constant energy contribution from the defect core is denoted $\mathcal{F}_c$. For unconfined nematic systems with head-to-tail symmetry, $\pm 1/2$ are energetically more favorable than ± 1 nematic defects as shown by equation 2.9. Indeed, in such materials the formation of $\pm 1/2$ is preferred [54], [57], [60].

The interaction between topological defects: The interaction of nematic defects with each other can be compared to the interaction of charged particles in electrostatics. Defect pairs of opposite charges will attract each other, while defect pairs of the same charge will repel each other. The conformation of two point defects of opposite charge $m = \pm 1/2$ and same size a , located at $\mathbf{r}_+ = (0, x_+)$ and $\mathbf{r}_- = (0, x_-)$, separated by a distance $\Delta = x_+ - x_-$, is energetically described by the term

$$\mathcal{F}_{\pm} = 2\pi K m^2 \ln \left(\frac{\Delta}{a} \right) + 2\mathcal{F}_c. \quad (2.10)$$

The free energy describing the conformation is minimized by reducing the distance Δ between the defects. Thus, each point defect experiences an attractive force $\mathbf{F} = -(\delta\mathcal{F}_{\pm}/\delta x_{\pm}) \hat{\mathbf{x}}$, moving the defect pairs towards each other. When nematic defects of opposite charge meet, they annihilate, leaving behind a perfectly aligned nematic director field [56], [59], [60].

Active nematic systems: Most biological systems are characterized by a constant input of energy ranging from very simple systems such as actin filaments, microtubules, or cellular monolayers that rely on the availability of ATP to more complex systems such as a flock of fish that requires food for survival. The inherent dynamics of those systems can be described with active nematic theory. In contrast to passive systems, active nematic systems are constantly maintained out of thermodynamic equilibrium by continuous energy input, resulting in the microscopic activity of the particles [55]. The activity of the particles is represented by dipole forces acting along their major axis. Active nematic systems are distinguished by the nature of those active stresses. An extensile nematic system is characterized by pushing forces, whereas, in contractile nematic systems, the force dipole corresponds to pulling forces. The microscopic activity of the constituting particles gives rise to a set of very distinct phenomena that can only be observed in active nematic systems. In active nematic systems, topological defects can spontaneously nucleate, and the defects can self-propel, properties that they do not possess in passive nematic systems

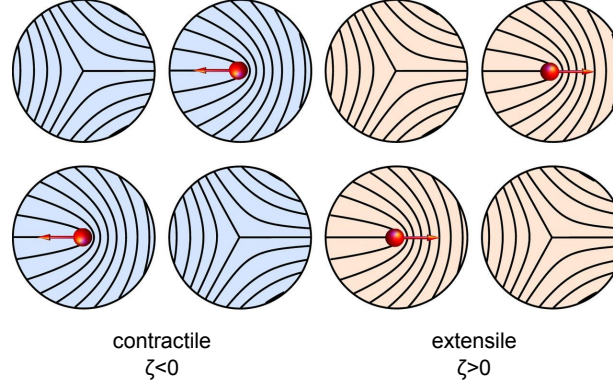


Figure 2.4: Impact of active nematics on defect interactions. For a contractile nematic system (blue panels), the motion of the $+1/2$ defect is directed toward the tail of its comet. Therefore, in the constellation of the upper row, annihilation is accelerated, whereas the defect pairs do not annihilate for the constellation displayed in the lower row. In an extensile nematic system (orange panels), the $+1/2$ defects move toward the comet head. In such a system, the topological defects in the constellation illustrated in the upper row move further apart, while the annihilation of the defects in the lower row is accelerated. Figure taken from [60].

[54], [55]. Let us take the previously mentioned example of two point defects at a certain distance Δ . In passive nematics, those defects experience an attractive force that moves them closer together, eventually resulting in their annihilation. In the case of active nematics, the conformation of the nematic defects and the nature of the active stresses decide whether the defect pairs will annihilate or move further apart (Figure 2.4). For instance, in a contractile nematic system, a $+1/2$ nematic defect moves in the direction of its comet tail. In such systems, assuming sufficiently strong activity, if a $+1/2$ defect faces a $-1/2$ nematic defect with its tail, the self-propulsion of the $+1/2$ defect accelerates their annihilation (Figure 2.4, contractile system, upper row). On the contrary, when the $+1/2$ defect faces the $-1/2$ defect with its head, the self-propulsion moves the $+1/2$ defect further apart from the $-1/2$ defect (Figure 2.4, contractile system, lower row). For an extensile nematic system, the constellations resulting in defect motion favoring or counteracting defect annihilation are reversed (Figure 2.4, extensile system, upper and lower panel). Hence, the nature of the active stresses is essential as it determines the behavior of the nematic system [60].

The origin of active forces in active nematic systems: In an active nematic system, the force exerted by those particles will generate active nematic stresses. Those active nematic stresses can, then, themselves interact with the particles contained in the medium. To better understand the nature of those active stresses, we closely follow a derivation first presented by Simha and Ramaswamy in 2002 [61]. Let us assume a rod-like particle α with its center of mass at the position $\mathbf{x}_\alpha$. The particle has a total length of $2a$, and its major

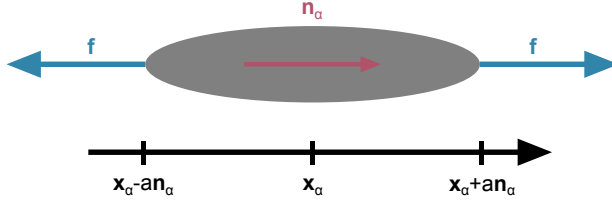


Figure 2.5: Active forces acting on a particle. Two point forces f act on a particle of length $2a$ at its extremities at $\mathbf{x}_\alpha - a\mathbf{n}_\alpha$ and $\mathbf{x}_\alpha + a\mathbf{n}_\alpha$. The particle is oriented along $\mathbf{n}_\alpha$.

axis is oriented parallel to $\mathbf{n}_\alpha$. Given that this is an active nematic system, we approximate the particle as a force dipole with point forces $\mathbf{f} = f\mathbf{n}_\alpha$ acting on the particle's ends at $\mathbf{x}_\alpha + a\mathbf{n}_\alpha$ and $\mathbf{x}_\alpha - a\mathbf{n}_\alpha$ (Figure 2.5). We can obtain an expression for the force density corresponding to the divergence of the stress

$$\nabla \Pi = \mathbf{f}(\mathbf{x}) = \sum_{\alpha} f\mathbf{n}_\alpha [\delta(\mathbf{x} - \mathbf{x}_\alpha - a\mathbf{n}_\alpha) - \delta(\mathbf{x} - \mathbf{x}_\alpha + a\mathbf{n}_\alpha)] \quad (2.11)$$

by summing over all the particles contained in the material. Using a Taylor expansion and integration by parts, we retain the following term:

$$f_i = -af \nabla_j \sum_{\alpha} n_{\alpha i} n_{\alpha j} \delta(\mathbf{x} - \mathbf{x}_\alpha). \quad (2.12)$$

We can further develop this expression, considering that the sum over all the particles yields a concentration c , the transformed expression reads $\Pi_{ij} = -afc(n_i n_j - 1/3\delta_{ij})$. The term af , representing the dipole moment, is combined with the concentration c into the constant ζ , which provides a measure for the activity of the system. Therefore, the active stresses in an active nematic system can be summarized as

$$\Pi^{\text{active}} = -\zeta \mathbf{Q}. \quad (2.13)$$

For extensile active nematic systems $\zeta > 0$, whereas in contractile nematic system $\zeta < 0$ [61]–[63].

Nematohydrodynamics equations for active nematic systems: Nematohydrodynamic equations are typically used to describe the behavior of passive and active nematic systems. As described previously, nematic materials can flow similarly to liquid, and their flow field is denoted by $\mathbf{u}$. This flow field can by itself influence the orientations of the particles in the medium and, therefore, alter the order parameter $\mathbf{Q}$. A change in the orientation of the particles can then, in turn, lead to flow field coupling in the material. Two equations are required to adequately describe the dynamics of the order tensor, the dynamics of the flow field, and the interplay of those two quantities with each other [56]. The Beris-Edwards equation captures the evolution of the structure tensor

$$(\partial_t + \mathbf{u} \cdot \nabla) \mathbf{Q} - \mathbf{S} = \Gamma \mathbf{H}. \quad (2.14)$$

The first term is the material derivative (equation 1.1) and describes the change of the order tensor. The rotational term, $\mathbf{S}$, accounts for the coupling between the flow field and the molecules. It is written as

$$\mathbf{S} = (\lambda \mathbf{E} + \boldsymbol{\Omega}) \cdot (\mathbf{Q} + \frac{1}{3} \mathbf{I}) + (\mathbf{Q} + \frac{1}{3} \mathbf{I}) \cdot (\lambda \mathbf{E} - \boldsymbol{\Omega}) - 2\lambda (\mathbf{Q} + \frac{1}{3} \mathbf{I})(\mathbf{Q} : \nabla \mathbf{u}). \quad (2.15)$$

Here, Ω and E denote the vorticity and the rate of strain tensors. The relative dominance of the rate of strain and the vorticity in influencing the alignment of the rod-like particles is captured by the parameter λ . The relaxation term $\Gamma \mathbf{H}$ corresponds to the derivative of the free energy with respect to the structure tensor $\mathbf{Q}$, where the rotational diffusivity is denoted as Γ . This molecular field $\mathbf{H}$ ensures that the system relaxes towards the minimum of the free energy when flow is absent. The expression for the molecular field $\mathbf{H}$ and the free energy $\mathcal{F}$ are given by:

$$\mathbf{H} = -\frac{\delta \mathcal{F}}{\delta \mathbf{Q}} + \frac{1}{3} \mathbf{I} \text{Tr} \left(\frac{\delta \mathcal{F}}{\delta \mathbf{Q}} \right) \quad (2.16)$$

$$\mathcal{F} = \frac{A}{2} \mathbf{Q}^2 + \frac{B}{3} \mathbf{Q}^3 + \frac{C}{4} \mathbf{Q}^4 + \frac{K}{2} (\nabla \mathbf{Q})^2, \quad (2.17)$$

where A , B , and C represent material parameters, and the last term describes the free energy associated with spatial inhomogeneities in the system assuming one single elastic constant K . Equation 2.14 shows a strong influence of the flow $\mathbf{u}$ on the dynamics of the structure tensor $\mathbf{Q}$ through the advection term and the rotational term. Imposing a constant density ρ , the dynamics of the flow field can be described by the Navier-Stokes equation (equation 1.11) for an incompressible fluid

$$\nabla \mathbf{u} = 0 \quad (2.18)$$

$$\rho (\partial_t + \mathbf{u} \cdot \nabla) \mathbf{u} = \nabla \cdot \mathbf{\Pi}. \quad (2.19)$$

The parameter π denotes the stress tensor. It accounts for the viscous, elastic, and active stresses. The viscous stresses describe the conventional viscous term from the Navier Stokes equations where η denotes the viscosity. The pressure p is absorbed into the term describing the elastic stresses that also account for the backflow induced by a change in the orientation of a liquid crystal molecule [54], [55], [62]–[64].

$$\mathbf{\Pi}_{\text{elastic}} = -p \mathbf{I} + 2\lambda \left(\mathbf{Q} + \frac{1}{3} \mathbf{I} \right) (\mathbf{Q} : \mathbf{H}) - \lambda \mathbf{H} \cdot \left(\mathbf{Q} + \frac{1}{3} \mathbf{I} \right) - \lambda \left(\mathbf{Q} + \frac{1}{3} \mathbf{I} \right) \cdot \mathbf{H} \quad (2.20)$$

$$- \nabla \mathbf{Q} \frac{\delta \mathcal{F}}{\delta \nabla \mathbf{Q}} + \mathbf{Q} \cdot \mathbf{H} - \mathbf{H} \cdot \mathbf{Q} \quad (2.21)$$

$$\mathbf{\Pi}_{\text{viscous}} = 2\eta \mathbf{E} \quad (2.22)$$

$$\mathbf{\Pi}_{\text{active}} = -\zeta \mathbf{Q} \quad (2.23)$$

The nematohydrodynamic equations describing a passive system can be recovered from those equations by imposing $\zeta = 0$. In an active nematic system, gradients in the director field generate active stresses and, therefore, flow which accounts for the different observed properties of active nematic systems, such as the spontaneous nucleation of topological defects [64].

Active nematic systems in subcellular systems: The emergence of non-polar nematic order has been reported for subcellular filaments such as actin and microtubules [54], [65]–

[69]. At first sight, this might seem counterintuitive, as both filaments have a polar structure with positive and negative ends. However, if these filaments assemble without their ends displaying a clear preference to point in a specific direction, they can be approximated to form a non-polar nematic phase [54]. The ability of subcellular filaments to form nematic structures has also been demonstrated inside cells. Cells cultured on substrates of varying stiffness displayed distinct cytoskeletal organization, ranging from isotropic arrangement of actin filaments on softer substrates to nematic alignment of actin stress fibers on stiffer substrates. The cellular morphology was also closely linked to the absence and presence of nematic order, with circular cell morphologies observed for cells with an isotropic cytoskeleton conformation, while elongated and polarized cell shapes were reported for cells with highly aligned actin stress fibers [70]. The ability of the cells to display nematic alignment of their stress fibers was linked to their ability to contract, which is characteristic of active nematic systems. [54], [70]–[72] In vitro experiments with gliding assays using either actin or microtubule filaments demonstrated the capacity of those systems to form topological half-integer defects. As predicted by active nematic theory, those nematic defects serve as a source for the generation of flow reflected in their capacity to self-propel [54], [68], [69].

Active nematic systems in multicellular assemblies: Similarly to the subcellular level, the emergence of nematic order was also observed for multicellular assemblies. Contrary to subcellular filaments, whose shape is perfectly approximated by rod-like particles, not all cells have an ellipsoidal shape. Only a few cell types display a shape that closely matches the rod-like shape of typical particles contained in nematic materials such as neural progenitor cells, fibroblasts, mesenchymal stroma cells, and endothelial cells [4], [51], [54], [73]–[75]. In that case, the nematic director field can be easily defined by the orientation of the cells' major axis. On the contrary, other cells, such as epithelial cells, lack a characteristic spindle-like shape and still have been reported to form active nematic phases. Every cell in such monolayers experiences either pushing or pulling forces from surrounding cells, inducing deformational changes. Those cell-cell interactions can be described by a deformation tensor for which the eigenvector associated with its biggest eigenvalue is pointing parallel to the cells' elongation. Thus, it is still possible to define an orientational field even for a monolayer made up of more circular-shaped cells, such as epithelial cells [3], [76]–[78]. Whether the cellular monolayer behaves as an extensile or contractile nematic system typically depends on the cell type. Epithelial cells and neuronal progenitor cells have a tendency to form extensile nematic systems [3], [4], whereas monolayers of fibroblasts are best described by contractile nematic systems [79]. The switch between an extensile and a contractile nematic system can be controlled by adjusting the strength of the cell-cell interactions and the cell-matrix interactions. Weakening the cell-cell junctions in MDCK monolayers by knocking out E-Cadherin resulted in the epithelial monolayer behaving as a contractile nematic system [78]. Moreover, simulations and experimental observations showed that patches of extensile and contractile nematic phases can coexist within the same cellular monolayer. The coexistence of contractile and extensile nematic cell layers arises from the decoupling of the cellular orientation from the force dipole [80].

Biological functions associated with topological defects: Cell-cell interactions, cell-substrate interactions, and cell proliferation act as active stresses that drive the nematic system away from equilibrium [81]. Like active nematic materials consisting of subcellular filaments, topological defects can also nucleate in cellular monolayers. Experimental observations suggest that unconfined cellular monolayers favor the formation of half-integer nematic defects [3], [54], [78], [79]. Only when surface patterning or geometrical boundary conditions guide the cellular alignment is it possible to observe the formation of full integer nematic defects in cellular monolayers [5], [54], [74]. In active nematic systems, the active stresses can lead to a range of phenomena, including spontaneous nucleation of defects and the motility of comet-shaped defects, which have been both observed in cellular monolayers [4], [78], [79]. The direction of motion of $+1/2$ topological defects indicates if the monolayer behaves as an extensile or contractile nematic system. For extensile systems, the $+1/2$ nematic defect should move in the direction from the tail to the head, whereas for contractile systems, the direction of motion is reversed [54], [81]. The threefold symmetry of $-1/2$ nematic defects prevents any motility of those defect types [81]. In the past ten years, it has become increasingly evident that topological defects in cellular monolayers serve as hotspots for cellular activity. Topological defects have been reported to orchestrate several biological processes relevant to morphogenesis, ranging from cell apoptosis, cell accumulation, and cell extrusion to cell differentiation [3]–[5], [33], [82]. In active nematic systems, mechanical stresses peak around topological defects. Epithelial MDCK monolayers have been shown to display an increase of compressive stresses around $+1/2$ nematic defects together with an increased occurrence of apoptotic events at those point singularities. Saw et al. demonstrated that the increased mechanical compression of epithelial cells at $+1/2$ nematic defects is accompanied by a translocation of YAP from the nucleus to the cytoplasm [3]. The suppression of YAP mediated through mechanical compression has been linked to the activation of proapoptotic pathways, further highlighting the role of topological defects in mechanotransduction pathways [3], [54]. Surface patterning can impose geometrical constraints on the cellular monolayers that control the type and the site of nematic defect formation [3], [5]. Guillamat et al. used circular patterns to control the formation of a $+1$ defect in the cellular monolayer growing on the patterned surface. The full integer point singularities resulted in compressive mechanical stresses that peaked at the center of the circular pattern. These compressive stresses correlated with the differentiation sites of C2C12 myoblasts into myotubes, accompanied by the emergence of cylindrical cellular protrusions reaching heights of several hundreds of micrometers [5]. The relevance of topological defects as organization centers for mechanical stresses and their significance in morphogenesis was further highlighted by Maroudas-Sacks et al. using regenerating Hydra. In regenerating Hydra, the presence of topological defects was directly linked to the emergence of specific morphological features at the disclination sites [82]. These two studies demonstrate the key role of topological defects in orchestrating mechanical stresses within biological tissues, driving essential processes in morphogenesis. Notably, this further highlights the potential of such disclination sites for applications in tissue engineering. In unconfined cellular monolayers, $+1/2$ nematic defects are favored over $+1$ nematic defects. In several studies, half-integer topological defects have also been linked to the accumula-

tion or depletion of cells within a cellular monolayer. This impact of point singularities on cellular dynamics has been observed in neuronal progenitor cells where cells accumulated at $+1/2$ defects, whereas $-1/2$ defects led to the disruption of the cellular monolayer [4]. Similar observations have been reported for bacteria colonies with new bacteria cell layers forming close to $+1/2$ defect and the depletion of an existing layer occurring at $-1/2$ defect sites [33]. In both studies, the cell accumulation at $+1/2$ defects arose from the interplay of anisotropic friction and active forces, resulting in a net positive influx of cells around the defect core. Similarly, around $-1/2$ defects, this causes a negative influx of cells, which in bacteria films resulted in the formation of holes at those disclination sites [4], [33].

Aim of this study

These studies successfully highlighted the importance of topological defects as catalysts of active flows within cellular monolayers, thus regulating cellular dynamics. While all of these studies emphasize the importance of topological singularities in cellular assemblies by linking them to specific biological functions, they only focus on the local impact of disclinations on cellular behavior, failing to describe how global cellular dynamics are affected by the presence and interplay of topological defects with each other. To address this gap, we used time-resolved and large-scale imaging to investigate how topological defects impact the global alignment dynamics of an endothelial cell monolayer exposed to shear stress. Leveraging the extensive field of view, we measured the alignment dynamics of 12,000 to 40,000 cells per field of view, thereby resolving the non-monotonous alignment behavior of the endothelial cells. Using physical modeling, we investigated the role of topological defects in mediating the emergence of long-range order.

2.3 Perfusion cell culture of endothelial cells

Experimental setup: As previously mentioned, endothelial cells exposed to shear flow reorient themselves so that their major axis points parallel to the flow direction. Endothelial cells cultured in a flow channel were perfused with cell culture medium to apply a shear stress on the adherent cellular monolayer. A laminar flow of constant velocity was generated in the channel using an air-pressure-driven pump system from ibidi. To monitor the alignment dynamics of the endothelial cell layer throughout the whole duration of the experiment, the flow channel was mounted on a microscope setup. A small incubation chamber installed on the microscope stage allowed to maintain the cells at 37 °C and 5% CO₂. Because of the limited size of the incubation chamber, the pump system was placed in an incubator next to the microscope setup (Figure 2.6 a). The flow channel was connected through over 1 m long tubings with the pump in the incubator that was maintained at the same temperature and CO₂ level as the incubation chamber. With this constellation, the tubing exited the incubator through a small opening in its rear to reach the incubation chamber on the microscope stage. This introduced a temperature gradient

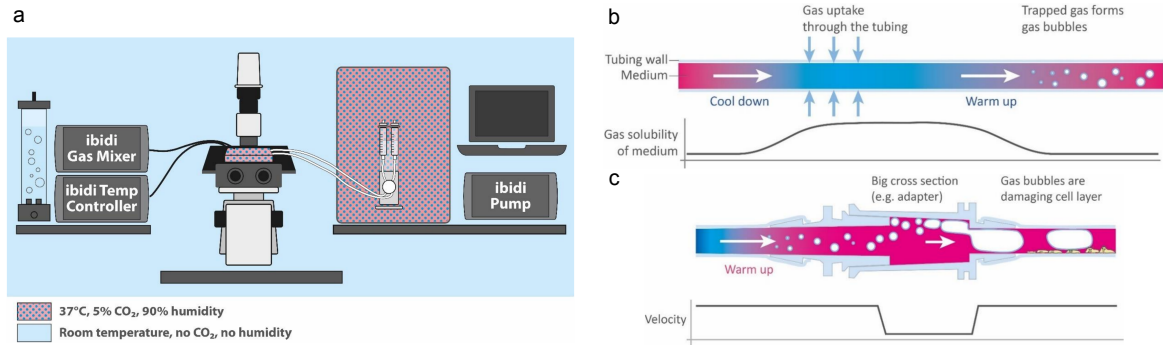


Figure 2.6: Live cell imaging of cell culture under perfusion. (a) The pump and the perfusion setup are placed in an incubator next to the microscope to maintain the medium reservoir of the pump at the same temperature and CO₂ saturation as the cells in the flow channel in the incubation chamber on the microscope stage. (b) The temperature gradient causes a gas uptake when the medium cools down while transiting between the incubator and the incubation chamber on the microscope stage. Gas bubbles nucleate when the temperature of the cell culture medium increases in the incubation chamber. (c) The bigger cross-section of the adapter decreases the flow velocity of the cell culture medium. Gas bubbles easily get trapped on small edges or cavities where they merge with other gas bubbles. The cellular monolayer is destroyed when a sufficiently big gas bubble is flushed into the flow channel. The images in (a-c) have been adapted from [83].

in the circulation of the cell culture medium that affected the solubility of gas in the liquid (Figure 2.6 b). The solubility of gas is highly dependent on the temperature of the liquid. At higher temperatures, the amount of gas dissolved in the liquid decreases, whereas at lower temperatures, the solubility of the gas increases. The silicone tubing is permeable to gas, which is essential to maintain the pH of the cell culture medium at physiological levels. However, this also leads to the formation of gas bubbles in the liquid caused by the temperature gradient present in the experimental setup. More precisely, as the tubing exits the incubator, the cell culture medium is exposed to a colder environment, resulting in gas uptake. When the cell culture medium enters the incubation chamber, its temperature increases again. Consequently, this reduces the gas solubility of the medium, triggering the nucleation of small gas bubbles (Figure 2.6 b). Those small air bubbles are often not an issue because they are sufficiently small to not interfere with the cellular monolayer in the flow channel. However, the tubing is connected to the flow channel via an adapter with a wider cross-section than the tubing, which decreases the fluid velocity in the adapter. This reduction in flow velocity causes the air bubbles to become trapped in small cavities within the adapter, where they accumulate over time to form a larger gas bubble. When this gas bubble enters the flow chamber, the cells directly exposed to it undergo cell death (Figure 2.6 c). Therefore, to avoid bubble formation, the tubing outside the incubator is wrapped with insulating material to prevent a drastic temperature drop and the associated excessive gas uptake by the liquid. The continuous perfusion of the cellular monolayer by

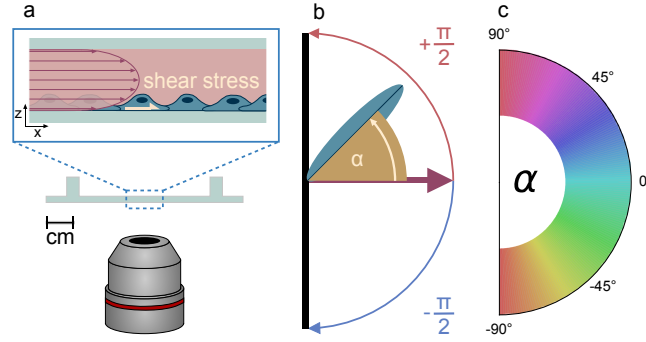


Figure 2.7: Experimental setup and analysis steps. (a) The endothelial cells were exposed to shear stress in a flow chamber mounted to a microscope stage. Using an automated microscope setup, we imaged a field of view that spanned several millimeters. (b) The nematic director field provided the orientation of the HAOEC monolayer throughout the field of view, allowing the computation of the angle α . (c) The angle α was set to zero along the axis parallel to the flow and was defined in the interval $\alpha \in [\pi/2, -\pi/2]$.

cell culture medium generates shear stress that acts on the cellular monolayer. The amount of shear stress the cells are exposed to depends on the viscosity of the cell culture medium, η , the dimensions of the flow channel, and the flow velocity of the cell culture medium, ϕ . The shear stress, π , can then be written as

$$\pi = \eta\gamma\phi, \quad (2.24)$$

where the factor γ accounts for the geometry and dimensions of the channel. For cell culture medium at 37°C , the dynamic viscosity is $\eta \approx 0.0072 \text{ dyn}\cdot\text{s}/\text{cm}^2$. To generate shear stress rates ranging from 10 to 20 dyn/cm^2 , we used the same flow channel but varied the velocity of the fluid [84].

Imaging of endothelial cell layer: To capture the response of the endothelial cell monolayer on a global scale, a field of view spanning several millimeters is imaged using an automated microscope setup (Figure 2.7 a). This big field of view allowed us to image the collective response of 12 000 to 40 000 cells to the exposure of shear stress. Because of the parabolic velocity profile of the fluid in the flow channel, only the cells in regions at least $200 \mu\text{m}$ from the channel walls were exposed to the same amount of shear stress. Therefore, to restrict the analysis to the part of the cellular monolayer that has been exposed to homogeneous shear stress levels, the field of view was cropped by $200 \mu\text{m}$ from each channel wall after image acquisition [84].

Analysis of the nematic director field: The nematic director field describing the orientation within the cellular monolayer was extracted from the bright field images by analyzing its intensity gradients. To compute the nematic director at a specific position, we considered a region of interest of $130 \times 130 \mu\text{m}$ centered in this particular spot. Thus,

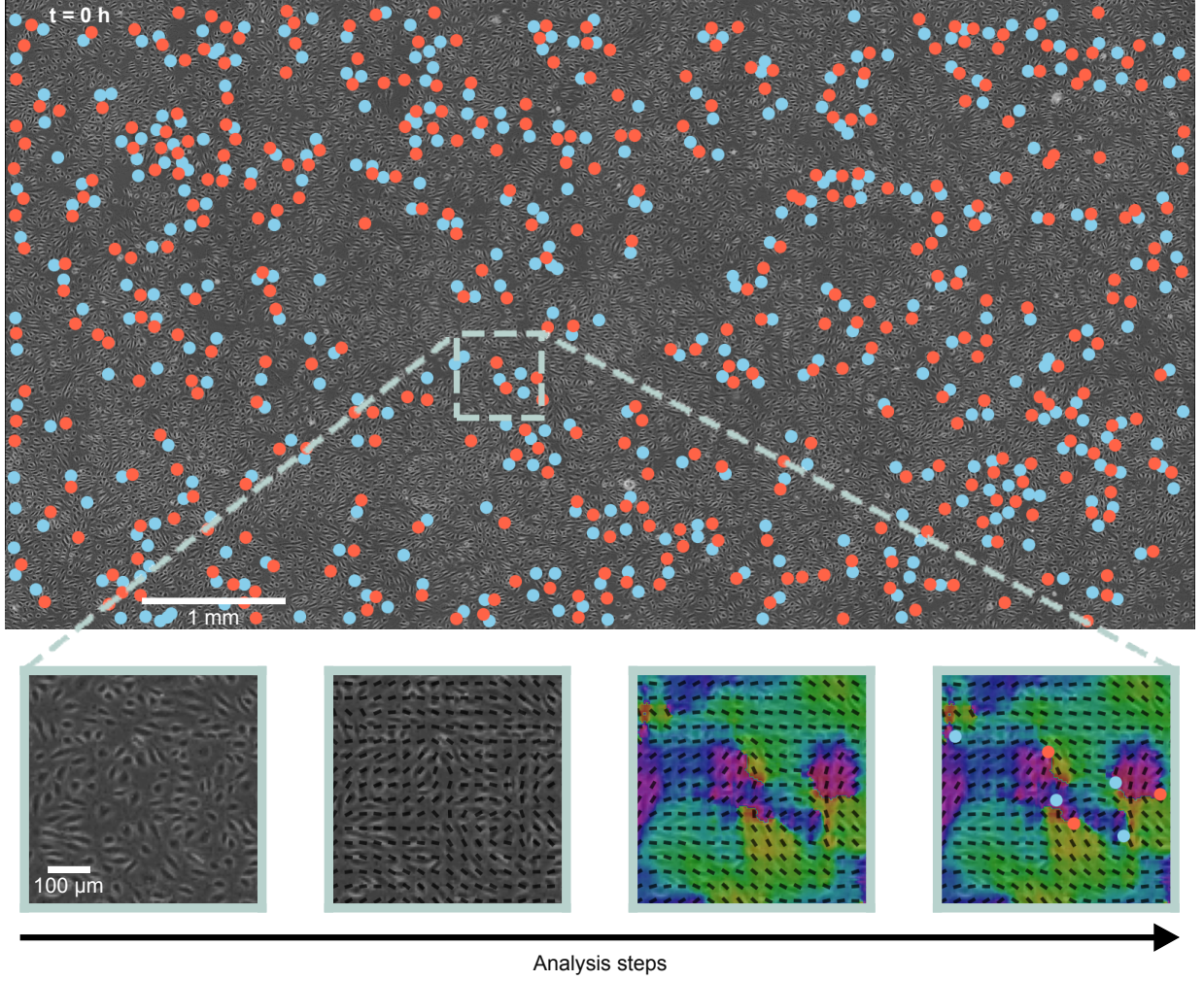


Figure 2.8: Analysis steps. Phase contrast image with $+1/2$ (red dots) and $-1/2$ (blue dots) nematic defects plotted in the upper row. A zoom-in illustrates the analysis steps from left to right. From the phase contrast image, we obtained the nematic director field. We color-coded the nematic director field to visually represent the orientation of the endothelial cell monolayer. The nematic defects were detected from the nematic director field.

the nematic director always provides the average orientation of the cells contained in this area. The nematic director $\mathbf{n}$ forms an angle α with the direction of the flow, which is also the preferred orientation axis of the cells (Figure 2.7 b). In our system, a dealignment of the nematic director by an angle α_0 is associated with a certain energy cost. Because of the symmetry of the system, a misalignment of $-\alpha_0$ should be associated with the same energetical cost as a misalignment of α_0 . This motivated our choice of the interval $\alpha \in [-\pi/2, \pi/2]$ deviating from the established choice of the angle $\alpha \in [0, \pi]$ for most nematic systems. To facilitate the visual representation of the nematic director field, we

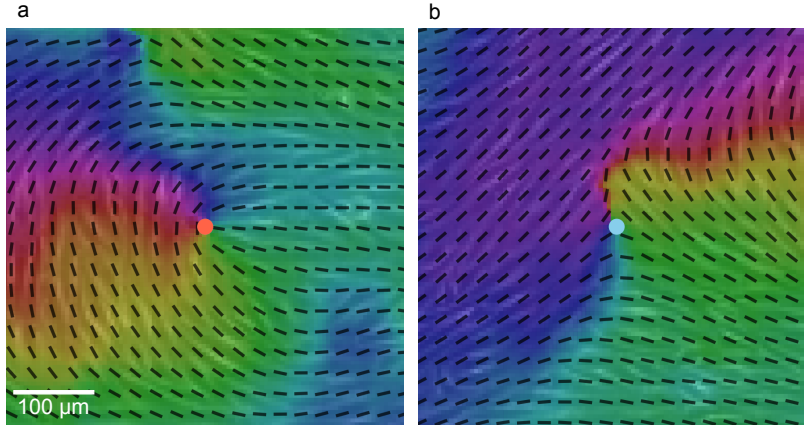


Figure 2.9: Representative images of nematic defects found in the HAOEC monolayer. (a) A $+1/2$ nematic defect indicated by a red dot. (b) A $-1/2$ nematic defect highlighted by a blue dot.

use a cyclic colormap to represent the director field as a color-coded heatmap (Figure 2.8 c). As previously mentioned, the nematic director field is obtained from the phase contrast image and captures the cellular alignment of the endothelial cells throughout the monolayer. However, the size of the field of view impairs an adequate visual representation of the nematic director field using a vector plot with sufficient spatial resolution. Therefore, we color code the angle α the nematic director $\mathbf{n}$ forms with the flow axis, which allows us to correctly represent the orientation of each pixel in a heatmap. From the nematic director field, we detect $+1/2$ and $-1/2$ topological defects indicated by red and blue dots (Figure 2.8).

Comparison of nematic phase formed by endothelial cell monolayers to other systems: As previously mentioned, the existence of a nematic phase formed by cellular monolayers has already been reported for many different cell types. Contrary to epithelial monolayers, where cell-cell interactions generate dipole forces that define the cellular alignment of the cells, endothelial cells have a rather elliptic shape that facilitates the association of a cellular orientation (Figure 2.8). Especially in contrast to epithelial cell layers, it is, therefore, very easy to correlate the cellular orientation with the nematic director field. This also helps with the identification of topological defects that can be easily identified from the cellular orientations alone (Figure 2.9 a and b). The topological defects arising from singularities in the cellular orientations vary in size but are typically comprised of dozens of cells. Those defects formed spontaneously when the cellular monolayer was exposed to flow, which is an indication of the presence of active stresses within the cellular monolayer, driving the system away from equilibrium. Compared to other experimental systems, the applied flow interfered with the cellular monolayer and initiated a transition from an isotropic disordered state with no clear preferred orientation to a highly aligned cellular monolayer exhibiting long-range order.

Simulation of active nematic systems with flow aligning field: To correctly describe the experimental observations, we simulated an active nematic system using nematohydrodynamic equations (Chapter 2.2). The intrinsic activity of the cellular monolayer

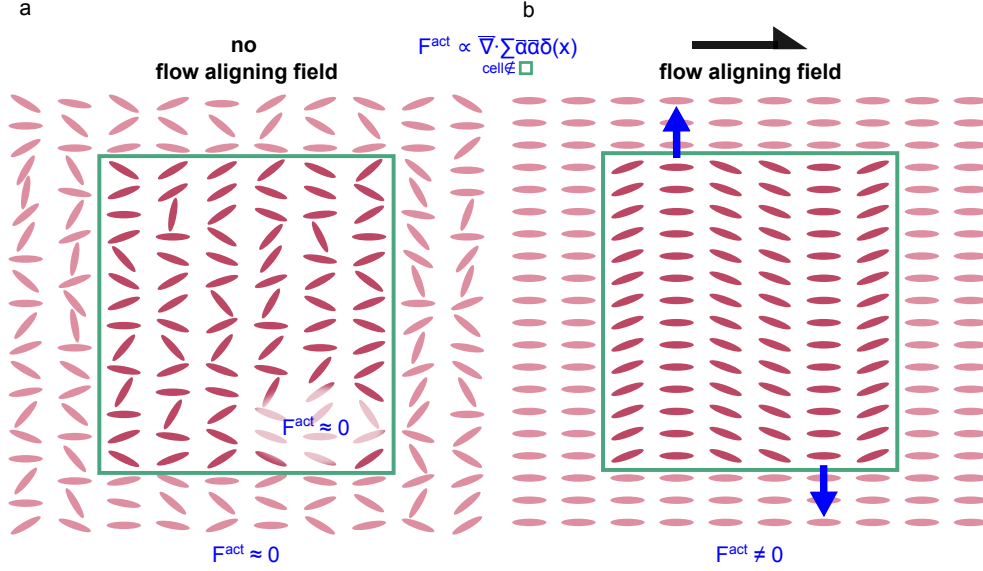


Figure 2.10: Effect of a flow-aligning field on active nematics. (a) Without a flow-aligning field, inter-cellular forces are randomly oriented and cancel each other out. (b) The shear in the x-z plane acts as an affine alignment field, inducing elongation and ordering of the cells along that direction. As local ordering is established, inter-cellular forces stop canceling out, resulting in mesoscopic active forces F^{act} (collective active stresses), approximated as extensile forces ($\zeta > 0$).

was assumed to be weakly extensile and set to $\zeta = 0.003$. Epithelial cells commonly display stronger cell-cell interactions than endothelial cells. Therefore, the resulting active stresses arising from cell-cell interactions should also be weaker, which justifies the choice of the activity parameter ζ . In the absence of an aligning field, the nematic director field lacks global order, resulting in a negligible small active force (Figure 2.10 a). To correctly describe how an external flow aligning field affects the dynamics of the active nematic system, we modified the free energy that describes the equilibrium state the system would relax to in the absence of any active stresses. As introduced in the section on nemato-hydrodynamics, the free energy consists of a term describing the Landau-de Gennes free energy $\mathcal{F}_{\text{LDG}}$ and the deformation free energy $\mathcal{F}_d$ (equation 2.16). In the experiment, the flow-aligning field imposes a preferred orientation for the cellular monolayer, resulting in a highly aligned state. To account for the superjacent flow, we introduce an additional energy term $\mathcal{F}_{\text{Field}} = -\epsilon_0 \mathbf{E} \cdot \mathbf{Q} \cdot \mathbf{E}$, where the matrix $\mathbf{E}$ provides the flow direction and ϵ_0 represents the strength of the flow. By expanding the free energy by a third term $\mathcal{F} = \mathcal{F}_{\text{LDG}} + \mathcal{F}_d + \mathcal{F}_{\text{Field}}$ the equations successfully describe the dynamics of the system where an external aligning field favors nematic alignment along the flow direction. Indeed, simulations taking into account the modified free energy yield a rapid nematic ordering of the nematic director field. Therefore, gradients in the nematic director field lead to active stresses within the nematic system. When these deformations become sufficiently

strong, spontaneous defect nucleation can occur (Figure 2.10 a). For more details on the values of all the parameters in the nematohydrodynamics equations, refer to the following publication [85].

2.4 HAOECs align under shear stress following a non-monotonous process

HAOECs do not align for low shear stress values: Endothelial cells sense shear stress through a complex manifold of mechanotransduction pathways [40], [86]. The signaling cascades triggered by the shear stress induce a cellular response, such as an increase in stress fibers, the rearrangement of the cellular cytoskeleton, and a change in cell shape and orientation [40]. Because the cellular response towards shear stress relies on mechanotransduction pathways, it is only logical that a threshold value exists below which the mechanical stimulation will not be sufficient to elicit a cellular response. Two studies have confirmed that endothelial cells no longer respond to shear stress below a certain threshold value [9], [87]. However, in the human body, the shear stress levels experienced by endothelial cells vary strongly depending on the type of blood vessel (Figure 2.1 b). Consequently, the threshold values required for cell alignment differ between distinct endothelial cell types. HAOEC are reported to be exposed to shear stress values ranging from 10 to 60 dyn/cm². However, in specific arteries, such as the descending aorta, the shear stress can be as low as 5 dyn/cm². Therefore, a shear rate of 1.4 dyn/cm² was selected to check if HAOEC fail to respond to low shear stress levels. To demonstrate that HAOECs only align at sufficiently high shear stress levels, we exposed them to 20 and 1.4 dyn/cm². To achieve such low shear stress levels, we used a flow channel with a height of 800 μ m, which allowed for the simultaneous perfusion of HAOEC in a flow chamber of 200 μ m height by connecting the two flow channels in series. In the first flow channel, the cells were exposed to 1.4 dyn/cm², while in the second flow channel, the cells were exposed to 20 dyn/cm² due to reduced channel height. After 24 hours of perfusion, the flow was stopped, and the cells were fixed with paraformaldehyde. We stained the actin filaments with phalloidin to visualize the cytoskeleton of the cells. The HAOEC exposed to low shear stress levels displayed no preferred orientation. The cells were randomly oriented and did not display the typical elongated shape characteristic of endothelial cells under flow. The nuclei also failed to align in flow direction. Moreover, the orientation of the nuclei did not always match that of the cell (Figure 2.11 a). The cells displayed the characteristic elongated morphology for the high shear stress rate and were increasingly aligned in flow direction, with their actin filaments being oriented parallel to the flow. Contrary to the cells exposed to low shear rates, the cells' nuclei were pointing in the same direction as the cells (Figure 2.11 b). Interestingly, those areas of high cellular alignment were interrupted by local regions of cellular misalignment. In those areas, the cells still displayed the characteristic elongated shape typical for endothelial cells exposed to shear

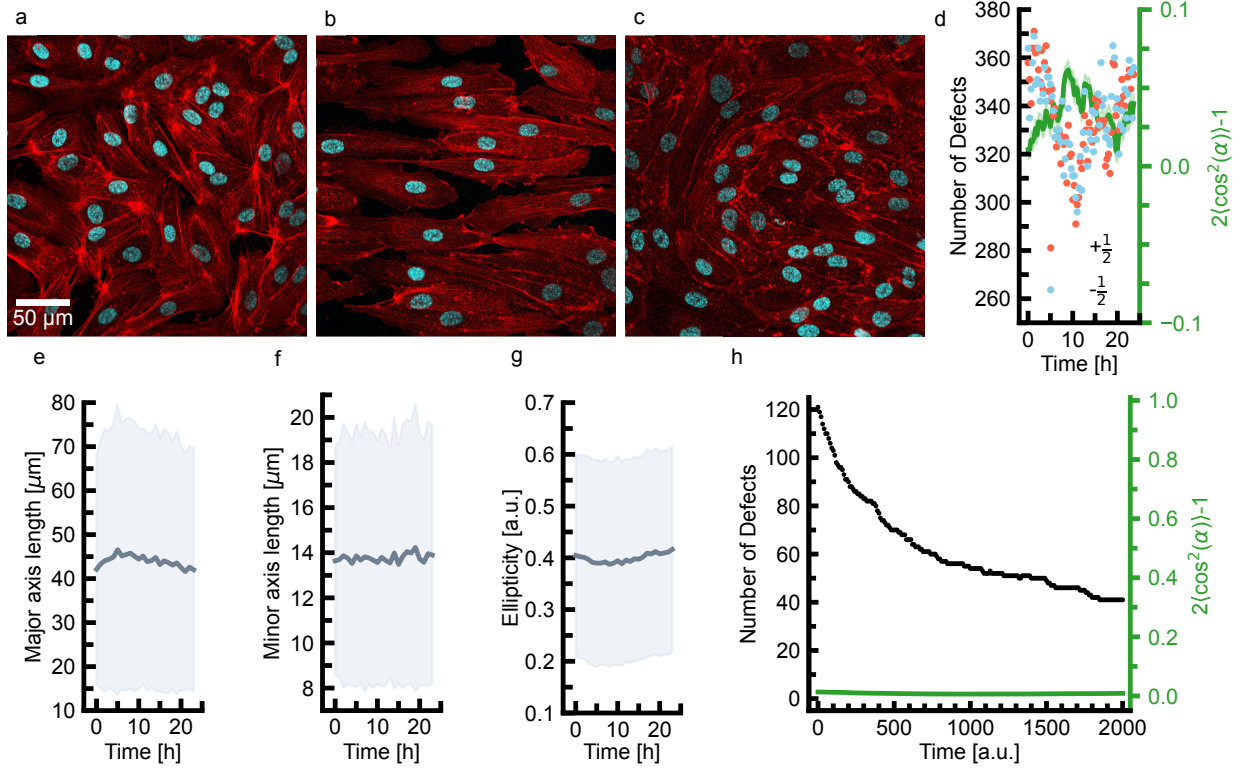


Figure 2.11: Comparison of HAOEC response exposed to distinct shear rate levels (a) HAOEC exposed to low shear stress levels of 1.4 dyn/cm² for 24 hours do not align under flow. (b) HAOEC exposed to physiological shear stress levels of 20 dyn/cm² for 24 hours display areas of increased alignment in flow direction. (c) A distinct region of the HAOEC monolayer is shown in (b). Here, the cells are locally misaligned. The cells within this region represent a +1/2 nematic defect. (d) The dynamics of +1/2 (red dots) and -1/2 (blue dots) nematic defects present in the endothelial monolayer under static conditions for 24 hours. The global order parameter S (green) measures the magnitude of the alignment in the system. The light green area indicates the 95% confidence interval. (e) The major axis of the cells' shape is plotted over time. (f) The minor axis of the cells' shape is plotted over time. (g) The circularity of the cells is plotted over time. The light gray area in (e, f, and g) corresponds to the standard deviation of the mean. (h) The simulation of an active nematic system without a flow-aligning field matches the experimental observations. The order parameter S is plotted in green, and black dots indicate the absolute number of topological defects in the system. As the number of +1/2 nematic defects corresponds exactly to the number of -1/2 nematic defects in the simulations, we do not distinguish between the charges for the simulation data.

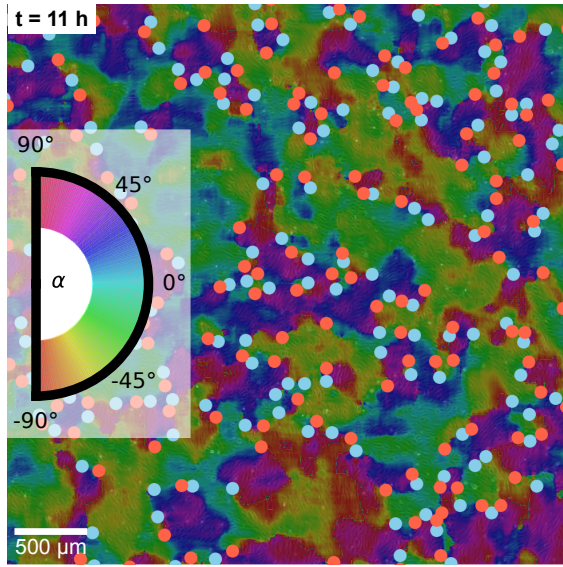


Figure 2.12: Cellular monolayer cultured under static conditions. Under static conditions, topological defects are also present within the cellular monolayer. The red dots represent $+1/2$ topological defects, and the blue dots correspond to $-1/2$ topological defects. The orientation field is superimposed on the phase contrast image of the cellular monolayer. The color code illustrating the orientations of the nematic director field is plotted in the insert on the left.

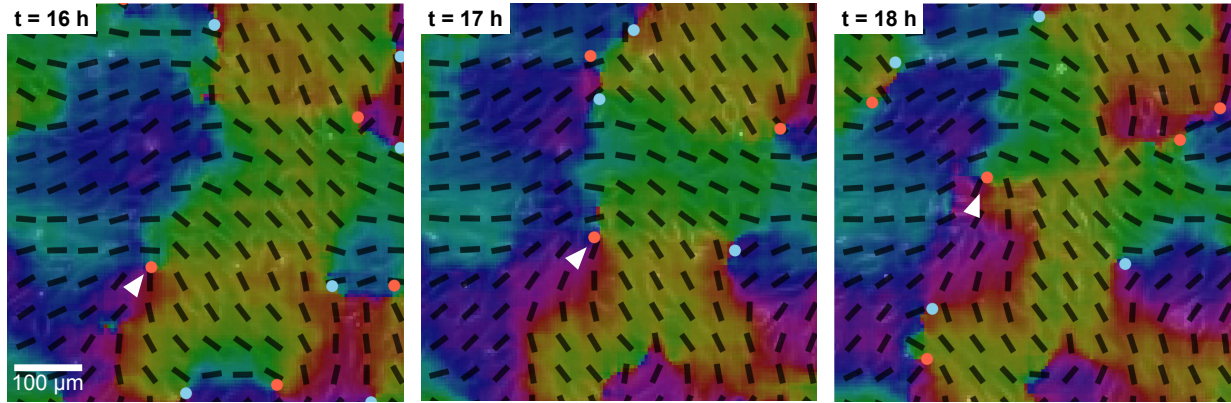


Figure 2.13: Trajectory of an isolated $+1/2$ topological defect under static conditions. The isolated $+1/2$ defect moves in the direction of its comet head. The cellular monolayer is cultured under static conditions.

stress. However, they failed to align in flow direction (Figure 2.11 c). The elongated shape of the endothelial cells facilitated the assignment of a clear orientation to each cell, allowing the identification of misaligned regions as topological defects within the cellular monolayer.

Characterization of cell culture under static conditions: To determine whether topological defects resulted from shear stress and to characterize the endothelial monolayer under static conditions, we imaged the cells cultured in the flow channel without flow. The global order parameter $S = \langle 2 \cos^2 \alpha \rangle - 1$ measured the magnitude of the order present within the cellular monolayer throughout the experiment. The parameter α indicates the angle of the nematic director field with the x-axis. During the full 24 hours of the experiment, the global order parameter remained close to zero, indicating an

isotropic state lacking global alignment. Similar to the perfused cell culture, we also observed topological defects in the static cell culture. However, the amount of these defects present in the observed cellular monolayer remained approximately constant throughout the experiment (Figure 2.11 d). As expected, the shape of the endothelial cells remained unchanged (Figure 2.11 e, f, and g). We examined zoomed-in areas to better understand how nematic defects can be present in the cellular monolayer. We observed that small areas of local cellular alignment existed (Figure 2.13). A topological defect is, by definition, the point at which those regions of different orientations meet. Therefore, we still observed topological defects in the cellular monolayer without global order due to these small regions of local alignment. Additionally, we observed the trajectory of isolated $+1/2$ nematic defects. These defects predominantly moved in the direction of their comet head (Figure 2.13). Therefore, the endothelial monolayer can be approximated as an extensile nematic system. Similarly, the simulations of an active nematic system, as defined in the previous section, without a flow-aligning field confirmed our experimental results. In the simulations, the system was also characterized by a low global order parameter indicating an isotropic state and a constant number of topological defects (Figure 2.11 h).

Alignment dynamics of HAOECs exposed to sufficiently high shear stress levels: When the endothelial cells are exposed to physiological levels of shear stress, they elongate and align in the flow direction. The experiments performed on endothelial cell layers under static conditions demonstrated that topological defects are already present in the unaligned isotropic state of the monolayer. In condensed matter physics, topological defects have been reported to play a crucial role in transitions from disordered to ordered states. Therefore, to investigate whether topological defects play a similar role in biological matter, we tracked the dynamics of the defects during the cellular alignment (Figure 2.14). At the onset of the flow, the endothelial cell monolayer displayed no clear preferred direction of orientation. As observed for the static cell culture, topological defects were already present from the beginning (Figure 2.14, $t = 0$ h). During the first 10 hours, the cells gradually aligned in flow direction, and the number of nematic defects decreased (Figure 2.14, $t = 10$ h). Yet, from 10 to 25 hours, the density of topological defects increased again. This was accompanied by increased misalignment within the cellular monolayer (Figure 2.14, $t = 25$ h). Eventually, as the cells continued to be exposed to shear stress, the nematic defects annihilated, and the cells aligned along the flow direction, establishing long-range order (Figure 2.14, $t = 64$ h). The cells did not monotonously align in flow direction but did so following an alignment process characterized by three distinct regimes. In the first phase, the initial ordering phase (I), the endothelial cells aligned and elongated along the flow direction (Figure 2.15 a, d, e, and f). During the second phase, the intermediate disordering phase (II), the endothelial cells misaligned, which resulted in a decrease in the global order parameter S (Figure 2.15 a). During this phase, the deformations in the director field led to the nucleation of new topological defects, thus increasing the defect density (Figure 2.15 a). Simultaneously, during the intermediate disordering phase, the cells again became increasingly round, which could indicate an increase in deformational forces within the cellular monolayer (Figure 2.15 d, e, and f). Eventually, during the last

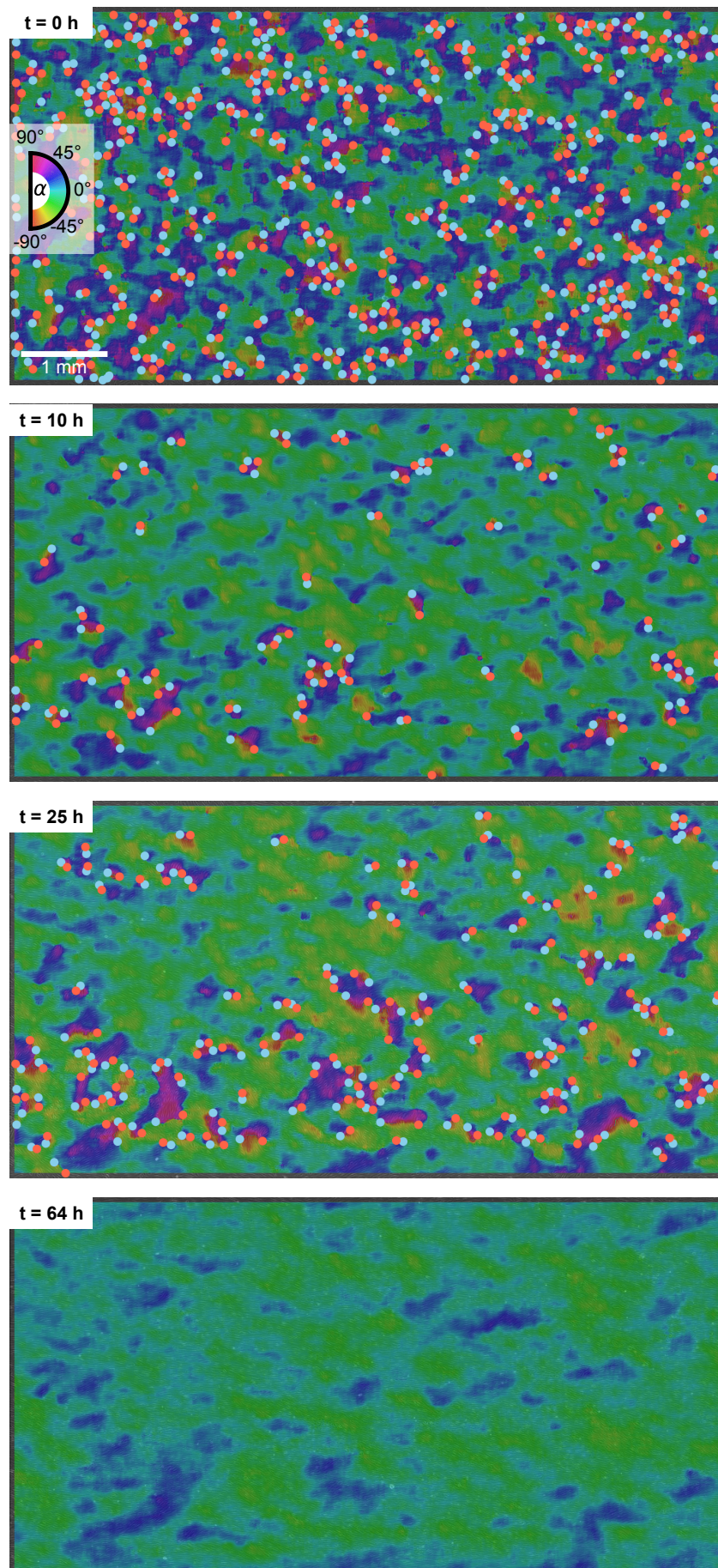


Figure 2.14: Caption next page.

Figure 2.14: Non-monotonous defect dynamics upon exposure to shear stress.

The images show the phase contrast images of the endothelial cell monolayer for the whole field of view. The flow is applied from left to right. The nematic orientation field is color-coded and overlaid on top of the phase contrast image. The color code attributes a color to each orientation and is plotted in the first snapshot at $t = 0$ h on the left. The nematic director field is only defined starting $65\ \mu\text{m}$ from the corner of the image, as it represents the local average of the orientation with a box of dimensions $130 \times 130\ \mu\text{m}$. Topological $+1/2$ defects are indicated by red dots and $-1/2$ defect by blue dots. The absolute number of nematic defects decreases in a first instance from $t = 0$ h to $t = 10$ h. Then, the number of nematic defects increases again as new nematic defects nucleate from $t = 10$ h to $t = 25$ h. Eventually, as long-range order is established, all nematic defects disappear at $t = 64$ h.

phase, the steady-state ordering phase (III), the endothelial cells realigned in the direction of the flow, and the nematic defects annihilated with each other (Figure 2.15 a). Equally, the cells elongated again, displaying the characteristic elliptic shape of endothelial cells under shear stress (Figure 2.15 d, e, and f). Only the simulation of an active nematic system with a flow-aligning field can capture those same non-monotonic alignment dynamics as observed in the experiments (Figure 2.15 c). A passive nematic system coupled to a flow-aligning field aligns monotonously without the emergence of an intermediate disordering phase because it lacks the active stresses that can nucleate topological defects (Figure 2.15 c). Yet, an active nematic system lacking the external flow aligning field does not align at all. The complex interplay between the alignment constraint imposed by the external field and the intrinsic activity of the system will be discussed in more detail in a subsequent paragraph. For now, the next paragraph focuses on the role the applied shear stress plays in driving the system toward an aligned state.

Dealignment dynamics of the endothelial cell monolayer: In the experimental setup, the superjacent flow continuously drives the biological system toward a highly aligned state. To assess whether constant exposure to shear stress is required to achieve cellular alignment during the steady-state ordering phase, we stopped the flow at two different time points: first, in a highly aligned state with almost no defects present within the cellular monolayer, and second, at an early stage during the steady state alignment phase. When the flow was stopped after the cells were perfectly aligned in flow direction, the global order parameter S of the cellular monolayer remained constant. Equally, no new topological defects nucleated during the first 10 hours after the flow was stopped. Eventually, the first defects started to nucleate, but the overall defect density remained low (Figure 2.16 a). Therefore, after the flow is stopped, the relaxation back to an isotropic state occurs very slowly in highly aligned systems. In contrast, when the flow was stopped early during the steady state alignment phase, a change in cellular dynamics was observed almost immediately (Figure 2.16 b). The cells stopped aligning and gradually began to

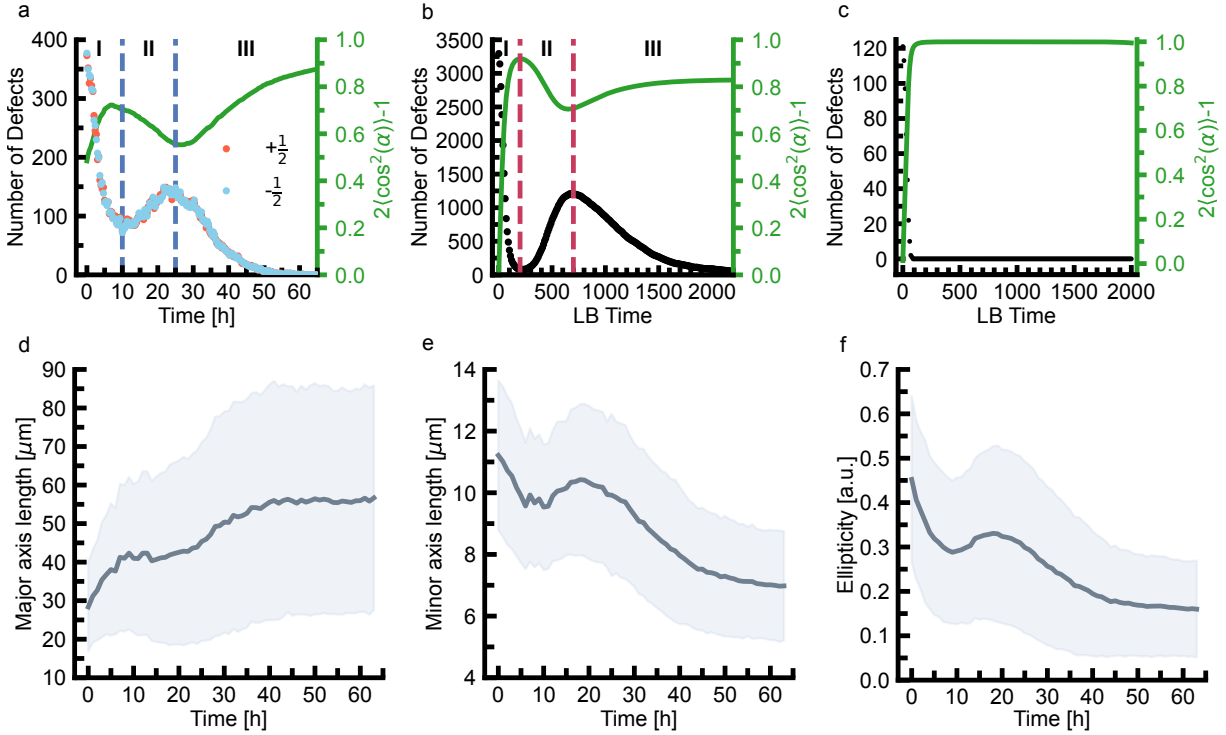


Figure 2.15: Alignment dynamics of endothelial cells match the simulation data. (a) The order parameter S (green) and the number of $+1/2$ (red dots) and $-1/2$ (blue dots) nematic defects as the cells align in flow direction. The light green area indicates the 95% confidence interval. (b) Simulation data for an active nematic system with an external aligning field. (c) Simulation data of a passive nematic system with an external aligning field. (d) The major axis describing the shape of endothelial cells increases under perfusion cell culture. (e) The minor axis of the endothelial cells decreases under flow conditions. (f) Consequently, the ellipticity of the cells decreases under shear stress. A circle is characterized by an ellipticity of 1.

dealign. Consequently, constant flow is required to achieve the establishment of long-range order within the cellular monolayer. Additionally, the relaxation of the monolayer back to the isotropic state depends strongly on its alignment.

The interplay of activity and the strength of the aligning field governs the alignment dynamics: The non-monotonous alignment dynamics observed in experiments and simulations only occurred within a defined regime: the aligning flow field needed to be strong enough to induce nematic ordering, and the activity of the system needed to be sufficiently strong to induce nucleation of topological defects from gradients in the nematic director field. The nucleation of these defects decreased the global order established in the system and gave rise to the emergence of the intermediate disordering phase (Figure 2.17 d and f). The region for which three-stage ordering dynamics was observed was designated

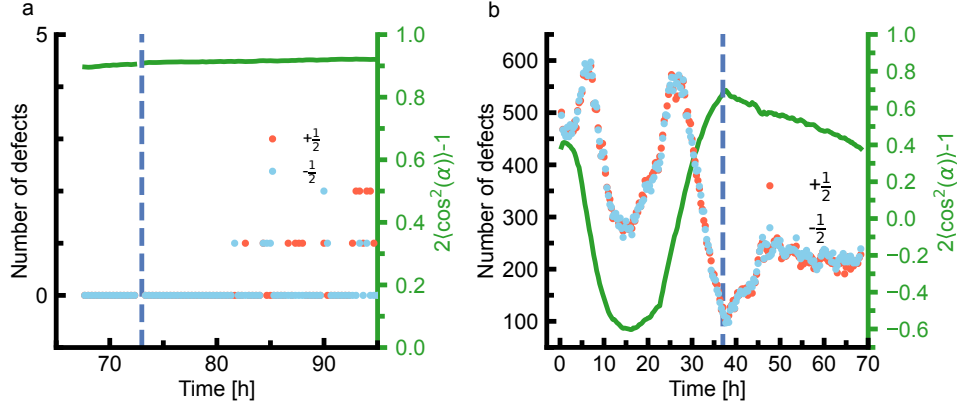


Figure 2.16: Cellular dynamics of HAOEC after the flow stopped. (a) At a stage where the cells have reached almost perfect alignment in flow direction, the cells relax only very slowly back toward an isotropic state. (b) If the flow is stopped early during the steady state ordering phase, the endothelial monolayer does not continue to evolve toward a highly aligned state but misaligns. (a-b) The light green area indicates the 95% confidence interval.

as the non-monotonous coarsening regime (Figure 2.17 d, blue region). For weaker activity, the external aligning field dominated, thereby resulting in monotonous alignment of the active nematic system without the emergence of a temporary stage of local misalignment (Figure 2.17 d and e, orange region). Therefore, this regime was denoted the monotonous coarsening regime. On the contrary, when the activity dominated the system, the system did not align, and no global order was established. Instead, the system evolved towards a state of weak local alignment (Figure 2.17 d and h, red region). When the activity and the aligning field were weak, the system remained in an isotropic state (Figure 2.17 d and g, green data point). This case corresponds closely to the biological equivalent of the dynamics of HAOEC exposed to very low shear stress levels discussed in a previous paragraph. For the biological system, it is much more difficult to precisely control the intrinsic activity of the cellular monolayer. In the case of an endothelial cell layer, the cell-cell interactions are at the origin of the intrinsic activity of the monolayer. As the density increases, the activity emanating from cell-cell interactions, such as volume exclusion and friction through cellular motion, increases. Therefore, the cell density is considered a rough estimate of the intrinsic activity within the cellular monolayer. This hypothesis is further confirmed by a previous study demonstrating the correlation between the activity within a monolayer of epithelial cells and their density. For lower cell densities, the global order parameter increased monotonously (Figure 2.17 a and b, orange region). Conversely, at elevated cell densities, the system displayed non-monotonous alignment dynamics (Figure 2.17 a and c). The measurements displaying this kind of behavior are classified by the non-monotonous alignment regime (Figure 2.17 a, blue region).

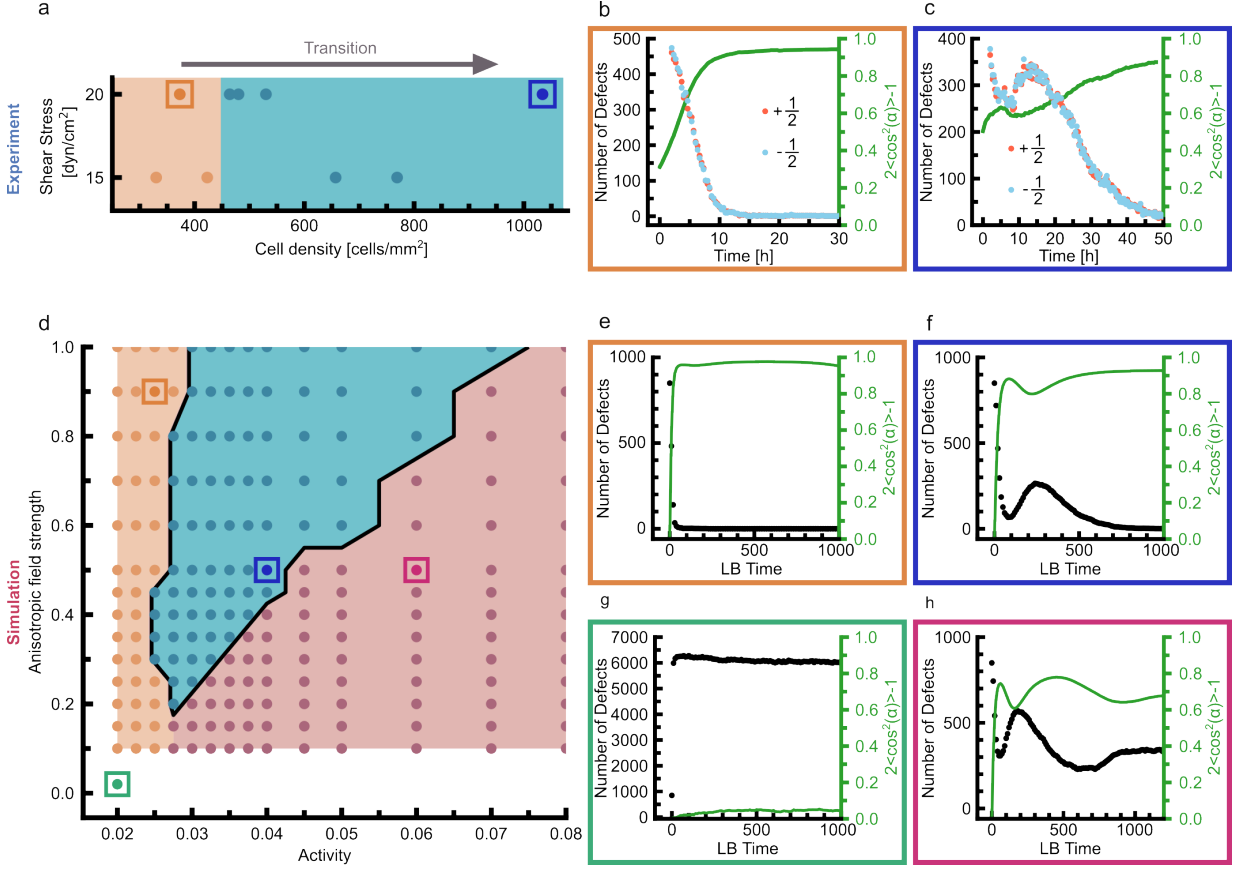


Figure 2.17: Interplay between cellular activity and external aligning field governs alignment dynamics. (a) The phase diagram for the experimental data obtained from several experiments at different cell densities shows a transition from the regime with monotonous alignment dynamics in orange towards the regime with a non-monotonous alignment behavior in blue. (b) At lower cell densities, the endothelial cells align monotonously along the flow direction, lacking the three-stage dynamics. The light green area indicates the 95% confidence interval. (c) At higher cell densities, the endothelial monolayer displays non-trivial alignment dynamics characterized by a non-monotonous alignment. The light green area indicates the 95% confidence interval. (d) The phase diagram obtained from simulations shows that the non-monotonous alignment regime, highlighted in blue, only exists within a very defined region. The orange region denotes the monotonous alignment regime, while the red region highlights the regime characterized by continuous defect generation. The green data point represents the isotropic state, where the force-aligning field is too weak to induce global order. (e) The system displays a monotonous alignment behavior in the trivial region for high anisotropic field strength and low activity within the nematic director field. (f) In the non-monotonous coarsening regime, the interplay between the anisotropic field strength and the intrinsic activity gives rise to a three-stage, non-monotonous alignment behavior. (g) When activity and flow anisotropic field are low, the cells don't display localized order, resulting in an isotropic state. The number of defects saturates as orientational order becomes ill-defined. (h) When the activity is the dominating parameter, the system fails to align and evolves towards a state with only local nematic alignment.

Anisotropic growth of domains: Although the cellular monolayer temporarily misaligned, with continuous exposure to shear stress, the cells eventually aligned in flow direction, thus establishing long-range order. To quantify the spatiotemporal growth of locally aligned regions, we measured the correlation length. Commonly, in nematic systems, the correlation length of the nematic director field is defined as $C_{2\theta}(r) = \langle \hat{\mathbf{n}}_{2\theta}(r) \cdot \hat{\mathbf{n}}_{2\theta}(0) \rangle$ with $\hat{\mathbf{n}} = (\cos(2\theta), \sin(2\theta))$. In nematic systems, the correlation function can be fitted to $C_{2\theta}(r) = Ae^{r/l_{\text{corr}}} + Bl$, where l_{corr} is the correlation length, and A and B are the fitting constants [33]. Without a preferred direction of orientation, the second fitting constant typically is very small $B \ll 1$. However, in our system, where the superjacent flow established a preferred orientation axis, the correlation function, as previously defined, did not drop to zero. Notably, during the intermediate disordering phase, this correlation function was insufficient to adequately capture the transient misalignment of the cells. To address this, we adjusted the interval in which α is defined. Therefore, in our study, the definition of the interval $\alpha \in [-\pi/2, \pi/2]$ deviates from the established convention (Figure 2.6 c). Additionally, we constructed a correlation function so that the contributions from two distinct points, both at a distance Δr with orientations α_0 and $-\alpha_0$, would cancel each other out. To this end, we considered the deviation of the nematic director from the preferred orientation axis $\delta \mathbf{n} = (\delta n_{\parallel}, \delta n_{\perp}) = (\cos(\alpha), \sin(\alpha))$ (Figure 2.18 a). As the contributions from α_0 and $-\alpha_0$ should cancel each other out, we decided that only the perpendicular component of the deviation from the preferred orientation axis $\delta n_{\perp}$ would be considered for the correlation function. To investigate if the applied flow along the x-axis sets a bias for the coarsening along this direction, we chose to separately compute the correlation function along the axis parallel and perpendicular to the flow axis (Figure 2.18 b and c). The correlation function for the decay of $\delta n_{\perp}$ along the axis parallel to the flow is defined as

$$C_{\delta n_{\perp \parallel}}(\Delta r_{\parallel}) = \left\langle \frac{\delta n_{\perp}(r) \delta n_{\perp}(r + \Delta r_{\parallel})}{\delta n_{\perp}^2} \right\rangle, \quad (2.25)$$

where $\Delta r_{\parallel}$ is the distance between the points r and $r + \Delta r_{\parallel}$. Similarly, the definition of the correlation function of $\delta n_{\perp}$ along the axis perpendicular to the flow is given by

$$C_{\delta n_{\perp \perp}}(\Delta r_{\perp}) = \left\langle \frac{\delta n_{\perp}(r) \delta n_{\perp}(r + \Delta r_{\perp})}{\delta n_{\perp}^2} \right\rangle. \quad (2.26)$$

For the experimental data, the correlation function parallel and perpendicular to the flow axis initially decayed to zero (Figure 2.19 a and b). As the cellular monolayer continued to align along the flow direction, the correlation function along the axis parallel to the flow $C_{\delta n_{\perp \parallel}}(\Delta r_{\parallel})$ decayed slower than the correlation function along the axis perpendicular to the flow $C_{\delta n_{\perp \perp}}(\Delta r_{\perp})$ (Figure 2.19 a and b). To quantify the size of similarly aligned regions along the axis parallel and perpendicular to the preferred orientation, we computed the correlation length. The correlation length ξ was defined as the length for which the correlation function dropped below a value of $C_{n_{\perp}}(\xi) \leq 1/e$. The correlation length parallel $\xi_{\parallel}$ to the flow axis increased faster than the correlation length perpendicular $\xi_{\perp}$ to the flow axis (Figure 2.19 c). This suggests that similarly aligned regions grow faster along the axis

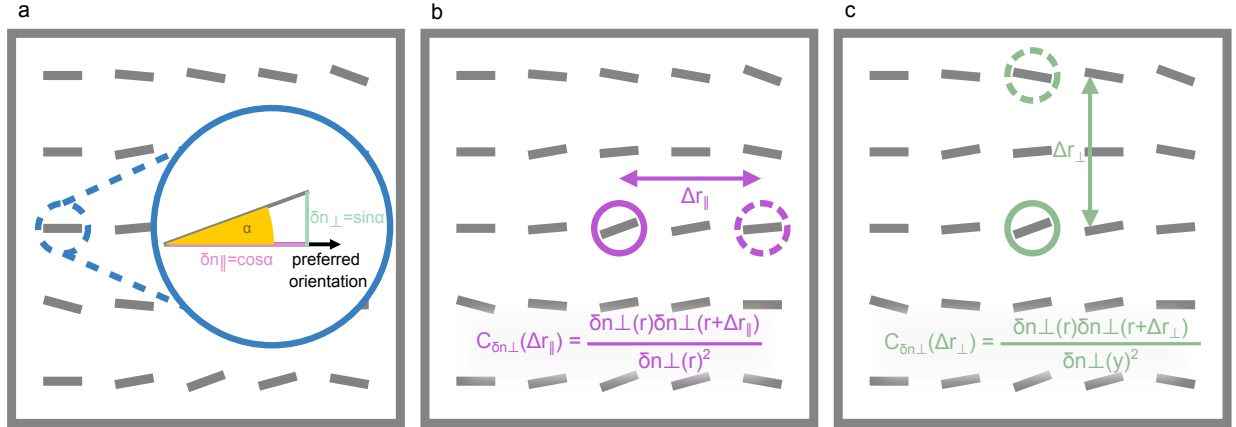


Figure 2.18: Definition of the correlation function of the deviation of the nematic director. (a) The deviation of the nematic director from the preferred orientation axis. (b) The correlation function of $\delta n_{\perp}$ along the axis parallel to the preferred orientation axis. (c) The correlation function of $\delta n_{\perp}$ along the axis perpendicular to the preferred orientation axis.

parallel to the flow than along the axis perpendicular to the flow. The shear stress induces a symmetry breaking, which generates a bias along the axis of the flow, resulting in faster growth of similarly aligned regions along that direction.

For the simulation data, the correlation lengths display a very similar anisotropic behavior than for the experimental data (Figure 2.19 e and f). This anisotropic behavior is also reflected in the correlation lengths (Figure 2.19 a and b). However, in the simulations, the correlation length along the axis perpendicular to the preferred orientation axis, $\xi_{\perp}$, did not increase, contrary to experimental observations. The correlation length along the axis parallel to the external aligning field, $\xi_{\parallel}$, on the other hand, evolved precisely as observed in the experimental data (Figure 2.19 g). The deviation observed for $\xi_{\perp}$ between simulations and experiments can be explained by an insufficient representation of the biological system in the theoretical model. Indeed, in the experiment, the cells continue to proliferate, which increases the cellular density. Moreover, with increasing alignment, the cells progressively elongate. The dynamic changes of those parameters have not been implemented into the theoretical model, which can account for the differences observed when comparing the simulations to the experimental results.

Although the simulation data along the axis perpendicular to the flow axis does not precisely match the experimental results, the theoretical model still reproduces the asymmetric growth behavior of similarly aligned regions observed in the cellular monolayer. Given the definition of the angle α together with the correlation functions, we chose to distinguish the similarly aligned regions by two distinct domains. In domain 1, the nematic director field forms an angle $\alpha > 0$ with the preferred orientation axis (Figure 2.20 a and b, brown

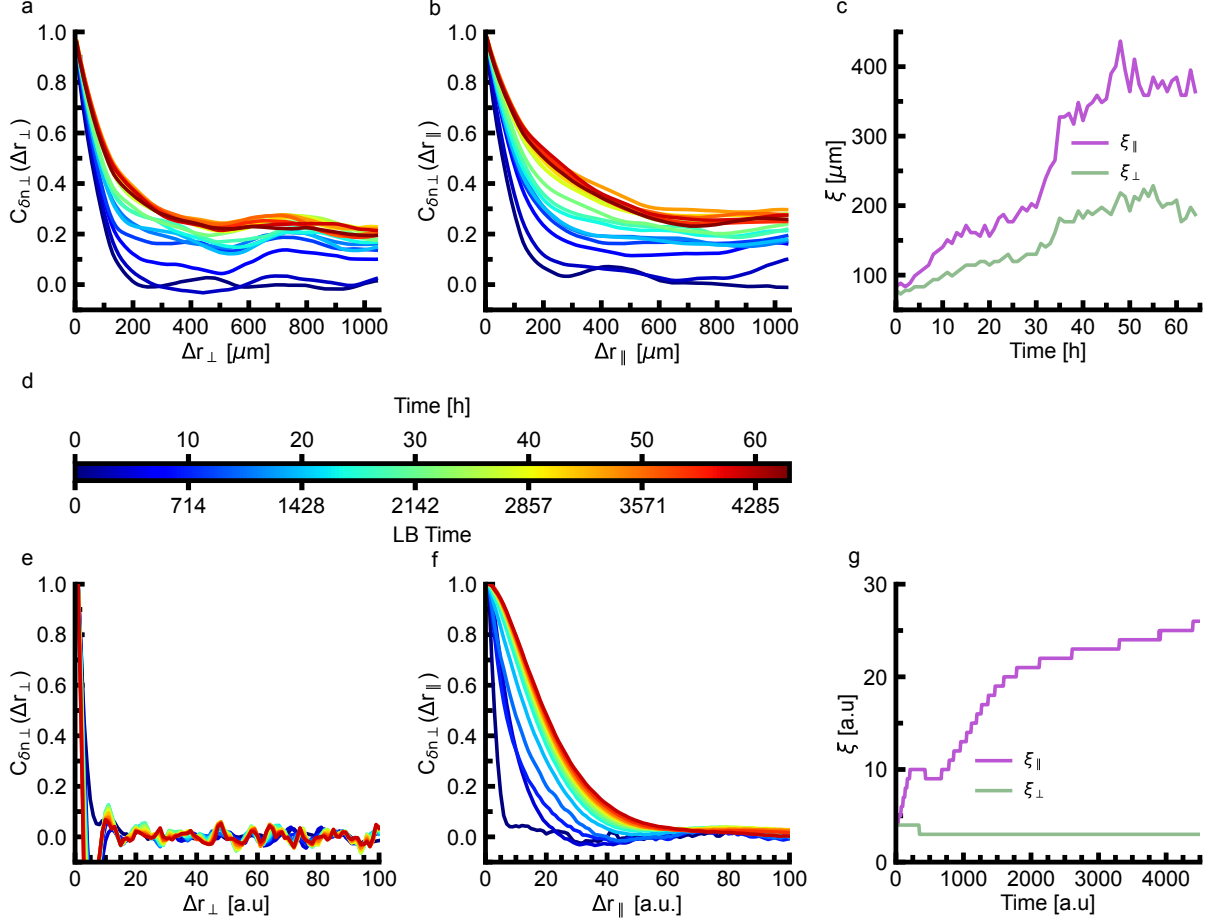


Figure 2.19: Correlation dynamics reveal anisotropic growth of similarly aligned regions. (a) The decay of the correlation function $C_{n_{\perp}}(\Delta r_{\perp})$ along the axis perpendicular to the preferred orientation axis for the experimental data. The different times are plotted according to a color code detailed in (d). (b) The decay of the correlation function $C_{n_{\perp}}(\Delta r_{\parallel})$ along the axis parallel to the preferred orientation axis for the experimental data. The color code representing the correlation function at different times throughout the experiment adheres to the same logic as in (a). (c) The evolution of the correlation lengths $\xi(t)$ along the axis perpendicular and parallel to the preferred orientation axis over time for the experimental data. (d) The color map for (a, b, e, and f) associates a time to the colors used in the plots displaying the correlation functions. (e-g) The corresponding figures to (a-c) for the simulation data.

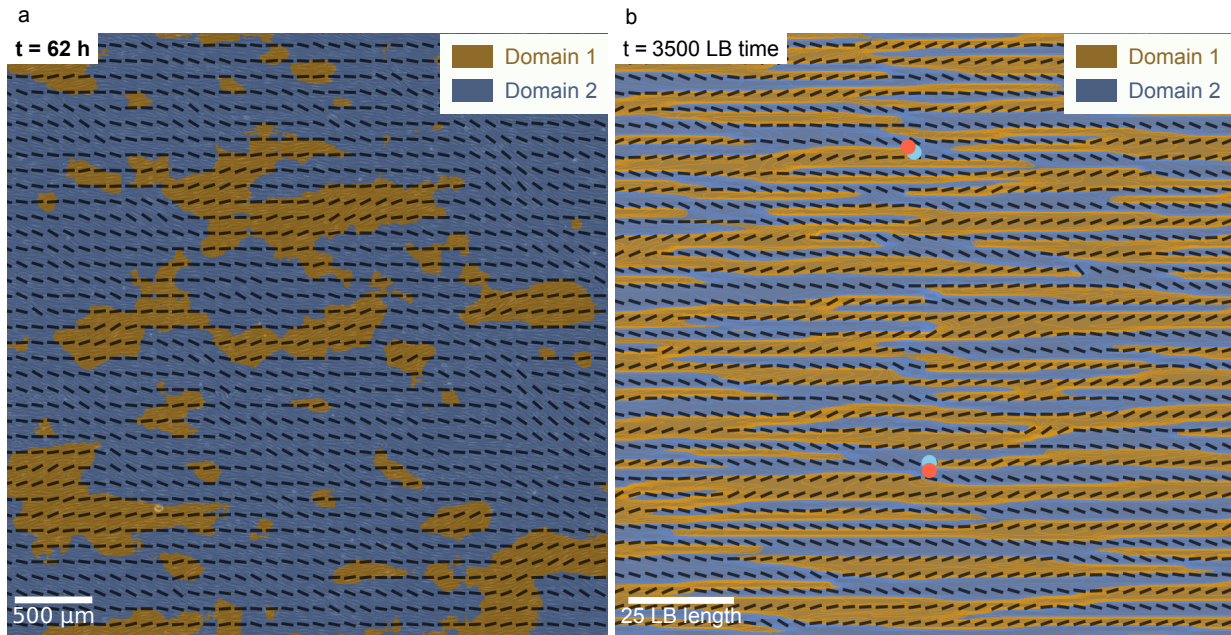


Figure 2.20: Asymmetric shape of the domains. (a) For the experimental system, the domains are further elongated along the axis parallel to the flow than along the axis perpendicular to the flow. The domain 1 (brown regions) is characterized by an orientation field with $\alpha > 0$, while in domain 2 (blue regions) the orientation field forms an angle $\alpha < 0$ with the preferred orientation axis. (b) For the simulation data, the anisotropy of the domains along the preferred orientation axis is more pronounced than in the experimental system.

regions). In domain 2, the angle formed by the nematic director field with the preferred orientation axis adheres to $\alpha < 0$ (Figure 2.20 a and b, blue regions). In both the experimental and the simulation data, the domains have a tendency to extend along the axis parallel to the flow direction rather than perpendicular to it, which reflects their anisotropic growth behavior. This effect is much more pronounced in the simulation data because of the abovementioned reasons (Figure 2.20 a and b). For the experimental data shown in this chapter, the cells tended to form an angle $\alpha < 0$ with the preferred orientation axis, displaying a bias towards domain 2 (Figure 2.20 a). Yet, in other experiments, the cells displayed a bias towards domain 1. From the experimental data alone, it is unclear what underlying mechanism provoked this bias towards one of the two domains.

Distinct growth behavior of the domains during the three different alignment stages: In a previous paragraph, we introduced the notion that the endothelial cells do not align monotonously in the direction imposed by the flow. More precisely, the alignment process is interrupted by a temporary misalignment, which motivated the distinction of the alignment process into three distinct phases. Interestingly, the coarsening of the domains also displayed three different phases characterized by distinct growth dynamics. To assess

the coarsening of the domains, we considered the time evolution of the correlation function together with the correlation length parallel to the flow axis $\xi_{\parallel}(t)$. For the experimental and simulation data, during the initial ordering phase (I), the correlation functions rapidly decayed to zero in agreement with the small size of the domains compared to later time points (Figure 2.19 e). During this phase, the domains grew exponentially which is reflected in the exponential increase of the characteristic correlation length $\xi_{\parallel}$ observed for the simulation data (Figure 2.21 d). Theoretical physics predicts that when a two-dimensional diffusive nematic system relaxes back to its isotropic state, its dynamics follow $\xi \propto t^{1/2}$ for the growth of its correlation length [88]. During the initial ordering phase (I), the growth behavior of the correlation length parallel to the axis of the flow, $\xi_{\parallel}$, adheres closely to those dynamics in the simulations (Figure 2.21 d). While for the experimental data, the growth dynamics of the correlation length parallel to the flow axis do closely follow the predicted power law, the data still suggest a rapid increase distinct from the growth dynamics observed in the other two alignment phases. The observed scaling dynamics of the correlation length suggest that the annihilation of the topological defects, reflected in the decrease of the defect density, does not significantly impact the coarsening dynamics of the domains. Additionally, during the initial ordering phase (I), the correlation functions did not collapse with the distance normalized by the correlation length $\Delta R_{\parallel}/\xi_{\parallel}$ (Figure 2.21 e). This is another indication that the ordering kinetics during this phase were not governed by long-range logarithmic interactions between the topological defects [89].

In the subsequent phase, the intermediate disordering phase (II), the correlation functions ceased to drop to zero for both the experimental and the simulation data, which indicated the establishment of long-range order (Figure 2.19 b and e). During this phase, the active stresses within the nematic director field increased, resulting in the nucleation of new topological defects. Consequently, during this phase, in the simulation data, the correlation length stopped growing, accompanied by a slight decrease (Figure 2.21 d). In the biological system, the growth of the correlation length significantly slowed down during this phase, reflecting the nucleation of new topological defects accompanied by an increase in cellular misalignment.

Eventually, in the last phase, the steady-state ordering phase (III), the correlation functions continued to converge towards a finite value reflecting the long-range order that has been established for the simulation data, as well as for the experimental data (Figure 2.19 b and e). Consecutively, the correlation length $\xi_{\parallel}$ resumed its growth. The collapse of the correlation function normalized by $r/\xi_{\parallel}$ indicated that the interactions between the topological defects affected the ordering dynamics in both the experiment and the simulation (Figure 2.21 c and f). Theoretical physics predicts that in such a case, the interaction of the nematic defects with each other introduces a logarithmic correction to the growth of the correlation length $\xi_{\parallel} \propto (t/\log(t))^{1/2}$ [89]. Therefore, we performed prolonged simulations to confirm that the growth of the domains followed the power law as predicted for kinetic ordering in passive materials (Figure 2.21 d). For the biological system, the limited available timespan made it challenging to confirm that the same scaling dynamics

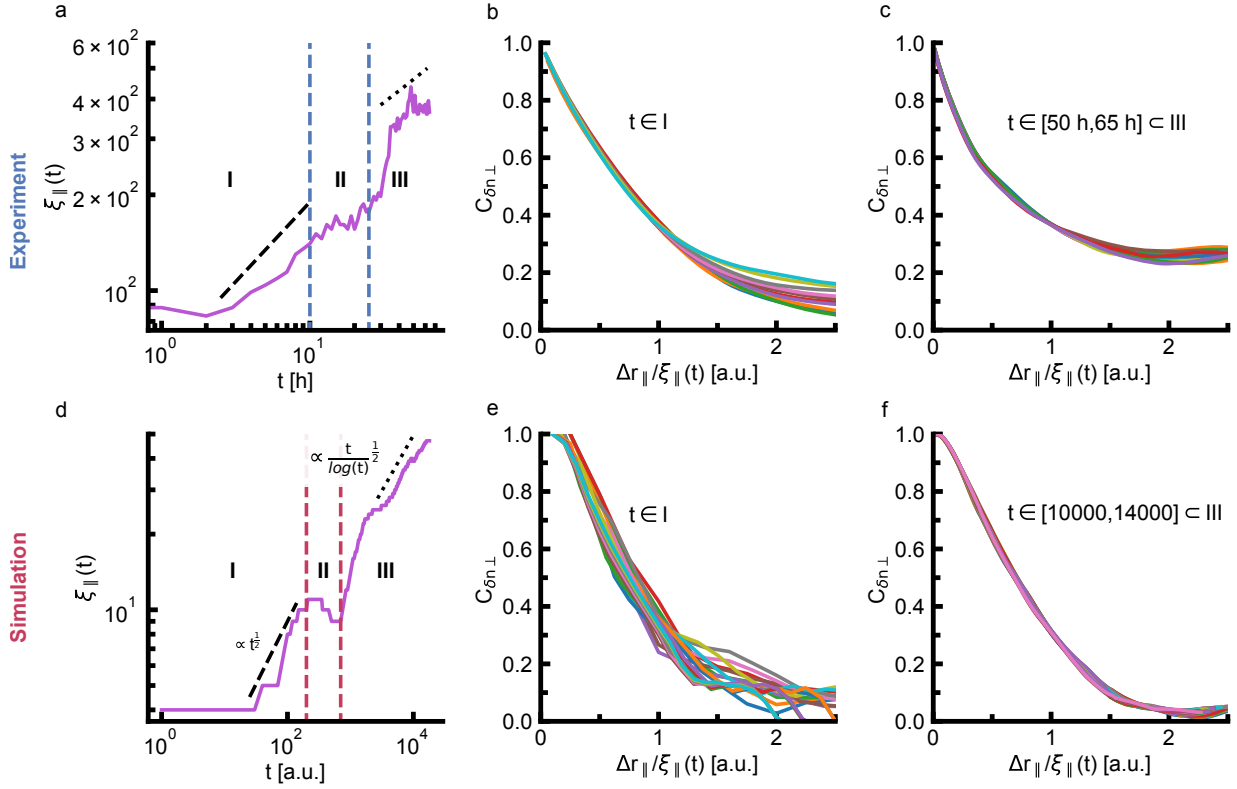


Figure 2.21: Growth dynamics of domains depend on the alignment phase. (a) For the experimental data, the growth of the domains is distinguished into three different stages that match the three distinct alignment phases. The dashed line highlights the rapid increase in the correlation length observed during the initial ordering phase. The dashed line shows the growth dynamics of the domains at late stages during the steady-state ordering phase (III), emphasizing the distinct growth dynamics observed during the first and third phase. (b) The correlation function for the experimental data normalized by the correlation length $\xi_{||}$ during the initial ordering phase (I). (c) The correlation function for the experimental data normalized by the correlation length $\xi_{||}$ for late times during the steady-state ordering phase (III) collapses. (d-f) The corresponding figures to (a-c) for the simulation data. In (d), the dashed and dotted lines, denote the $1/2$ -power law scaling and the $1/2$ -power law scaling with a logarithmic correction.

$\xi_{||} \propto (t/\ln(t))^{1/2}$ hold true. However, it is very clear that the growth of the correlation length $\xi_{||}$ follows different dynamics in the final phase compared to the two previous phases.

By definition, the distinct growth dynamics of the correlation length $\xi_{||}$ were also reflected in the growth dynamics of the domains. Indeed, during the initial ordering phase, the area of domain 2 increased significantly in only eleven hours, mirroring the stark growth of the correlation length $\xi_{||}$ during this phase (Figure 2.22, $t = 0$ h and $t = 11$ h). The active instabilities within the cellular monolayer catalyzed the nucleation of topological defects

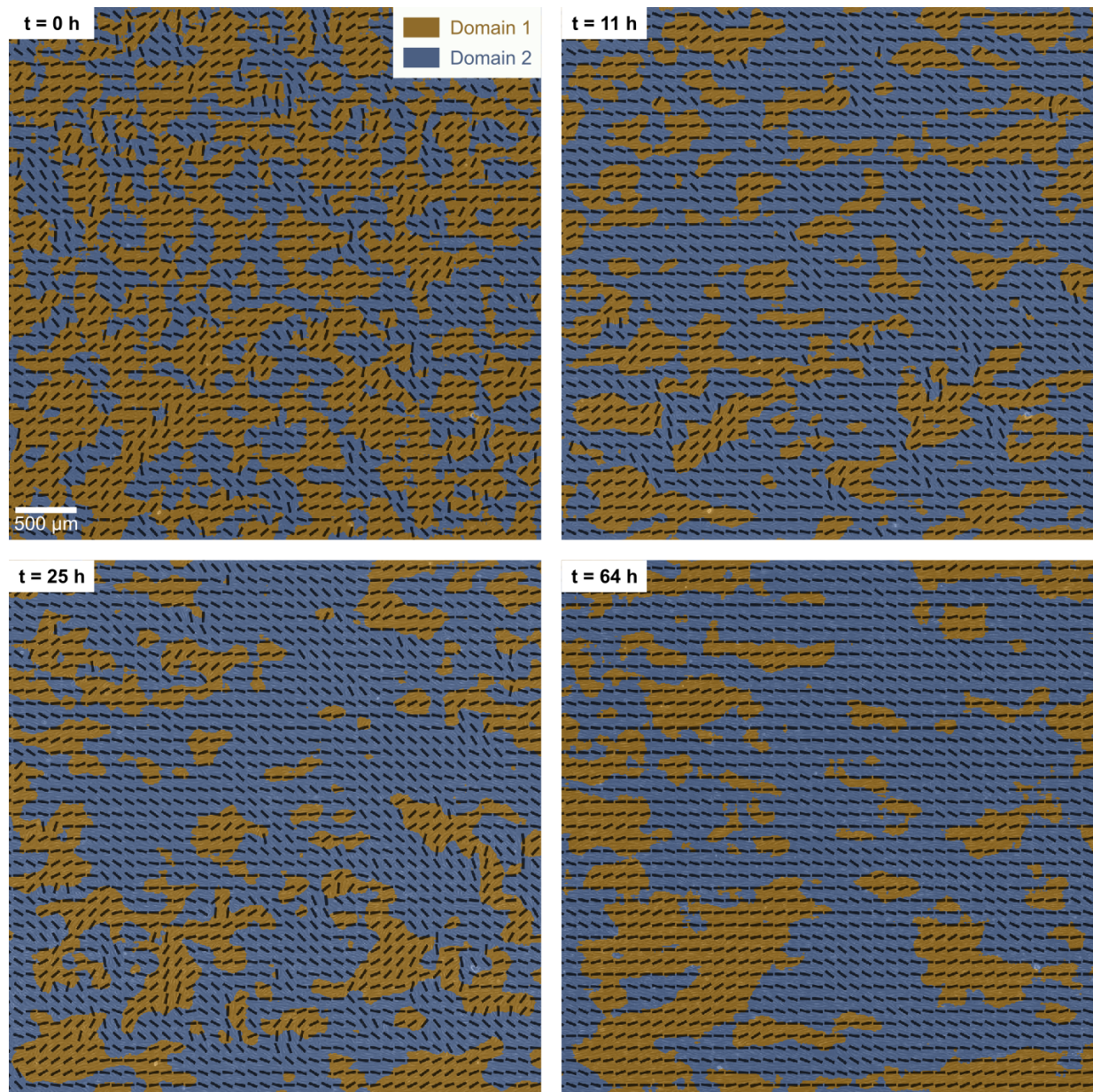


Figure 2.22: Distinct coarsening dynamics of domains. The distinct coarsening dynamics can be distinguished in the experimental data from the different growth rates of the domains. In the initial ordering phase (I), the area of domain 2 displays a stark increase from the onset of the flow, at $t = 0$ h, to the onset of the intermediate disordering phase (II), at $t = 11$ h. During the intermediate ordering phase (II), the size of the domains does not significantly increase nor decrease between the onset of the phase, at $t = 11$ h, to the end of the phase, at $t = 25$ hours. During the final phase, the steady-state ordering phase, the size of the domains only slowly increases. The domain size displayed only moderate growth from $t = 25$ h to $t = 64$ h compared to the growth rate during the initial ordering phase (I).

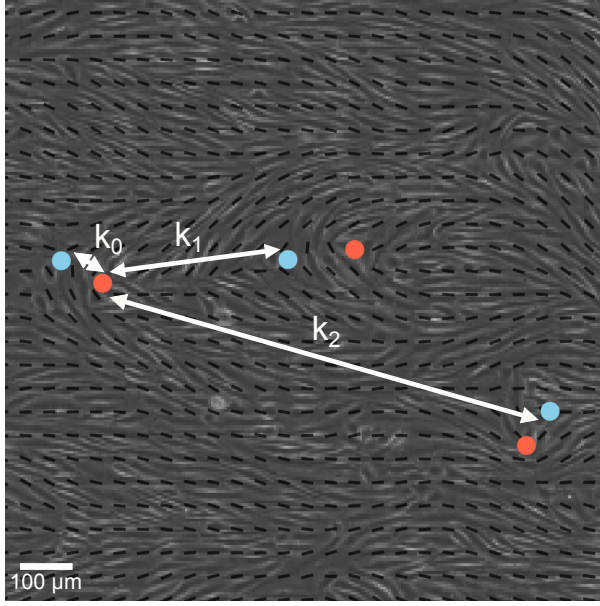


Figure 2.23: The distance to the k^{th} nearest neighbor. The distance to the nearest neighbor of opposite charge for the $+1/2$ defect is denoted by k_0 , k_1 represents the distance to the second nearest neighbor, and k_2 corresponds to the distance to the third nearest neighbor.

during the intermediate disordering phase, which halted the growth of the domains. Thus, the size of the domains remained approximately constant during this phase, as did the correlation length $\xi_{\parallel}$ (Figure 2.22, $t = 11$ h and $t = 25$ h). Eventually, during the steady-state ordering phase (III), the domains continued to grow. However, compared to the fast growth rate during the initial ordering phase (I), the domains only grew moderately in size. After 39 hours, the size of the domains increased only minimally compared to their significant growth during the first phase in less than ten hours (Figure 2.22, $t = 25$ h and $t = 64$ h).

Heterogenous distribution of defect pairs: The previous paragraph showed that the ordering kinetics during the steady-state ordering phase (III) were affected by long-range interactions between topological defects. In contrast, the ordering kinetics during the initial ordering phase (I) were characteristic of diffusive isotropic-nematic transitions. To better characterize the role defect interactions play during the alignment process, we decided to assess the spatial distribution of the defects throughout the alignment process. The large field of view, capturing the dynamics of the cellular monolayer, proved to be crucial in obtaining a statistically significant characterization of the spatiotemporal dynamics of the topological defects. During the onset of the intermediate disordering phase (II), active stresses present within the cellular monolayer lead to the nucleation of new topological defects. Interestingly, the nucleation sites of those defects were not homogenously distributed throughout the whole field of view but were locally restricted to certain areas. Defect nucleation often occurred in close proximity to already existing defects (Figure 2.14, $t = 25$ hours). Therefore, we measured the distance to the k^{th} nearest neighbors for oppositely charged defects (Figure 2.24). After a defect pair nucleated, the defects typically remained close to each other. From previous studies, we would expect that newly nucleated defect pairs would either move further apart from each other as expected in traditional active

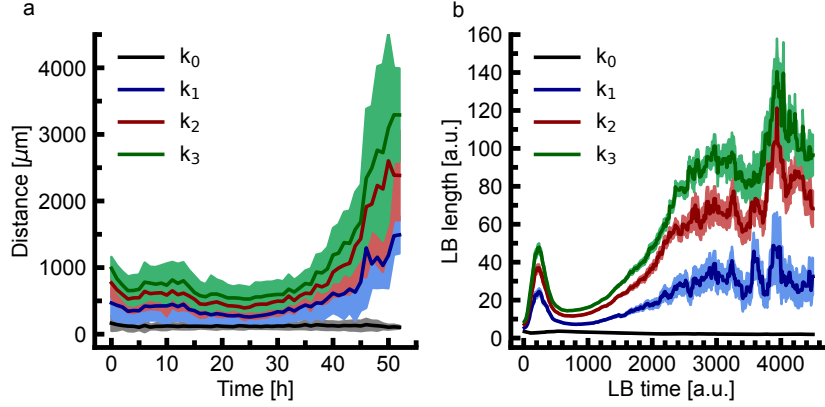


Figure 2.24: Heterogeneous distribution of defect pairs. (a) The time evolution of the distance between the k^{th} nearest neighbors for the experimental data. The brighter areas indicate the standard deviation from the mean for the corresponding color. (b) The time evolution of the distance between the k^{th} nearest neighbors for the simulation data. The brighter areas indicate the standard deviation from the mean for the corresponding color.

turbulence or instantaneously annihilate as observed in flow-tumbling active systems with external fields [60], [90]. However, throughout the coarsening of the system, the distance to the nearest neighbor of the opposite charge remained constant in both the biological system and the physical model (Figure 2.24 a and b, $k < 1$). Together, with the locally restricted nucleation of new topological defects during the intermediate disordering phase (II), this resulted in a heterogeneous distribution of topological defect pairs throughout the system. The defects were concentrated in specific areas. These areas of high defect density were intersected by regions where no defects were present (Figure 2.14, $t = 25$ hours). We identified these areas of high defect density as the domain boundaries between domain 1 and domain 2. The topological defects only existed along the interface between these two domains (Figure 2.25). Consequently, this constraint imposed the spatial heterogeneity observed in defect density. During the steady-state ordering phase, the defect pairs annihilated with each other. As the existence of the defects was restricted to the domain boundaries, the distance between the remaining nearest neighbors of higher order $k \geq 1$ increased as the system coarsened (Figure 2.24 a and b, $k \geq 1$, and Figure 2.25). Neither the simulations of an active nematic system without an external aligning field nor those of a passive nematic system with an external aligning field were able to reproduce the spatial heterogeneity in defect distribution observed in the experiment. This indicates that the spatial heterogeneity of defect density results from the complex coupling between cellular activity, the superjacent flow field, domain interfaces, and the topological defects existing on them.

String excitations between topological defect pairs: Even as more and more defect

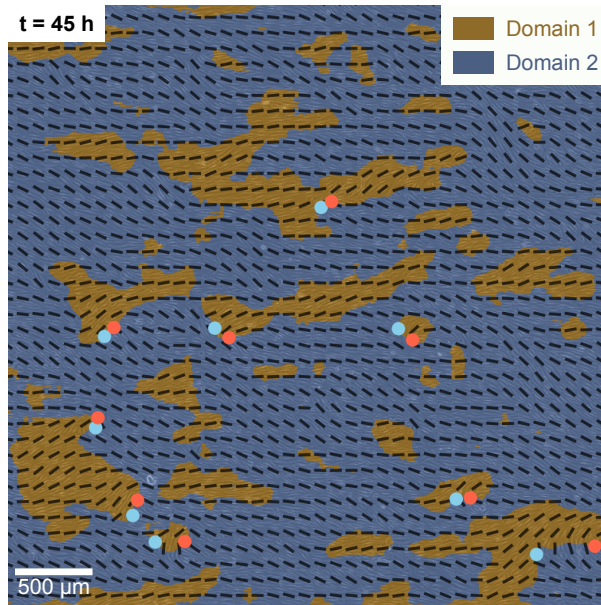


Figure 2.25: Nematic defects only exist on domain boundaries. The nearest neighbor pairs remain in close proximity to each other, while the distance to the second nearest neighbor increases during coarsening.

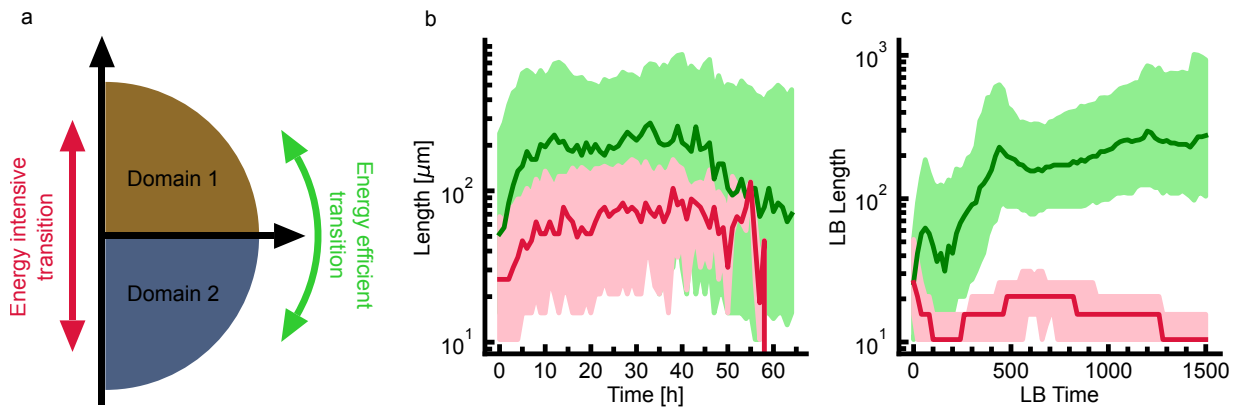


Figure 2.26: Energetic cost of misaligned regions caused by the superjacent flow. (a) The transition from one domain to the other can be energy intensive, indicated by the red arrow, or energy efficient, indicated by the green arrow. (b) The average length of the strings with perpendicular orientation (red) decreases to zero as the system coarsens. The length of the green string initially increases and eventually plateaus during coarsening for experimental data. The brighter areas illustrate the interquartile range for data of the corresponding color. (c) The average length of red and green the strings for the simulation data. The brighter areas illustrate the interquartile range for data of the corresponding color.

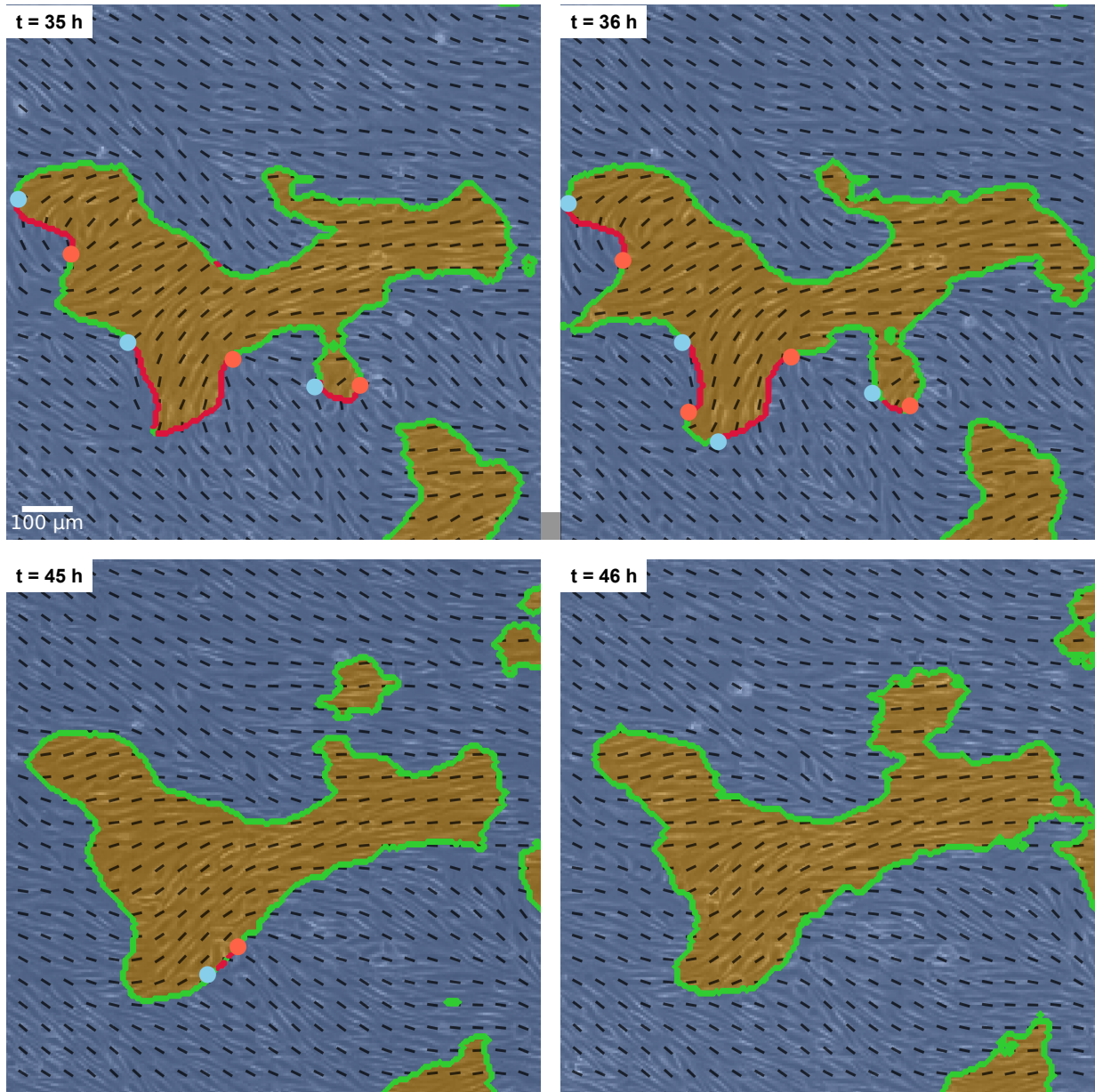


Figure 2.27: Topological defects interact through string excitations. Topological defects reside on the domain boundaries. They are connected by alternating patterns of energy-efficient (green) and energy-intensive (red) strings. When a new defect pair is created, they are connected to each other by a string, as well as to their immediate neighbors on the domain boundary ($t = 35 \text{ h}$ to $t = 36 \text{ h}$). Defect annihilation contributes to eliminating the red strings ($t = 45 \text{ h}$ and $t = 46 \text{ h}$).

pairs annihilated, the distance between the nearest neighbors $k < 1$ remained constant, indicating that throughout the entire alignment process, those defect pairs remained bound (Figure 2.24 a and b, $k < 1$, and Figure 2.25). Typically, in classic active nematic systems, a newly formed defect pair either annihilates or quickly separates driven by the intrinsic activity of the system [60]. In contrast, for the biological system and the physical model, the distance between the nearest neighbors remained constant throughout the alignment process. In the cellular monolayer, the distance between nearest neighbors measured $\approx 123 \pm 11 \mu\text{m}$. This was equivalent to up to 11 cells separating the defect pairs from each other. Contrary to most active nematic systems, the intrinsic activity of the cellular monolayer was insufficient to disrupt the topological defect pairs. They remained bound throughout the steady-state ordering process long after defect nucleation occurred. Notably, only the combination of an active nematic system and a superjacent flow gave rise to this particular defect constellation. Therefore, we explored whether the enhanced stability of these defect pairs arose from the complex interaction between cellular activity and the external flow-aligning field. The applied flow imposes an orientational constraint on the cells, clearly favoring an orientation that points parallel to the flow axis. The topological defects are produced by local areas of misalignment and sit at the boundaries between the two domains. Assuming that only one defect pair resides on the domain boundary, there are two possible ways to connect these defect pairs. One possible connection is through the misaligned region. The superjacent flow associates an energetic cost for deflecting from the preferred orientation axis. Consequently, this transition is energetically costly to the system. Therefore, it is called the energy-intensive transition (Figure 2.26 a). The other option is to transit through the almost perfectly aligned region to move in between the defects. Along this trajectory, the nematic directors only deviate slightly from the preferred orientation axis. Therefore, this transition is called the energy-efficient transition (Figure 2.26 a). The energy-intensive transition represents the path along the nematic directors with the maximum gradients with respect to the preferred orientation axis, while the energy-efficient transition corresponds to the path with the minimum gradients. Interestingly, we found that these strings perfectly connected the topological defects with each other while simultaneously running along the domain boundaries (Figure 2.27). The red string represents the energy-intensive transitions, and the green strings represent the energy-efficient transitions. As the red strings indicated areas of local misalignment and were associated with an energetic cost, we expected the length of the red strings to decrease over time as the system coarsened. Indeed, the average length of the red strings dropped to zero as long-range order was established for the experimental and the simulation data (Figure 2.26 b and c). We expected the average string length to gradually increase during coarsening, but it decreased. We attributed this trend to the nucleation of small domains by splitting events from bigger domains (Figure 2.27, $t = 45 \text{ h}$). Moreover, we observed a continuous growth of domain 2, resulting in the decrease of the interface between the two domains. This observation likely contributed to the measured reduction of the average length of the green strings.

The external flow provides a physical significance to the different strings connecting the

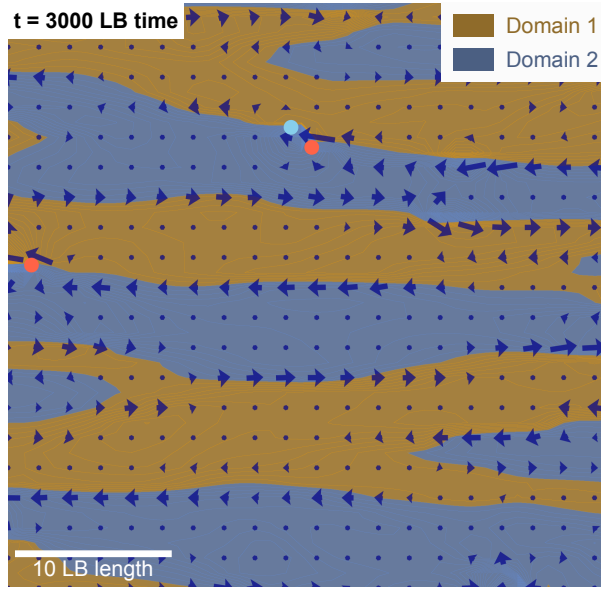


Figure 2.28: String excitations as a potential path for long-range force transmission. The force field obtained for the nematic director field from the simulation. The blue arrows represent the forces. The forces are highest along the domain boundaries.

defects. Commonly, an isoline represents a path along which the nematic director field remains constant. Without an external aligning field, there exists an infinite number of possibilities for connecting two defects of opposite charge via isolines. However, none of these paths have an associated physical constraint that would allow one to distinguish one isoline from the other. In our system, the superimposed flow provides physical significance by penalizing transitions from domain 1 to domain 2 via the red strings, as these transitions involve an energy-intensive dealignment from the preferred orientation axis. Indeed, topological defects of opposite charge were connected with each other by red and green isolines. Although the nucleation of new defect pairs disrupted existing isolines, it continued to respect the existing pattern of connecting oppositely charged defects through alternating red and green strings (Figure 2.27, $t = 35$ hours and $t = 36$ hours). The interactions between oppositely charged defects occurring along the red and green strings stabilized the defect pair conformations until the late stages of the steady-state ordering phase (III). Additionally, the defects generally only moved along the isolines (Figure 2.27). Thus, the isolines mediated defect annihilation. During the steady-state ordering phase, defect pairs annihilated, which simultaneously eliminated the energy-intensive isolines (Figure 2.27, $t = 45$ hours and $t = 46$ hours). After all the defects annihilated in the experimental system, only the green string remained, fully extending along the domain boundaries. From the simulation data, it was possible to interfere the distribution of forces along these strings. The simulation data showed that the forces were highly concentrated along the domain boundaries. The localization of the forces to the strings, together with their extensive length, may provide a mechanism for long-range transmission of mechanical forces in such systems. Unfortunately, the current experimental setup did not allow the measurement of cell-substrate interactions. To measure the mechanical forces exerted by the cells on the substrate it would be necessary to introduce a polyacrylamide substrate in the flow channel

to perform traction force microscopy.

2.5 Conclusion and Outlook

Impact: The extensive field of view allowed to globally resolve the dynamic response of HAOECs to exposure to shear stress. The HAOECs did not monotonously align in flow direction but adjusted their orientation to the flow following a three-stage alignment process with an intermediate phase of misalignment. The results obtained from simulations of an active nematic system matched the experimental observations and highlighted the importance of the interplay between cellular activity and the external alignment field for the ordering process. The coupling between those two parameters catalyzed the nucleation of new topological defects and temporarily disrupted cellular alignment. Therefore, the framework of active nematic liquid crystals has helped to understand the underlying mechanisms giving rise to the intermediate disordering phase. Together with simulations, we defined a phase diagram that correctly captures the distinct alignment dynamics of an active nematic system depending on the balance between the external alignment field and the intrinsic cellular activity. Those findings reconcile previously contradictory observations that reported temporary orthogonal alignment of endothelial cells exposed to elevated, yet physiological, shear stress levels $\pi > 20$ dyn/cm² [6]–[9].

Future directions: During the alignment process, the topological defects of opposite charge formed highly stable defect conformations. These defect pairs persisted over an extended period, which is very uncommon for classic active nematic systems [90]–[92] and impacted the ordering dynamics during the steady-state ordering phase. These stable defect constellations were maintained by the interaction of the external flow aligning field with the intrinsic activity of the cellular monolayer. We identified strings extending between topological defects of opposite charge reinforcing the defect constellations. We understood those strings as the biological analogs of the pair-superfluid phase predicted for two-dimensional antiferromagnetic condensates under a magnetic field [93]. The strings described in classical XY models of magnetism exhibit both nematic and polar interactions. Besides the logarithmic Coulomb potential, those strings mediate a linear interaction potential between the defects. The linear interaction potential can be understood as a line tension acting between the defects with a strength that is proportional to the string length [93]–[95]. Indeed, the simulation data showed that the mechanical stresses within the active nematic system localized around those strings. Although we could not confirm this observation in the biological system, our findings show the relevance of such string excitations in mediating mechanical forces within a cellular monolayer. Typically, in the HAOEC monolayer, the strings spanned several hundreds of micrometers and may provide a mechanism for long-range force transmission over dozens to hundreds of cells. Previously, topological defects have already been reported to act as local hubs for mechanotransduction [3]. Yet, our data suggests that the interaction between nematic defects through topological string

excitations could control mechanotransduction pathways on a global scale. Therefore, further experiments are required to investigate how topological defects impact the local and global gene expression profiles within cellular monolayers. The relevance of these findings is not only restricted to the ordering dynamics of endothelial cells under flow but may be important in a different context. Notably, shear stress can also arise from two tissue layers migrating against one another, as reported for ectoderm and mesoderm progenitors observed during gastrulation in zebrafish embryos, where it contributes to embryo development [96], [97]. Moreover, recent studies identified a nematic phase during embryogenesis across multiple species, along with the observed presence of nematic defects during this process. This highlights the potential significance of topological defects in development [98]. Thus, our findings may set a benchmark for a wide range of biological phenomena in morphogenesis, tissue remodeling, and disease progression, where spatiotemporally localized mechanical effects have consequences over long distances and time scales.

Chapter 3

Microfluidic chip for angiogenesis

Introduction

The previous chapter demonstrated the importance of topological defects in modulating collective cell dynamics within a cellular monolayer. Similarly, previous studies recognized the central role of nematic defects in promoting specific morphological events such as the formation of protrusions. Additionally, in vivo, nematic defects have already been reported to occur at distinct morphological features, particularly at bifurcation points. Therefore, we sought to develop a microfluidic chip to investigate endothelial cell dynamics during angiogenesis with the ultimate goal to establish a co-culture system to investigate vascularization of organoids. This chapter will first provide a brief overview of the biology underlying angiogenesis, followed by a summary of the current advancements in vascularized organoids. Subsequently, we will present the design of a microfluidic chip conceived as a minimalistic model to study angiogenesis, primarily relying on mechanical stimulation through interstitial flow and a gradient of VEGFA.

3.1 Sprouting angiogenesis

Sprouting angiogenesis: Blood vessel formation occurs through very distinct processes [99]. The formation of new blood vessels, also called angiogenesis, is initiated through a process called the angiogenic switch. New tissue can only form up to a volume of $\approx 1 \text{ mm}^3$ without a deficit in oxygen and nutrient availability [99]. An insufficient supply of oxygen and nutrients triggers the release of proangiogenic growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which promotes the vascularization of the unvascularized tissue [99]–[101]. Sprouting angiogenesis is the fastest process of new blood vessel formation and, thus, the predominant mechanism responsible for the vascularization of newly formed tissue [99], [102]. During sprouting angiogenesis, new capillaries are established from pre-existing blood vessels. It occurs in a physiological and pathological context [99], [100], [103]. Physiological conditions during which sprouting angiogenesis takes place are embryonic development or wound healing, and pathological

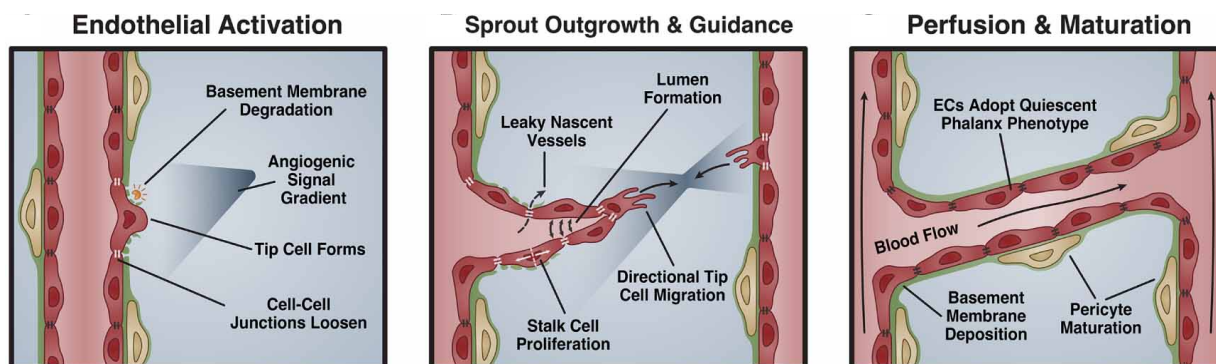


Figure 3.1: Sprouting Angiogenesis Tissue with a deficit in oxygen and nutrient supply will release proangiogenic stimuli that induce tip cell selection. A specialized endothelial tip cell with an enhanced migratory phenotype will invade the tissue leading the stalk cells toward the origin of the angiogenic stimuli. Polarization of the stalk cells creates a lumen, which will mature after two EC tip cells anastomose. Graphic adapted from [104].

conditions could be diabetes or cancer [99], [100]. During sprouting angiogenesis, a quiescent endothelial cell is activated through a gradient of angiogenic growth factors [99], [101]. Those angiogenic growth factors, such as FGF and VEGF, initiate the specialization of one endothelial cell into a tip cell [99], [100]. This tip cell displays a specialized phenotype characterized by the enhanced secretion of metalloproteinases involved in the degradation of the extracellular matrix and the basement membrane [100], [101]. Additionally, tip cells also form filopodia and lamellipodia that mediate their migration towards the source of the angiogenic signal [100], [101], [103]. Typically, tip cells are characterized by a reduced proliferation rate [101], [103]. The stalk cells migrate behind the invading tip cell and, contrary to the tip cell, are characterized by an increased proliferation rate to guarantee the elongation of the nascent blood vessel [99], [101], [103]. The polarization of the stalk cells leads to lumen formation, and an unperfused nascent blood vessel is established [100]. When two tip cells from distinct nascent blood vessels encounter each other, anastomose occurs, and a perfused blood vessel is established [101]. The perfusion of the freshly formed blood vessel ensures its continued maturation and stabilization through the recruitment of pericytes and the deposition of basement membrane [101], [103]. The sufficient supply of the newly formed tissue with oxygen and nutrients stops the release of proangiogenic stimuli, allowing the endothelial cells to return to a quiescent state.

3.2 Vascularized Organoids

In vivo vascularization of organoids: In vivo, newly formed tissue requires a well-established vascular network, as the availability of oxygen and nutrients is restricted by diffusion to a few hundred micrometers from capillaries [105], [106]. In vitro, organoid

culture applications experience a similar issue. Organoids closely recapitulate the morphological complexity of *in vivo* tissue and, in some cases, even display functionality similar to *in vivo* organs. Therefore, organoids could offer significant improvements in regenerative medicine. Nevertheless, the insufficient vascularization of 3D organoids still limits the size and complexity achieved with organoid culture. To overcome these constraints and to thrive towards organoid culture systems with a highly realistic resemblance to *in vivo* tissue and organs, many efforts have been dedicated to establishing vascularized organoid culture systems [106]. Vascularization of organoids can be achieved using a broad spectrum of techniques. Those techniques are distinguished by *in vivo* and *in vitro* applications. *In vivo* vascularization of organoids describes the vascularization of *in vitro* grown tissue after transplantation into a host animal [105], [107]. Although the vasculature maturation achieved with this approach surpasses what can be obtained with *in vitro* applications to date, this approach is very work-intensive. Therefore, it suffers from low throughput and poor scalability, which makes it unattractive for high-throughput applications such as drug screening [106], [107]. *In vitro* applications commonly offer a markedly better throughput. To achieve vascularization *in vitro*, the distinct methods rely upon the cells' ability to self-assemble or on a templating approach where cellular assembly into a vascular network is controlled via a scaffold [105].

Templating approach: The scaffold used in the templating approach can be as simple as a hollow channel formed by removing a needle from a polymerized hydrogel matrix. Endothelial cells seeded into this channel can form a lumen, giving rise to a small blood vessel [108]. More sophisticated scaffolds can be generated with 3D bioprinting [104]. Combining distinct bioinks allows the printing of tissues interwoven with a complex vascular network. While 3D printing can rapidly generate 3D cellular structures that outperform conventional organoid culture regarding the size of the construct [104], it also faces several limitations. Not only is 3D printing technologically complex, but it also merely guides the cellular assembly into predefined structures. As a result, 3D printed tissues often lack the capacity for tissue remodeling with limited formation of smaller capillaries beyond the pre-printed vessels [104], [105]. Conversely, techniques relying on cellular self-assembly usually achieve a finer vasculature, but are more limited regarding their throughput. Four of these methods will be presented in the following paragraphs.

Organoid co-culture with vascular cells: Another approach to achieve vascularized organoids exploits the potential of endothelial cells to self-assemble into a vascular network under the correct biochemical and mechanical cues when seeded in a hydrogel matrix. The organoid is cultured separately, and only after the desired maturity is achieved, the organoid is mixed with endothelial cells, together with other cell types such as fibroblast, smooth muscle cells, or pericytes that secrete growth factors to help with the formation of a vasculature [106]. Pham et al. coated brain organoids derived from induced pluripotent stem cells with matrigel containing endothelial cells. iPSC-derived brain organoids can be cultured for up to a year. However, their growth slows down after a few months of culture because the organoids reach a size of 200-400 μm where sufficient oxygen supply cannot be

guaranteed throughout the organoid. Therefore, Pham et al. coated the brain organoids with endothelial cells after 34 days of organoid culture. To further enhance the maturation of the immature vasculature, they transplanted the organoids with the matrigel coating into mice for another 2 weeks, where the organoid grew to a size of 2 mm and achieved good vascularization [105], [109], [110]. The transplantation step into a host animal was required as the mechanical cues in vitro are insufficient to establish a fully matured vasculature [106], [110], [111].

Co-culture of vascular organoids with organoids: Vascularized organoids can also be achieved by culturing the organoid and the endothelial cells separately. The vascular organoid and the tissue organoid are then assembled at a later stage [106], [107], [112]. Oh et al. cultured vascular networks formed by human dermal microvascular endothelial cells and human glioblastoma spheroids separately. After the endothelial cells assembled into a vasculature, Oh et al. introduced the tumor spheroid, which was positioned on top of the network of blood vessels using a membrane with micropores. In this study, the authors successfully showed that the tumor spheroids triggered sprouting angiogenesis. The newly formed microvessels extended towards the tumor, and the cancer cells migrated towards the vessels, forming a capillary bed [113]. In a recent publication, Shaji et al. demonstrated the vascularization of a cerebral organoid using a similar approach. To ensure robust vascularization of the brain organoid, the authors developed a cocktail of growth factors that significantly enhanced sprouting angiogenesis [111].

Co-differentiation of vascularized organoids: Patient-derived organoids from human pluripotent stem cells hold great potential for personalized medicine as they allow for in-vitro drug screening and the possibility of tailoring the treatment to each individual. However, stem cell-derived organoids still lack sufficient maturity because of poor vascularization, which limits their growth. To obtain a vascularized organoid from human pluripotent stem cells, the correct biochemical cues need to be precisely timed to tightly control the simultaneous differentiation of stem cells into both vascular cells and tissue-specific cells [106]. Abilez et al. combined precisely timed growth factor administration with micropatterning to achieve cellular self-assembly of spatially organized cardiac vascular organoids differentiated from human pluripotent stem cells [106], [114]. It can be very challenging to correctly time the addition of growth factors to achieve the desired heterogeneous cell culture population [115]. Therefore, another approach consists of controlling the expression of transcription factors to reprogram already differentiated cells within the organoid [106]. Cakir et al. achieved vascularized human cortical organoids by targeting the expression of the transcription factor ETV2 in 20% of the organoid population [106], [116]. They showed that this approach provides consistent results over a broad range of different differentiation conditions [116].

Vascularized organoids on a chip: Seizing the available biological tools, such as combining different cell types and using growth factors to guide cellular behavior, allows the generation of vascularized organoids. However, biochemical stimuli alone are insufficient

to achieve a fully mature vasculature. For further maturation, immature blood vessels require additional mechanical stimuli [110], [112]. Many of the approaches presented above would require transplantation into a host animal to achieve enhanced maturation of the vasculature, which would drastically decrease the throughput [105], [107], [110]. Microfluidic devices have been engineered to act as the interface between the biological system and the experimental setup to effortlessly administer mechanical stimuli, such as blood flow. Microfluidic chips can be connected to pumps that allow the tight control of experimental parameters, such as perfusion with cell culture medium. Previous studies used microfluidic chips as a model system to investigate vasculogenesis and angiogenesis. Endothelial cells seeded in fibrinogen were exposed to a gradient of growth factors secreted by fibroblasts to trigger cellular self-assembly into a vascular network [117], [118]. Recently, more and more studies have focused on establishing vascularized organoid systems using microfluidic chips [119], [120]. Arslan et al. achieved the formation of a vascularized cardiac microtissue with beating contractions that transported microbeads through the perfusable microvessels through differentiation from human pluripotent stem cells within a microfluidic chip. These human-derived model systems are particularly promising as they offer the scalability and biological relevance required for disease modeling [119]. Recently, besides demonstrating the feasibility of vascularization for different types of organoids, the focus has started to shift toward setups that allow robust downstream analysis. Lee et al. developed a protocol enabling transcriptomic analysis of sprouting microvessels, allowing the distinction between tip and stalk cells by single-cell RNA-sequencing [121]. Similarly, Quintard et al. built a peelable microfluidic device that provides access to the vascularized organoid for any downstream analysis [122].

3.3 Angiogenesis on a chip

Co-culture with different cell types: Endothelial cells seeded on matrigel can self-assemble into a tubular network [123]. Similarly, when seeded into a fibrinogen hydrogel, endothelial cells can self-organize and form microvessels. This process is called vasculogenesis. During angiogenesis, endothelial cells give rise to new nascent microvessels by forming angiogenic sprouts from an already existing vessel [108], [110]. In most applications involving microfluidic devices, the pre-existing blood vessel is the microfluidic channel coated with endothelial cells. The endothelial cells line the wall of the microfluidic channel, and the sprouting angiogenesis occurs in a neighboring microfluidic chamber filled with hydrogel [110]. Many different endothelial cells display the capacity for angiogenesis [113], [120], [124]–[126], while the most commonly used type of endothelial cells is human umbilical vein endothelial cells (HUVECs) [111], [117], [118], [127], [128]. Although endothelial cells can form sprouting angiogenesis stimulated by mechanical and biochemical signals, co-culturing endothelial cells with stromal cells significantly enhances the stability of the lumen formed by the microvessels [108], [110]. Especially in applications that require a prolonged cell culture period, the presence of stromal cells is essential to the maintenance of stable microvessels [110], [120]. Stromal cells such as fibroblasts have been shown to interact with

endothelial cells by the secretion of growth factors, metalloproteinases, tissue inhibitors of metalloproteinases, and the deposition of fibronectin and collagen to help with maturation of blood vessels [108], [128]. Moreover, cancer-associated fibroblasts have been reported to interact mechanically with endothelial cells to enhance the formation of angiogenic sprouts [129]. The presence of stromal cells is only crucial during the initial phase of microvessel establishment; after a functional vascular network has been formed, the depletion of stromal cells does not negatively impact the vasculature [128]. Stromal cells are not required to be in the same microfluidic chamber as the endothelial cells for microfluidic chips of moderate height ($\approx 100\text{-}200\ \mu\text{m}$); they can also be spatially separated from endothelial cells [128]. Indeed, many microfluidic chips integrated an additional channel for the stromal cells [111], [113], [117], [118]. However, for setups with an increased height, the co-culture of endothelial cells and stromal cells in the same hydrogel chamber is beneficial [120], [128].

Matrix composition: The ability of endothelial cells to establish a vascular network within hydrogel depends strongly on the type of the hydrogel. While endothelial cells have been widely reported to form microvessels in fibrinogen [113], [118], [122], [128], [130] or collagen hydrogels [125], [127], they fail to establish microvasculature in matrigel [110], [131]. Fibrinogen or fibrin is a naturally occurring hydrogel. It plays a crucial role during wound healing as it stops the bleeding and provides structural support for angiogenesis to occur [132]–[135]. During later stages of wound healing, collagen is deposited mainly by stromal cells to restore the initial tissue and close the wound [136], [137]. While collagen has been reported to display weaker angiogenic capacity, the mixture of collagen and fibrin shows strong angiogenic potential [137]. Indeed, many applications use a mixture of fibrinogen and collagen for its strong potential to induce angiogenesis as this is not only relevant in the context of wound healing but also faithfully recapitulates the environment formed by solid tumors [111], [117], [138]. Increasing the density of both fibrin and collagen reduces the invasion depth of angiogenic sprouts while at the same time decreasing the number of single invading cells and increasing the number of multicellular invading sprouts [139].

Mechanical stimuli: Besides the matrix composition, mechanical stimuli such as interstitial flow have also been shown to enhance angiogenic sprout formation. In vivo, the interstitial flow is caused by the flow generated through vascular leakage and lymphatic drainage. The interstitial flow typically has values ranging from $\approx 1\text{-}10\ \mu\text{m}$, and its biological function is the transport of nutrients and signaling molecules and the removal of waste [108]. In microfluidic chips, the interstitial flow has been shown to promote and direct sprout formation [118], [140]. The endothelial cells will grow toward the source of the interstitial flow. Kim et al. showed that the strength of the interstitial flow correlates with the length of the invading sprouts [140]. While interstitial flow can promote the outgrowth of microvessels, reversing the interstitial flow has the opposite effect and leads to a regression of the formed microvessels [141]. A pressure gradient commonly creates an interstitial flow in a microfluidic chip. A pressure gradient can be achieved by imposing a velocity gradient in the two opposite side channels flanking the tissue chamber or by filling two opposing reservoirs with a different amount of medium [118], [127]. Most applications

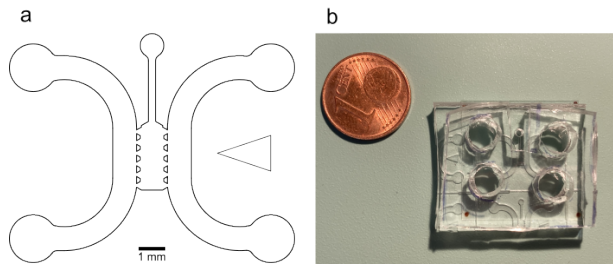


Figure 3.2: Angiogenesis chip.

(a) CAD drawing of the angiogenesis chip.
 (b) The assembled microfluidic chip with an additional top layer of PDMS to increase the capacity of the medium reservoir.

prefer the approach with differently filled medium reservoirs that generate a hydrostatic pressure to give rise to the interstitial flow, as this does not limit throughput and scalability. Although the setup proposed by Chang et al. offers better control over the magnitude of the interstitial flow and avoids the need for reservoir refills every 12 hours [118], the connection to a microfluidic pump that can operate at such low velocities also limits the number of microfluidic chips that can be processed simultaneously. It adds an extra layer of complexity to readouts, such as live cell imaging of angiogenic processes.

Aim of this study

Fibroblasts conditioned medium can exhibit variability in the secreted growth factors which can negatively impact reproducibility. To address this, we established a minimal model for angiogenesis driven primarily by an interstitial flow as an external stimulus, along with a gradient of VEGFA. Most angiogenesis chips achieve vascularized organoids by placing spheroids on top of the hydrogel chamber, typically at least $200\ \mu\text{m}$ from the bottom of the slide. This makes it challenging to image the vascularized organoid at higher magnifications. Additionally, as the spheroids are only placed on top of the gel, they may fail to provide critical mechanical cues to the angiogenic sprouts due to a lack of interaction with the hydrogel. To overcome this limitations, we designed a microfluidic device with an increased height of $\approx 300\mu\text{m}$ to provide sufficient space for accommodating a branched organoid.

3.4 Chip design

To ensure good scalability and parallelization, the microfluidic chip was designed so that the interstitial flow arises from the hydrodynamic pressure of differently filled medium reservoirs¹. Three parallel channels are separated by micropillars that enable filling the center channel with hydrogel to create the tissue chamber (Figure 3.2 a). To increase the volume capacity of the medium reservoirs, we added an additional layer of PDMS (Figure 3.2 b). To maintain good accessibility to the tissue chamber's inlet, the top PDMS layer is

¹The design was developed with the support of Jean Carlos Serrano Flores from the Weitz Lab at Harvard University.

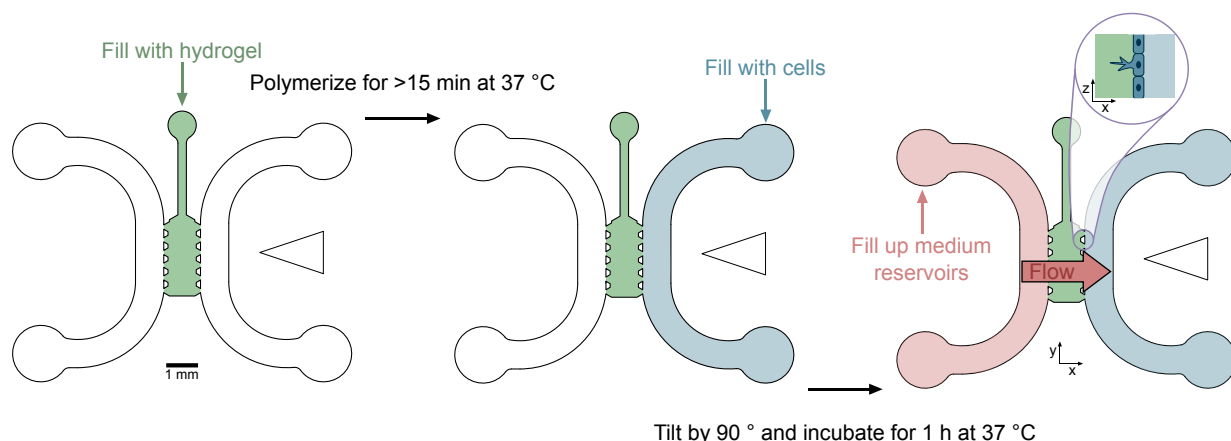


Figure 3.3: Seeding of the angiogenesis chip. The tissue chamber of the angiogenesis chip is filled with hydrogel. After hydrogel polymerization, the channel highlighted by the triangle is coated with fibronectin, and endothelial cells are seeded into the channel. To ensure cell adhesion at the interface to the adjacent tissue chamber, the microfluidic chip is incubated for one hour and tilted by 90° so that gravity settles the cells at the channel wall. After cell adhesion, the reservoirs opposite the cell seeding channel are filled with cell culture medium to create an interstitial flow that guides angiogenic sprout formation.

cut out and aligned accordingly. The micropillars separating the three neighboring channels are spaced at a distance of $150\ \mu\text{m}$ from each other. When the liquid hydrogel is filled into the tissue channel, the surface tension prevents it from leaking into the adjacent channels (Figure 3.3, left panel). During seeding, caution must be taken not to apply too much pressure to the liquid. This would disrupt the surface tension, and the hydrogel would flow into the neighboring channels, clogging the device. The micropillars allow spatial control over the positioning of the hydrogel. The hydrogel must first polymerize before any additional liquid can be loaded into the adjacent channels. After hydrogel polymerization, the channel for the endothelial cells is coated with fibronectin. Afterward, the endothelial cells are seeded into the channel (Figure 3.3, mid-panel). To ensure the formation of a continuous monolayer at the channel's interface with the tissue chamber, the microfluidic device is flipped by 90° during the incubation step. During incubation, the cells sink to the wall facing the hydrogel chamber, where they adhere. After cell adhesion, the channel opposite the tissue chamber is filled with cell culture medium. To create a hydrostatic pressure, the reservoirs connected to this channel are filled with more medium than the reservoirs connected to the cell culture channel. This gives rise to an interstitial flow through the tissue chamber, which stimulates angiogenic sprouting of the endothelial cells (Figure 3.3, right panel).

3.5 Interstitial Flow induces endothelial cell invasion

To accommodate an organoid introduced into the microfluidic chip, we designed the chip so that the channels have a height of 300 μm . Usually, microfluidic chips used for angiogenesis have a height of $\approx 100 \mu\text{m}$ and a reservoir diameter of 4 mm which allows to create an interstitial flow of $\approx 1 \mu\text{m/s}$ [118]. The interstitial flow generated within those devices ranges between 1-4 $\mu\text{m/s}$. However, increasing the channel height increases the cross-section of the hydrogel chamber facing the flow, thereby, according to Darcy's law (equation 1.19), the flow velocity decreases more rapidly. To compensate this effect, we increased the cross-section of the reservoirs to 6 mm. We produced microfluidic chips with 4 mm and 6 mm diameter reservoirs to assess which diameter best promotes angiogenesis. Simultaneously, we screened different types of hydrogels for their angiogenic potential to stimulate endothelial cell invasion. We tested endothelial cell sprouting in collagen, fibrinogen-collagen mixture, and fibrinogen (Figure 3.4 a, b, and c). Both, collagen and the fibrinogen-collagen mix did not support endothelial cell invasion for any of the two diameters. While in fibrinogen, no angiogenesis occurred in the microfluidic chips with a reservoir of 4 mm diameter, the endothelial cells cultured in the microfluidic chips with reservoir diameters of 6 mm adopted an invasive phenotype (Figure 3.4 d and e). The mechanical stimulus provided by the interstitial flow was sufficient to induce an invasive phenotype for the endothelial cells. However, it failed to promote enhanced angiogenic sprout formation. Indeed, the cells did not invade collectively or establish a lumen.

3.6 VEGF-A promotes sprouting angiogenesis

The mechanical stimulus provided by the interstitial flow was insufficient to promote angiogenesis. Therefore, we decided to supplement the medium with an additional growth factor as a biochemical stimulus to trigger angiogenesis. We added 25 ng/ml VEGF-A to the cell culture medium to support endothelial sprout and lumen formation. In vivo, secretion of VEGF-A strongly promotes angiogenic sprouting. Because angiogenesis also occurs in the context of cancer, we used PDAC conditioned medium to test its capacity to induce angiogenic sprouting. PDAC conditioned medium was obtained from PDAC cells cultured in 2D at 80% confluency. However, the PDAC conditioned medium did not trigger endothelial cell invasion into the fibrinogen matrix (Figure 3.5, upper row, left panel). The endothelial cell culture medium without VEGF-A only supported the invasion of individual endothelial cells but did not promote collective sprout invasion (Figure 3.5, upper row, mid panel). The endothelial cell culture medium, supplemented with VEGF-A, induced angiogenic sprout invasion (Figure 3.5, upper row, right panel). One day after seeding, the endothelial cells displayed a migratory phenotype with individual cells invading the fibrinogen matrix (Figure 3.5, lower row, left panel). Two days after seeding, the cells adopted a collective invasive behavior. The sprouts consisted of several cells invading the matrix. Some sprouts even had formed a lumen, with the endothelial cells lining the walls formed by the invading sprout (Figure 3.5 lower row, mid panel). On day 3, specific

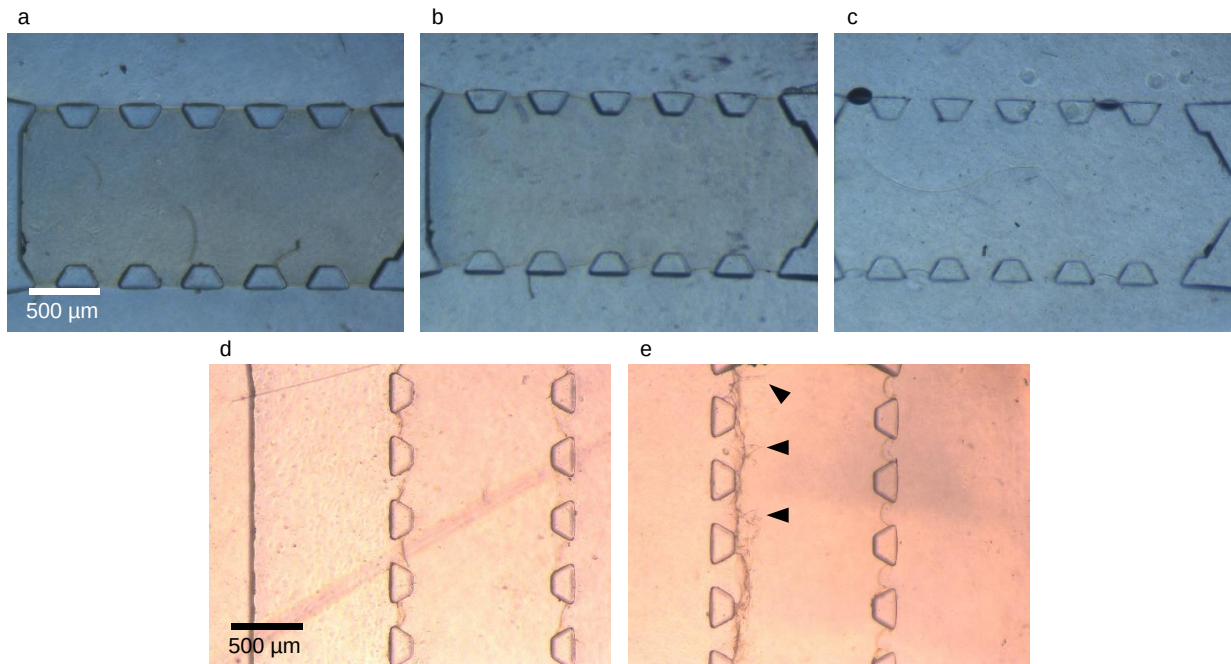


Figure 3.4: Angiogenesis chip filled with different hydrogels. (a) Collagen hydrogel polymerized in the tissue chamber. (b) A fibrinogen-collagen mixture in the microfluidic chip. (c) Polymerized fibrinogen in the hydrogel chamber. (d) The interstitial flow created by 4 mm diameter reservoirs cannot induce endothelial cell invasion into the fibrinogen. (e) Black arrows indicate the locations where endothelial cells invade the fibrinogen matrix stimulated by interstitial flow in the angiogenesis chip with a medium reservoir of 6 mm diameter.

sprouts grew to a length of several hundreds of micrometers. However, those very prominent sprouts did not succeed in developing a lumen (Figure 3.5, lower row right panel). After 3 days, the angiogenic sprouts formed by the endothelial cells did not invade much further, and they failed to grow sufficiently to form a connected vascular network with the opposite microfluidic channel. The endothelial cells might lack important biochemical cues to support the maturation of the vascular network. Although the mechanical stimulus provided by the interstitial flow is sufficient to introduce endothelial angiogenesis [127], many microfluidic applications studying angiogenesis co-culture fibroblast in a separate chamber because those cells secrete different growth factors that especially support the growth and maintenance of the vascular network [111], [113], [118], [121], [122], [140]. According to a previous study, vascularizing a tissue chamber of increased height compared to most microfluidic applications requires co-culture of endothelial cells with stromal cells in the same chamber [128]. The stromal cells can deposit proteins such as collagen that support further vasculature maturation. Although VEGF-A is reportedly sufficient to initiate angiogenesis, the presence of fibroblast has been linked to the maturation of the microvessel

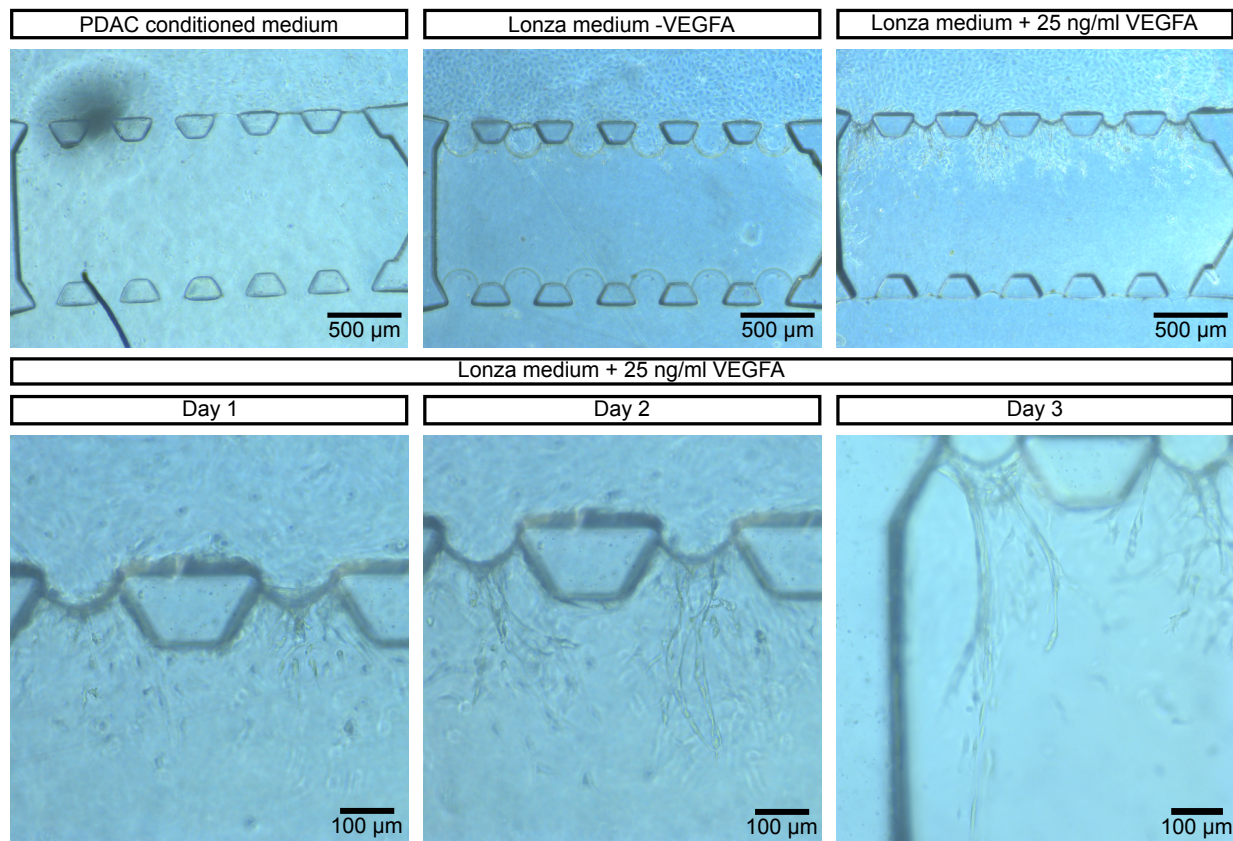


Figure 3.5: Impact of cell culture medium on sprout formation. (Upper row) Endothelial cells cultured in PDAC conditioned medium do not develop an invasive phenotype nor angiogenic sprouts. The interstitial flow stimulates endothelial cells cultured in endothelial cell culture medium (Lonza medium) to adopt an invasive phenotype. The addition of VEGF-A to the endothelial cell culture medium and the interstitial flow promote sprout invasion into the fibrinogen matrix. (Lower Row) On Day 1, the endothelial cells invade the fibrinogen matrix as individual cells. Two days after cell seeding, the first sprouts formed and developed a lumen. On day 3, individual sprouts developed to a length of several hundreds of micrometers.

to a perfusable network [117]. Additionally, exploring the benefits of adding other growth factors such as sphingosine-1-phosphate (S1P), phorbol 12-myristate 13-acetate (PMA), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF) and endothelial-specific molecule 1 (ESM1) could further support microvessel maturation. An extensive overview of growth factor cocktails used in the presented studies is provided in table A.1 in the appendix.

3.7 Outlook

Impact: In this chapter, we presented a microfluidic chip and established a protocol for achieving angiogenesis triggered exclusively by the mechanical stimulus of interstitial flow and a gradient of VEGFA. Many angiogenic chips rely on co-culture with fibroblasts that secrete growth factors to promote angiogenic sprouting. However, the variability in growth factors secreted by fibroblasts can impede maintaining consistent experimental conditions which can negatively affect the reproducibility of results. For instance, particularly, in dose-response studies the reliability and robustness of the readouts depends on homogenous conditions. Our minimal angiogenesis model system offers a valuable alternative, especially for applications requiring tight control over experimental conditions.

Co-culture with organoids with different hydrogel requirements: Many applications have already demonstrated the potential of using microfluidic chips to achieve vascularized organoids [111], [113], [120], [122]. However, most microfluidic systems use fibrinogen, collagen, or a fibrinogen-collagen mix to sustain microvascular network formation. In contrast, many 3D cell culture applications rely on matrigel, which has been reported to fail in supporting microvascular vessel formation. Therefore, the optimal hydrogel composition required for angiogenesis assays can differ from the one required for 3D cell culture conditions imposing a challenge for certain types of vascularized organoids. A workaround is to generate a mixture of the two distinct hydrogels [110], [131]. This may be applicable to some simple 3D cell culture models. Yet, organoids derived from human pluripotent stem cells are much more sensitive to changes in hydrogel composition. Therefore, altering the matrix's mechanical properties may interfere with the success of the differentiation protocol. To address this challenge, we developed a droplet-based organoid culture system that allows the encapsulation of any number of cells into hydrogel droplets with dimensions of a few hundred micrometers in diameter. These droplets, which are the focus of the next chapter, can be effortlessly integrated into the angiogenesis chip to overcome the challenge of combining two different hydrogel matrices for angiogenesis assay.

Chapter 4

Faster maturation of PDAC organoids in collagen droplets

Introduction

Beata Halassy, a virologist at the University of Zagreb, gained recognition for self-administering virus therapy to treat her tumor after conventional chemotherapy failed to cure her cancer [142]. Halassy was first diagnosed in 2016 with an aggressive form of breast cancer. Initially, she underwent conventional cancer therapy that involved mastectomy followed by chemotherapy. However, after her second recurrence in 2020, she decided against another round of chemotherapy based on its poor prognosis and instead opted for oncolytic virotherapy (OVT). Over two months, Halassy injected viruses directly into her tumor. This treatment successfully decreased the size of her tumor, allowing its surgical removal at the end of therapy. Post-surgical analysis revealed the presence of immune cells at the tumor site, suggesting that OVT helped recruit immune cells that combat the virus and successfully eliminated cancerous cells [143]. The potential benefits of virus-based therapy in cancer treatment have been known to the scientific community for over half a century. However, ethical considerations restricted their testing to patients for whom conventional treatments failed. Unfortunately, in such cases, the participants' immune systems are often compromised, limiting the efficacy of OVT. Under such conditions, the cancerous cells are primarily eliminated by the virus itself and not by the patient's own immune response [143], [144]. While Halassy's approach was both innovative and courageous, it cannot serve as a model for administering risky therapies to willing patients with early-stage cancer due to ethical concerns. Nevertheless, her case highlights the need for better preclinical models to test and develop new, promising treatment options, enhancing their safe translation into the clinic.

In this chapter, we explore how organoids may bridge this gap and discuss the current limitations that restrict their broad application in research. We present droplet-based microfluidics as a promising emerging technique in organoid culture. We use pancreatic

ductal adenocarcinoma (PDAC) cells to demonstrate how confining organoid growth to small volumes can impact organoid development through altered mechanical microenvironments. We conclude by placing our findings in a broader context and discussing their implications. Finally, we provide an outlook on how the field might evolve with the novel possibilities opened by droplet-based organoid culture.

4.1 Bulk organoid culture

Basics of organoid culture: Organoids are derived from stem cells or tissue-specific progenitor cells and faithfully recapitulate the architecture and, to some extent, the function of native tissues *in vivo* [1], [12]. Organoids have been successfully employed to create *in vitro* models for distinct human organs and tissues such as the heart [145], [146], the brain [147], [148], the lung [149], [150], the mammary gland [151], [152], and kidneys [153], [154]. Organoids can be distinguished into scaffold-free or scaffold-based approaches. Organoids cultured without scaffold are generated by culturing a suspension of adherent cells in an environment that does not promote cell adhesion to a substrate. Consequently, the cells aggregate to each other [155]. This can, for example, be achieved using microwells that offer no adhesion sites to the cells. At the same time, the small cavities ensure spatial confinement, bringing the cells into close contact with each other. This approach benefits from a rapid and efficient generation of many cellular aggregates [156]. However, those organoids often lack structural complexity compared to scaffold-grown organoids, as there is no supporting matrix to reinforce complex morphologies. For scaffold-based organoid culture, cell aggregates or single cells are seeded into a hydrogel, which acts as a supporting matrix [155]. The most prevalent hydrogel used in this context is matrigel, a natural scaffold isolated from Engelbreth-Holm-Swarm mouse sarcoma [1], [157]. Matrigel consists of a complex mixture of extracellular matrix proteins, among which are laminin, collagen type IV, and entactin [158]. Collagen is another hydrogel frequently used for 3D cell culture because of its abundance *in vivo* [1], [158]. Interestingly, cells seeded in collagen display a distinct phenotype from those seeded in matrigel. In matrigel, organoids display an increased rounded morphology, while organoid culture in a collagen matrix promotes an invasive phenotype [159]. Buchmann et al. used rheological studies to characterize the distinct mechanical properties of collagen and matrigel. In contrast to matrigel, collagen displays plastic deformation in response to mechanical deformation [160]. This aligns with the observation of mechanical crosstalk between individual cells seeded in collagen gels. The pulling forces exerted by the cells on the collagen fibers induced collagen fiber alignment, resulting in a locally increased collagen density [161]. Moreover, tumor cells have been shown to migrate along fibers displaying radial alignment to the tumor [162]. Those observations translate to the collective cell behavior observed during organoid growth. The collective pulling forces exerted by the organoid on the collagen fibers lead to the local alignment of collagen fibers and collagen cage formation which enhances the invasive phenotype displayed by those organoids [163], [164].

Organoid growth governed by self-organization: Organoids arising from individual cells and cell assemblies display an impressive capacity to self-assemble and organize into highly sophisticated morphologies containing distinct cell types. An individual cell seeded into a hydrogel first goes through an initial step of cell expansion, during which it proliferates to increase the number of cells constituting the nascent organoid. Cell assemblies seeded into a hydrogel usually do not require this step, as they already consist of sufficient cells to initiate cellular self-patterning immediately. The onset of the second phase is typically marked by symmetry breaking [1]. Symmetry breaking occurs when a population of cells is exposed to a spatially homogenous signaling cue, and only a subset of those cells responds to the trigger. The naturally occurring cell-to-cell variability within a population will stochastically select cells that become activated. Typically, this process entails the transition towards a cell population composed of various differentiated phenotypes through a complex cascade of downstream signaling events [165]. Symmetry breaking plays a role in many complex biological processes, such as morphogenesis and organogenesis. However, it can also be as simple as the tip cell selection process during angiogenic sprouting through the increased availability of surface VEGF receptors (VEGFR) for VEGF binding in the tip cell compared to its neighboring cells [166], [167]. Symmetry breaking leads to self-patterning within a cell population. Self-patterning describes the auto-induced spatial organization of cell types into a specific pattern. In a broader context, self-patterning describes many distinct concepts such as Turing reactive-diffusion dynamics or bistability of regulatory networks [1], [168]. Again, tip cell selection during angiogenic sprouting is a simple yet easily accessible example of self-patterning. In the tip cell, the activation of VEGFR entails the upregulation of the expression of Delta-like ligand 4 (Delta), a transmembrane ligand that binds to the transmembrane receptor Notch of the adjacent cells. Notch activation in the neighboring cell results in its proteolytic cleavage, producing the cleaved intracellular domain of Notch (NICD). The translocation of NICD into the nucleus promotes the downregulation of VEGFR and Delta, which consolidates the selective differentiation of quiescent endothelial cells into one single tip cell and several lateral stalk cells [166], [167]. During the morphogenetic rearrangement, the distinct cell types that emerged during self-patterning drive the development of the organoid toward a complex morphology. The differences in cellular behavior regarding cell migration, cell contractility, and cell-cell interactions induce the spatial self-organization of the organoid into an intricate 3D structure [1], [169]. In sprouting angiogenesis, morphogenetic rearrangement corresponds to the formation of a nascent blood vessel resulting from the migratory phenotype adopted by the tip cell that invades the surrounding matrix towards the angiogenic signal. The stalk cells characterized by a less migratory but enhanced proliferative phenotype follow behind the tip cell [166], [167].

Developmental phases of pancreatic ductal adenocarcinoma organoids: In this chapter, we use a murine pancreatic ductal adenocarcinoma (PDAC) organoid model system to characterize how volume confinement impacts developmental timescales during organoid growth. Randriamanantsoa et al. previously characterized this PDAC organoid model system in two seminal works. This characterization will be the foundation for the

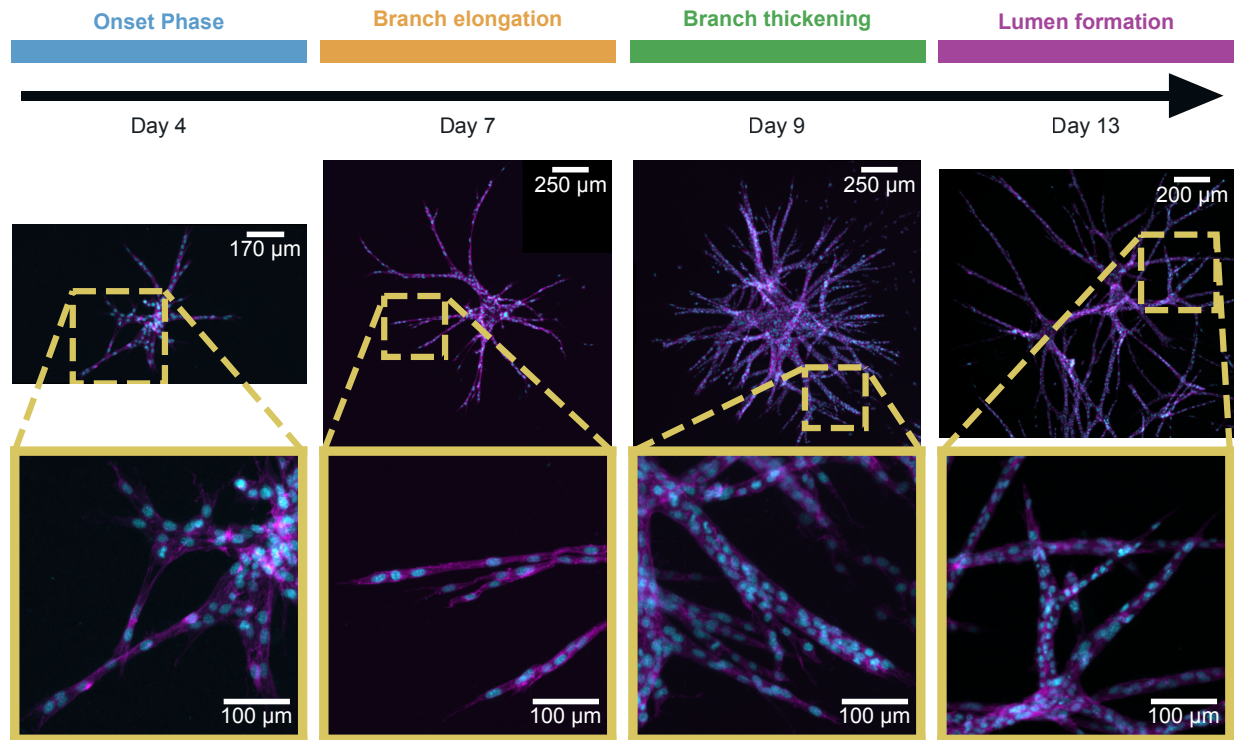


Figure 4.1: Developmental phases in conventional bulk organoid culture. As the organoids mature, they progress through distinct developmental phases: the onset phase, the branch establishment phase, the branch thickening phase and the lumen formation phase.

subsequent section [164], [170]. To obtain PDAC organoids, single cells are seeded into a bulk collagen gel. Over 14 days, those cells grow into highly branched structures with functional lumens that closely resemble the morphology of PDAC in mice. Analysis of transcriptomic data and morphological features showed that developing PDAC organoids undergo mesenchymal to epithelial transition (MET), culminating in lumen formation. MET describes the reverse process of epithelial to mesenchymal transition. During MET, a mesenchymal cell characterized by a strong migratory behavior and weak cell-cell adhesions adopts an increasingly epithelial phenotype characterized by enhanced basal-apical polarity and strengthening of cell-cell adhesions [171]. The development of PDAC organoids can be distinguished into four distinct phases (Figure 4.4). During the onset phase, starting with cell seeding up to day 5, seeded single cells proliferate and break symmetry, adopting an elongated organoid morphology. From day 7 to 9, the epithelial cells increasingly invade the surrounding matrix, leading to branch elongation during the consecutive elongation phase. During this phase, the epithelial cells are characterized by a strong mesenchymal phenotype, which manifests in a rapid increase in branch length. The elongated shape of the cells during this phase also matches their strong migratory behavior. Eventually, the extension speed of the branches reduces, and the organoids increasingly deform the surrounding col-

lagen, generating a collagen cage as the organoid progresses through the thickening phase from day 9 to 11. The final stage in organoid development starts on day 11, with lumen nucleation sites emerging and subsequent lumen opening. During those two final phases, the endothelial cells adopt an increasingly epithelial phenotype characterized by a less invasive migratory behavior. Therefore, the rate at which the branches elongate slows down. The enhanced epithelial phenotype is also reflected in a distinct cellular organization within the branches. While the epithelial cells displayed a more elongated phenotype during the extension phase, during the final two developmental phases, the epithelial cells organized in a cobblestone pattern within the branches. Lumen nucleation sites are characterized by a local increase in F-actin and E-cadherin localization to the apical side of the epithelial layers. Lumen formation occurs through two distinct processes. These mechanically separate the two opposing epithelial cell layers. Lumen opening occurs through the extrusion of apoptotic cells or fluid influx through the epithelial cell layer into the nascent lumen.

Potential applications of organoid culture: The complex spatiotemporal developmental stages present during organoid culture cannot be recapitulated in simple 2D cell culture, which fails to accurately capture the complexity of cellular architecture, cell-cell, and cell-matrix interactions encountered *in vivo*. The transition into the third dimension significantly alters cell migration, cell-cell interactions, and mechanotransduction pathways [172]–[174]. Furthermore, compared to cells cultured in 3D, 2D cell culture profoundly influences cellular shape, as shown by Meyers et al., who demonstrated through physical modeling that cell geometry significantly impacts signaling pathways within the cell [155], [174], [175]. Therefore, cells cultured in 3D can display a different drug response than those cultured in 2D [155], [176]. Notably, cells in 2D exhibit decreased resistance to drugs, reducing the reliability of the obtained drug screening results [176]. Consequently, such results cannot be directly translated to *in vivo* systems. By contrast, 3D cell culture mimics cellular behavior *in vivo* more accurately, providing more accurate outcomes. Therefore, organoid culture is much better suited for drug screening applications, which aim to preselect a small subset of promising agents from a vast pool of drug candidates. Beyond drug screening applications, organoid culture could provide more realistic biological model systems and find applications in personalized medicine. For instance, Volmert et al. demonstrated their predictive potential as a model system by demonstrating the adverse side effect of ondansetron, a drug frequently administered against pregnancy sickness, on cardiomyocyte differentiation and maturation during heart development in congruence with clinical studies [146]. Similarly, Vlachogiannis et al. used patient-derived organoids from metastatic gastrointestinal cancer to accurately predict patients' response to specific treatments [177], [178]. Patient-derived cancer organoid models could help physicians make faster and more informed decisions on treatment strategies. Especially for fast-progressing tumors, such as pancreatic cancer, patient-derived organoids, and personalized medicine could significantly enhance patient survival [179]. Moreover, organoids serve as a valuable model system for disease and embryo development. *In vitro* organoid culture allows researchers to assess and quantify biological processes that otherwise would be difficult to assess *in vivo* [1], [148].

Limitations of organoid culture: Conventional bulk organoid culture suffers from poor compatibility with high throughput applications. The lack of practical and scalable solutions to address these challenges prevents its broad implementation in a clinical setting and the pharmaceutical industry [178], [180]. Many organoid seeding protocols rely to this day on manual handling and cannot easily be performed by liquid handling robots. Besides slowing down the organoid generation process and introducing an additional source of error, this significantly limits the throughput and scalability of organoid culture [178], [181], [182]. To ensure the economic viability of high throughput organoid screens, organoid seeding efficiency must be drastically improved. Similarly, consumables, such as hydrogels or medium supplements, substantially increase the cost of organoid culture. Reducing the required amount of consumables by downscaling benefits the economic efficiency of organoid culture and allows the adaptation to already existing high throughput pipelines established for 2D cell culture [178], [183], [184]. High throughput screens require fast analysis pipelines, which creates additional difficulty for organoid culture. Typically, cells seeded into a hydrogel are randomly positioned. Therefore, acquiring visual readout can be incredibly time-consuming as not all the cells are located in the same focal plane [178]. Many efforts have been dedicated to establishing automated and controlled organoid culture systems that range from using microfluidic chips and controlling organoid position in the gel through micropatterns to 3D patterned organoids [185]. While those approaches offer improvements regarding precise control over organoid position and shape, they often lack good compatibility with automated workflows. Combining organoid culture with droplet-based microfluidic has shown increasingly promising results and will be introduced in the following section.

4.2 Droplet-based microfluidics

Basics of microfluidics: Microfluidics describes the study of fluid flow in systems referred to as microfluidic chips with dimensions ranging from a few micrometers to several hundreds of micrometers [186], [187]. The liquid is transported through the microchannels using a pressure-driven flow generated by a pump [186]. Conventional microfluidics typically operate at low Reynolds numbers, such that $Re \approx 0$, where viscous forces dominate and the flow can be approximated as a Stokes flow. However, laminar flow occurs up to a Reynolds number of $Re < 2000$, although inertia cannot be neglected anymore in those systems. In the regime, where inertial forces become relevant, $Re > 1$, many interesting phenomena such as flow focusing occur [188], [189]. For $Re > 2000$, turbulent flow emerges. Nowadays, microfluidics is widely used in several distinct fields, such as chemistry and biology, due to its small size, cost efficiency, capacity for parallelization, and automation [190].

Droplet-based microfluidics: Droplet-based microfluidics is a specific area in microfluidics focusing on the formation and handling of pico- to nanoliter droplets generated from

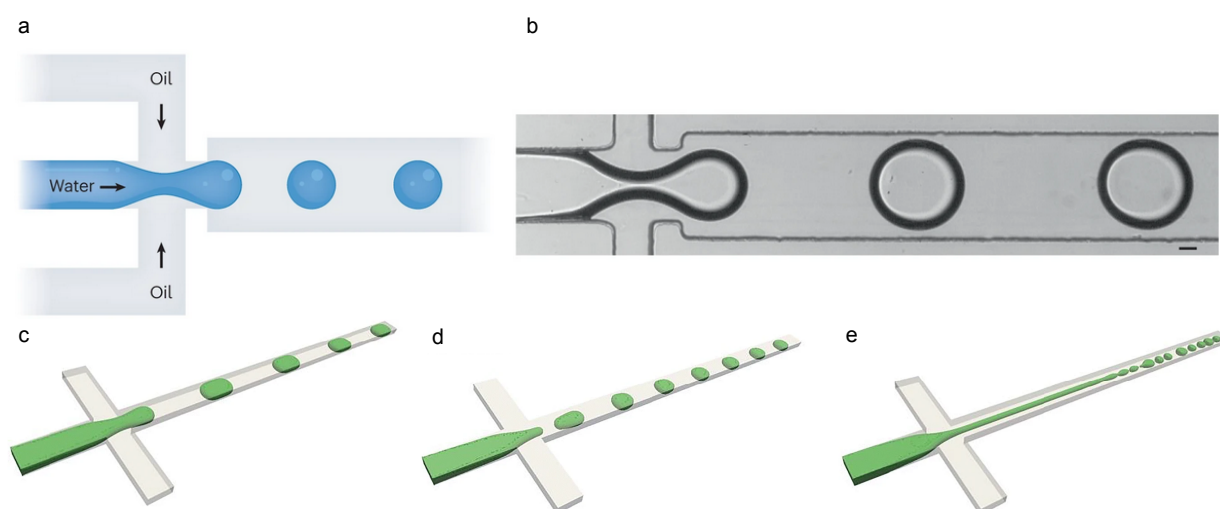


Figure 4.2: Droplet production in flow-focusing geometry. (a) and (b) Microfluidic chip design using flow-focusing to generate droplets. The scale bar is $50\ \mu\text{m}$. (a) and (b) are adapted from [193]. (c) In the squeezing regime, the inner fluid (green) flows through the orifice, blocking the outer fluid (beige), which leads to a pressure build-up that eventually pinches off the droplet. (d) In the dripping regime, droplet production is governed by the interplay between viscous drag and capillary instabilities. (e) In the jetting regime, the narrow jet extends downstream, and droplet production occurs at the end of the stream. (c) - (e) are reproduced from [194].

two immiscible fluids [191], [192]. The potential of this specific branch in microfluidics lies in its capacity to create small compartments that act as microreactors. The efficient splitting of samples into separated, well-defined fluid volumes allows the generation of a large number of droplets within a short amount of time in an automated fashion, representing a significant increase in throughput [191]. Moreover, the high surface-to-volume ratio allows efficient heat transfer throughout the droplet. Combined with effective mixing and the possibility to determine the incubation time from the spatial information of the droplet within the chip, these droplets constitute good microreactors [195]. Droplets can be generated within microfluidic chips made from PDMS through a set of distinct channel geometries. One of those is flow focusing (Figure 4.2 a and b). In a flow-focusing device, a central stream of a dispersed liquid is met by a continuous fluid carried by two lateral channels. Droplet generation takes place in the squeezing regime at low flow rates of the continuous fluid compared to the dispersed fluid. In this regime, the dispersed fluid flows into the constriction, blocking the continuous fluid and resulting in a pressure build-up. The rising pressure builds at the interface with dispersed fluid until it is eventually pinched off. In the droplet squeezing regime, the droplet size is commonly bigger than the size of the constriction (Figure 4.2 c). As the flow velocity of the continuous fluid increases, droplet formation transitions from the squeezing regime to the dripping regime, where, due to the increased viscous stresses, the dispersed face forms an elongated, narrow stream flowing

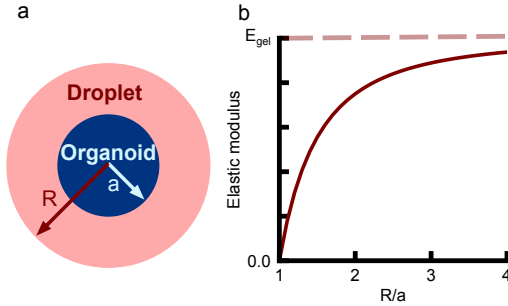


Figure 4.3: Elasticity modulus of collagen droplets compared to bulk gels.

(a) The radius of the collagen droplet is R , and the organoid, which is positioned in the center of the droplets, is characterized by a radius a . (b) The elastic modulus of the droplets changes with R/a . For an infinitely big collagen droplet compared to the size of the organoid, the elastic modulus of the droplet approaches that of a bulk gel.

through the constriction. Droplet formation occurs in close proximity to the constriction at the end of the stream and is governed by viscous drag and capillary instabilities (Figure 4.2 d). Viscous jetting occurs at even higher flow velocities of the continuous phase. The droplet formation is governed by a similar mechanism as in the dripping regime, with the only difference that the continuous fluid flow stabilizes the downstream extension of the thin fluid thread over multiple lengthscales of the dimensions of the constriction (Figure 4.2 e) [192], [196], [197]. Droplet-based microfluidics has revolutionized omics analysis by providing scalable, cost-effective technologies that enable high-throughput applications. [198] Initially applied in single-cell RNA sequencing (scRNA-seq) [199]–[201], this technology has since expanded its utility to multi-omics approaches such as scATAC-seq and CITE-seq. [198], [202] Similarly, organoid culture holds great potential for studying complex biological systems in vitro. [203]–[206] Still, it continues to face significant limitations, including cell heterogeneity, poor reproducibility, high costs, and scalability issues. [10]–[13] To address these limitations, recent research has explored the application of droplet-based microfluidics for organoid culture.

Combining organoid culture with droplet-based microfluidics: Using droplet-based microfluidics to encapsulate a large number of cells allows to benefit from the high production rate and a reduced cost due to reducing the amount of consumables. Moreover, Cohen et al. demonstrated that hPSC encapsulated in a matrigel-alginate core-shell structure can be expanded using a commercially available bioreactor. Compared to conventional 2D cell culture, this culture condition is 20 times more efficient. While the alginate shell protects the stem cells from mechanical damage, the matrigel provides important cues to enhance the efficacy of stem cell culture [207]. Similarly, Ding et al. successfully grew cancer spheroids within matrigel droplets from tumor cells obtained from a biopsy. Furthermore, they demonstrated the compatibility of their pipeline to help with treatment decisions in the clinical routine [208]. While Ding et al. performed their screening in commercially available microwell plates, Tomassi et al. used a custom-made microfluidic device to parallelize their screen. By keeping cell aggregates in emulsion, they analyzed the drug response of spheroids to 27 distinct conditions within the same microfluidic device [209].

Aim of this study

Those studies impressively demonstrate the compatibility of droplet-grown organoids for high throughput screenings. However, current applications are mostly limited to studying spheroids formed by the aggregation of several cells. Although those spheroids still represent 3D cell culture, they do not form through a complex spatiotemporal differentiation mechanism and lack morphological resemblance to native tissue. Therefore, they might not adequately represent the cellular response in vivo. Moreover, confining cells to droplets of only a few hundred micrometers in diameter represents a significant down-scaling of culture conditions, which impacts the mechanical properties of the supporting matrix. In 2D, the effective elastic modulus experienced by an organoid of size a in a droplet of size R is defined by $E_{\text{eff}} = E(1 - a^2/R^2)$, where E is the elastic modulus of the material (Figure 4.3 a).¹ Therefore, the smaller the droplet, assuming that the size of the organoid a remains constant, the smaller the effective Elastic modulus sensed by the organoid. On the contrary, as R increases, the effective Elastic modulus approaches the value sensed by an organoid cultured in a bulk collagen gel (Figure 4.3 b). Altering the mechanical properties of the supporting matrix could affect organoid development, as mechanical forces exerted during organoid development are a key developmental hallmark present in several branched organoid models [163], [164]. Lee et al. showed that reducing the dimensions of cell aggregates used for cardiac differentiation affected differentiation efficiency. While 1 mm diameter cardiac organoids expressed cardiac markers at day 35, smaller sized organoids of 500 μm diameter did fail to do so even after 49 days [210]. The knowledge of how altered culture conditions affect developmental phases is essential to adapt the time points of drug administration in component screening. To address this gap, we used droplets of different dimensions to assess how the modified mechanical properties of the supporting hydrogel affected organoid growth. Furthermore, by comparing morphological and transcriptomic data, we demonstrate that the maturation of droplet-grown organoids is accelerated compared to organoids cultured in conventional culture conditions.

4.3 Automated Organoid Seeding

Microfluidic pipeline for automated organoid seeding: We used droplet-based microfluidics to automate the organoid seeding process. PDAC cells cultured in 2D were brought into suspension and mechanically separated by syringe aspiration to ensure the encapsulation of single cells. The singularized cell solution was mixed with a collagen solution and filled into a syringe connected to the microfluidic chip with two inlets: one for the collagen solution mixed with PDAC cells and another for the fluorinated oil with surfactant. The collagen droplets were generated at the chip's cross-section, where the

¹These equations resulted from a very fruitful discussion with Fridtjof Brauns from the Kavli Institute for Theoretical Physics.

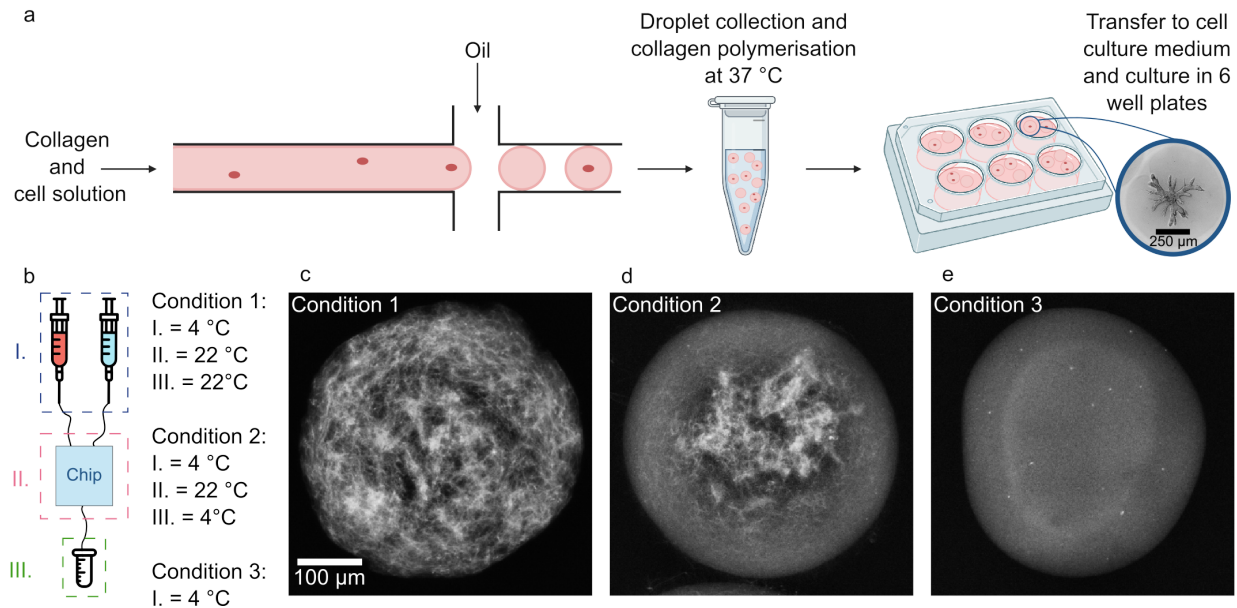


Figure 4.4: Characterization of the microfluidic chip. (a) The automated organoid seeding pipeline encapsulates single PDAC cells into collagen droplets. (b) Different temperature gradients were applied across the microfluidic setup to assess their impact on collagen polymerization. (c) Polymerization at room temperature leads to the aggregation of collagen fibers. (d) When the cooling of the collagen is disrupted temporarily, the collagen polymerizes prematurely. (e) Only collagen droplets with the entire microfluidic pipeline maintained at 4 °C and the collecting reservoir at 37 °C display homogenous collagen polymerization.

oil flowing in from both sides pinched off collagen droplets, creating a dispersed emulsion. The collagen droplets were collected in a reservoir heated at 37 °C to initiate collagen polymerization. The collagen droplets were maintained at 37 °C for 1 hour to achieve full polymerization of the droplets. Afterward, the emulsion was broken to recover the droplets, and the droplets were transferred to the cell culture medium. PDAC cells were cultured in the collagen droplets for up to a week and grew into branched organoid structures (Figure 4.4 a). The microfluidic chip was operated at 4 °C in the cold room to ensure correct collagen polymerization. We assessed the impact of distinct temperature gradients on the collagen polymerization of the droplets. We operated the whole setup at room temperature, where only the syringes were cooled to 4 °C, and collagen polymerization occurred at room temperature (condition I). In a second setup, we cooled the syringes and the collecting reservoir to 4 °C. However, the microfluidic chip remained at room temperature (condition II). For condition II, the droplets were transferred to 37 °C for polymerization after droplet collection. The third condition consisted of the protocol presented in the first part of this paragraph: the microfluidic pipeline was maintained at 4 °C except for the collecting reservoir, which was heated to 37 °C (Figure 4.4 b). To ensure homogenous

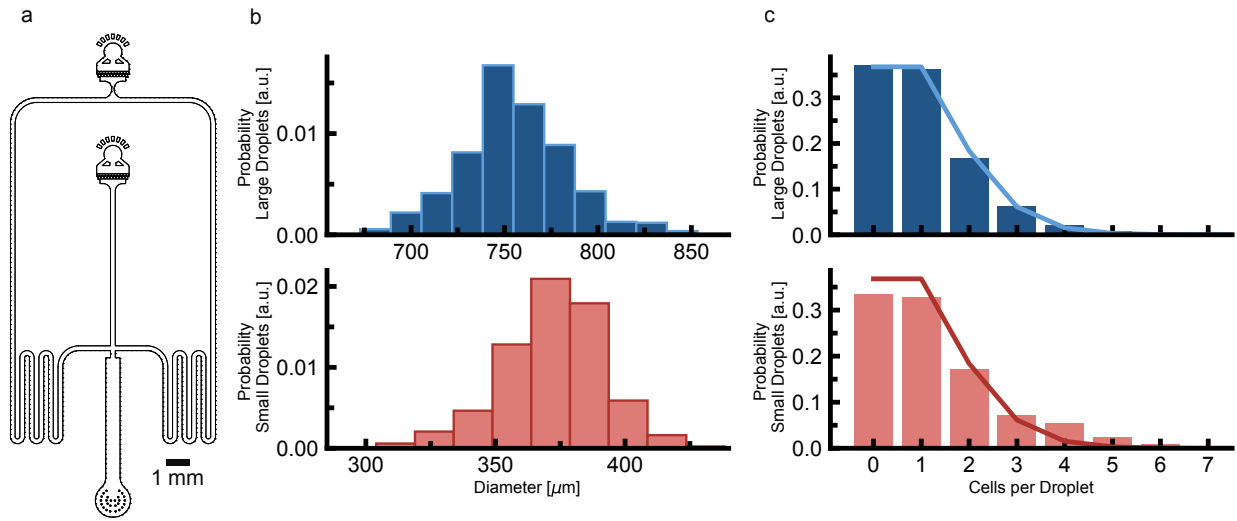


Figure 4.5: Characterization of the microfluidic chip. (a) CAD design of the microfluidic chip. (b) The microfluidic chips of channel height $600\ \mu\text{m}$ generate droplets of $\approx 750\ \mu\text{m}$ diameter (blue) ($n = 666$ droplets). The devices with a decreased channel height of $200\ \mu\text{m}$ produce droplets of $\approx 370\ \mu\text{m}$ (red) ($n = 907$ droplets). (c) The incorporation frequency follows Poisson statistics. The cell incorporation frequency for large droplets is shown in red ($n = 951$), and the one for small droplets in blue ($n = 906$). The line plot represents the theoretical Poisson distribution probabilities, assuming an average of one cell per droplet.

collagen polymerization, it was essential to perform polymerization at $37\ ^\circ\text{C}$. Although collagen also polymerizes at room temperature, it forms aggregates of collagen fibers in contrast to the evenly polymerized collagen at $37\ ^\circ\text{C}$ (Figure 4.4 c and e). Moreover, it was important to maintain the whole microfluidic setup at $4\ ^\circ\text{C}$ to prevent premature collagen polymerization. In condition II, where the cooling of the collagen was disrupted, part of the collagen polymerized prematurely and formed collagen fiber aggregates (Figure 4.4 d).

Generation of collagen droplets of two distinct sizes: We used two different microfluidic chips to generate droplets of distinct sizes. A microfluidic chip of a channel height of $600\ \mu\text{m}$ was used to achieve droplets of $\approx 750\ \mu\text{m}$ diameter (Figure 4.5 a and b). The same design was used to generate smaller droplets, but the channel height was decreased to $200\ \mu\text{m}$ (Figure 4.5 a). The droplets produced by this chip measured $\approx 370\ \mu\text{m}$ (Figure 4.5 b). The production rates were 10 Hz for the small droplets and 1 Hz for large droplets. The diameter of the larger droplets corresponds to twice the diameter of the smaller-sized droplets. Therefore, a cell seeded into a large droplet experienced a microenvironment eight times bigger in volume than a cell seeded in a smaller collagen droplet. The encapsulation frequency of PDAC cells into the collagen droplets follows a Poisson distribution. We used PDAC-H2B cells to visualize cell encapsulation frequency. For large droplets, 37% were empty, 36% contained one cell, and 27% contained two or more cells (Figure 4.5 c).

Similarly, 33% of small droplets were empty, 33% contained one cell, and 34% contained more than one cell (Figure 4.5 c). To evaluate organoid growth efficiency for droplet-based organoid culture, we assessed the proportion of droplets that contained at least one developed three-dimensional structure on day 4. For large droplets containing, on average, 1.1 cells per droplet, $47 \pm 5\%$ (mean $\pm$ std) of droplets contained at least one organoid on day 4. Small droplets contained, on average, 0.8 cells per droplet and displayed a slightly lower organoid growth efficiency with $40 \pm 8\%$ of droplets containing at least one organoid on day 4. Consequently, droplet-based organoid culture yielded approximately one organoid for every second seeded cell, which represented a significantly higher efficiency than compared to bulk, where extreme limiting dilution analysis showed that only every fifth cell developed into an organoid [164].

4.4 Comparison of organoid growth in small and large droplets

Comparison of organoid morphology: For bulk organoid culture, singularized PDAC cells are commonly seeded into a bulk gel of approximately 1 ml volume. Over two weeks, bulk-grown organoids develop into complexly branched structures with dimensions reaching several millimeters. Notably, on day 5, PDAC organoids typically measure $542 \pm 155 \mu\text{m}$ (mean $\pm$ s.d.) along their major axis [163], [164]. Encapsulating PDAC cells into droplets of only several hundreds of micrometers in diameter provides a significantly altered mechanical microenvironment for the growing organoids. To investigate the impact of volume confinement, we analyzed organoid development in small ($\approx 370 \mu\text{m}$ diameter, 27 nl) and large ($\approx 750 \mu\text{m}$ diameter, 221 nl) collagen droplets. While branched organoids were present in small and large droplets, the organoids that grew in the small droplets rather developed into spheroids. On the contrary, organoids grown in large droplets displayed an increased tendency to evolve into branched and elongated structures (Figure 4.6). To investigate this hypothesis further, we analyzed the shape of 2D projected organoids and computed their compactness over time by comparing the parameter of the organoid's shape to that of a circle with the same area. The organoids cultured in small droplets exhibited reduced compactness after 6 days in culture compared to organoids grown in large droplets (Figure 4.7 a). This further supports the hypothesis that the organoids in small droplets rather exhibit a spherical shape. Next, we assessed the branching efficiency of organoids grown in large versus small droplets by quantifying the proportion of branched versus spheroid structures on day four of organoid culture. In bulk organoid culture, the first 5 days are characterized as the onset phase, where the organoid displays moderate branching and invasive behavior. Therefore, we classified the organoids into two categories: spheroids and invasive phenotypes. Spheroids were characterized by a major axis less than $a < 60 \mu\text{m}$, a minor axis greater than $b > a/2$, or fewer than two branches with lengths of at least $30 \mu\text{m}$. Structures not meeting those criteria were classified as branched. Those organoids exhibited at

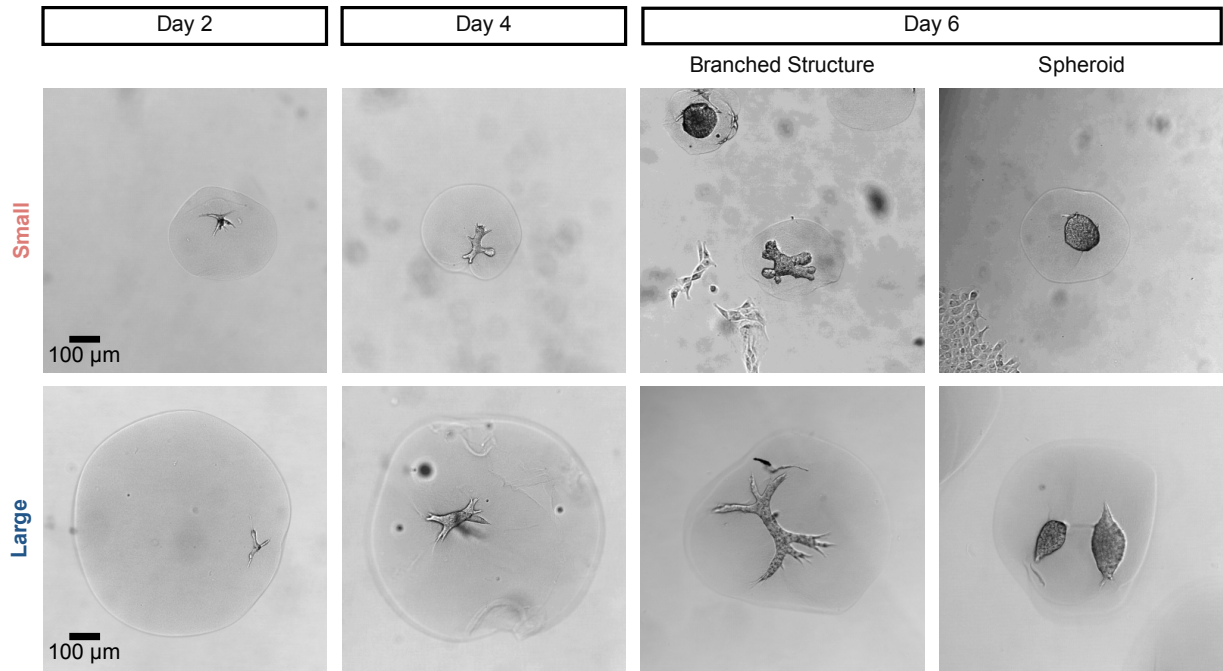


Figure 4.6: Morphology of organoids grown in small versus large droplets. In small and large droplets, the organoids grow into branched structures or spheroids. In large droplets, the branched structure is more prevalent, whereas in small droplets, the spheroids are more common.

least two branches or an invasive phenotype with the potential for further branching in later developmental stages. Organoids grown in small droplets were increasingly classified as spheroids, and only $44 \pm 5\%$ displayed a branched or invasive phenotype. In contrast, $59 \pm 5\%$ of organoids cultured in large droplets exhibited characteristics of a branched or invasive phenotype (Figure 4.7 b). Furthermore, the major axis of organoids cultured in large droplets increased faster than those cultured in small droplets (Figure 4.7 c). Notably, on day 5, organoids grown in small droplets measured $150 \pm 42 \mu\text{m}$ along their major axis, whereas the organoids cultured in large droplets reached a mean major axis of $266 \pm 89 \mu\text{m}$. Although the organoids in large droplets displayed a more pronounced growth, they only attained half the size of those cultured in bulk collagen gels. Organoids cultured in bulk are commonly characterized by a strong heterogeneity within the organoid batch. Therefore, to test whether confinement to smaller volumes decreases organoid heterogeneity, we determined the polydispersity index for organoids grown in small droplets, large droplets, and bulk collagen gels. The polydispersity index was computed from the area of the 2D projected organoids: $PDI = \sqrt{(\langle A_{\text{proj}}^2 \rangle - \langle A_{\text{proj}} \rangle^2) / \langle A_{\text{proj}} \rangle}$ [211]. The polydispersity index for bulk-grown organoids was $PDI_{\text{Bulk}} = 63\%$. Culturing the organoids in large collagen droplets reduced the polydispersity index to $PDI = 47\%$. The most significant decrease in the polydispersity index was achieved by culturing the droplets in small collagen droplets,

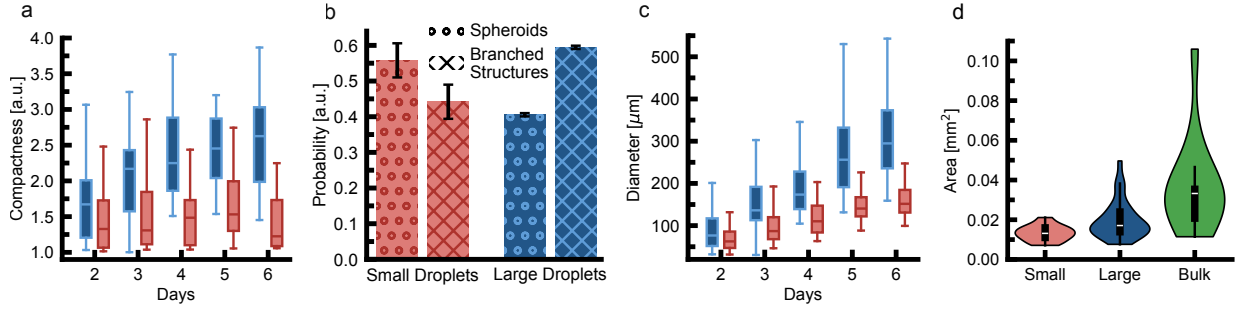


Figure 4.7: Comparison of organoid growth in small versus large droplets. (a) Organoids grown in small droplets exhibit a more rounded morphology, resulting in a reduced compactness compared to the branched organoids obtained from organoid culture in large droplets. (b) Organoids cultured in small droplets rather grow as spheroids, whereas organoids grown in large droplets have an increased tendency to develop into branched structures. (c) The organoids grown in small droplets remain smaller than those grown in large droplets. (d) The morphological homogeneity increases with decreasing droplet size.

which achieved a polydispersity index of $PDI = 28\%$ (Figure 4.7 d). Consequently, confining organoid growth to collagen droplets reduced their morphological complexity while simultaneously improving organoid homogeneity. For this reason, organoids cultured in the small droplets displayed the highest homogeneity but lacked complex morphological features such as organoid branches, thereby failing to closely recapitulate in vivo tissue.

Comparison of droplet deformation: Mechanical interaction of bulk-grown organoids with the supporting collagen matrix enables the growth of those highly branched organoid structures. Bulk-grown organoids have been shown to plastically deform their surrounding matrix, leading to the build-up of a collagen cage, which further enhances the development of a branched morphology [163], [164]. The comparison of organoid morphologies showed that large droplets promote the development of branched structures. Therefore, we quantified the impact of droplet size on the mechanical interaction between organoids and the surrounding collagen matrix. To investigate this, we monitored changes in droplet diameter over time. Large droplets underwent visible deformation after one week in culture (Figure 4.8 a and b). To confirm the significant decrease in the diameter of large droplets, we measured their 2D-projected shapes. Additionally, to verify that the mechanical forces exerted by the organoids on the collagen matrix caused this reduction in droplet diameter, we compared the decrease in droplet dimensions of large droplets to those of small droplets (Figure 4.8 c). Our findings indicate that small and large droplets experienced similar compression up to day 5. However, by day 6, large droplets were compressed by 12%, while the diameter of small droplets was only decreased by 5%, indicating that branched organoids deform the supporting matrix more strongly. Next, we compared organoid morphology and droplet deformation. Assuming the organoids grew in the center of the droplets, we computed the relative distance between the organoid and the boundary

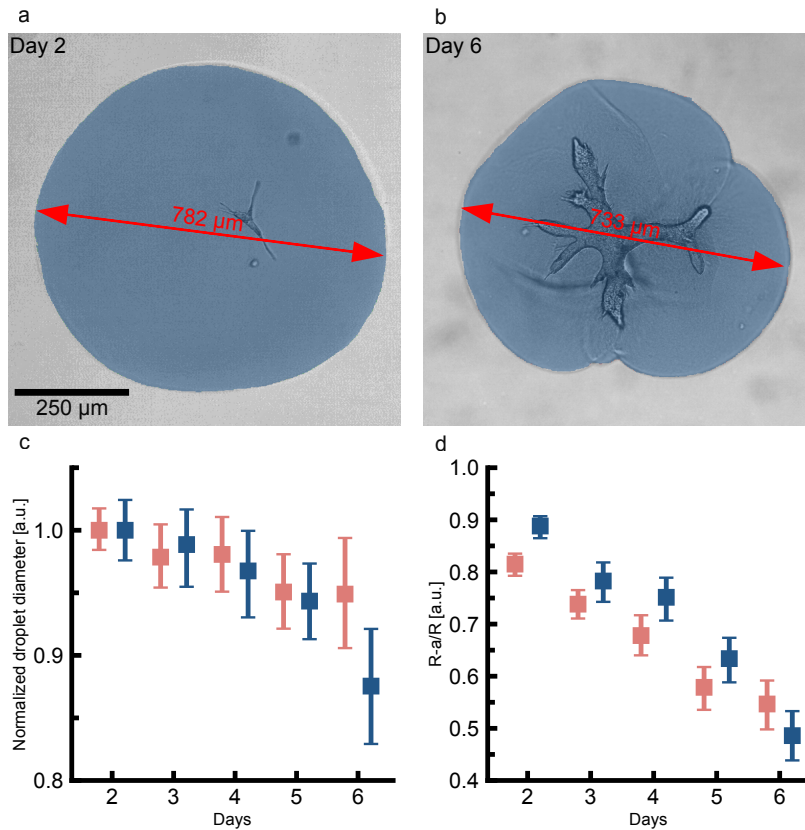


Figure 4.8: Organoids mechanically deform collagen droplets. (a) On day 2, the collagen droplet still exhibits a homogenous surface. (b) On day 6, the growing organoid increasingly deforms the droplet. Therefore, the collagen droplets typically display a reduced diameter than on day 2. (c) The normalized droplet diameter over time. Large droplets display a more pronounced decrease in droplet diameter than small droplets. (d) The mean relative distance between the organoid and the droplets' surface for small droplets (red) and large droplets (blue) over time ($n_{\text{Large}} \geq 23$ and $n_{\text{Small}} \geq 23$ for each time point). The error bars show the 95% confidence interval.

of the droplets, defined as $(r - a)/r$. The parameter r corresponds to the diameter of the droplet, whereas a denotes the major axis of the 2D projected shape of the organoid. Our analysis revealed that organoids in small droplets were initially located closer to the droplet boundary than organoids cultured in large droplets. However, by day 6, organoids cultured in large droplets were relatively closer to the droplet boundary than those cultured in small droplets (Figure 4.8 d). The decreased relative distance for organoids cultured in large droplets is likely not only caused by the more prominent growth of organoids in large droplets but also by their enhanced mechanical interaction with the surrounding collagen. We used fluorescently labeled collagen and visualized cell nuclei using SiRDNA to investigate the impact of a branched organoid morphology on droplet deformation. By day 5, the branched structures significantly deformed the collagen droplets (Figure 4.9). Notably,

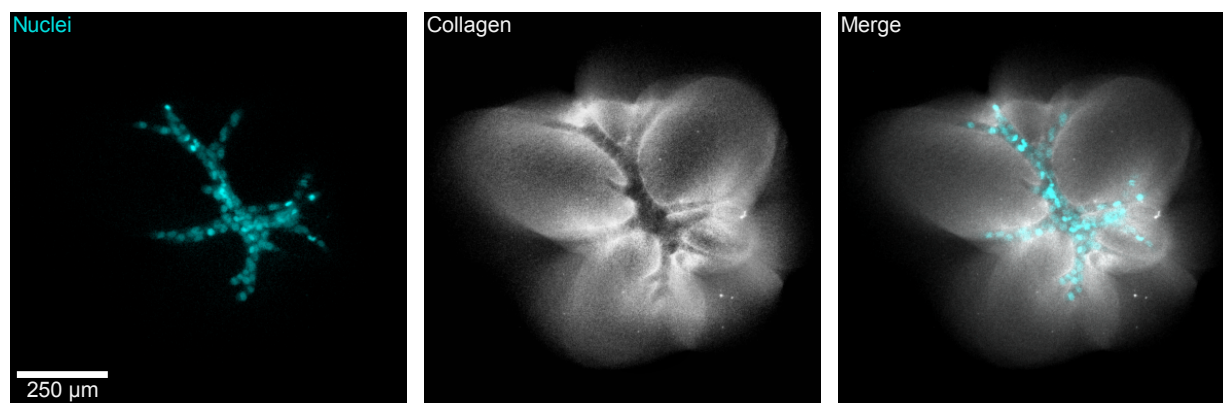


Figure 4.9: Droplet-grown organoid form a collagen cage. The PDAC organoid was labeled with sirDNA to visualize the nuclei. The collagen is made visible by conjugation to Atto488. The location of the organoid branches correlates with an increased collagen deformation.

the sites exhibiting the strongest mechanical deformation coincided with the location of organoid branches. Furthermore, the increased collagen intensity at the organoid boundary suggested the formation of a collagen cage, which is crucial for the development of a branched organoid morphology.

Comparison of transcriptome: We used bulk RNA sequencing to investigate whether the altered mechanical microenvironment experienced by the organoids growing in differently sized droplets affected their transcriptome. We analyzed the transcriptome of organoids cultured in bulk collagen gels at a density of 1.3 mg/ml and 3 mg/ml and of organoids cultured in large and small droplets. All conditions were analyzed on day 4 and day 7. The principal component analysis (PCA) of the data set suggested good reproducibility within the distinct batches of the same conditions (Figure 4.10 a). Furthermore, the PCA plot indicated significant similarity among organoids cultured in bulk at different collagen densities. Likewise, droplet-grown organoids displayed close proximity regardless of droplet size. This suggests that organoids cultured in droplets exhibit a similar transcriptomic profile independent of the droplets' dimensions. To confirm this hypothesis, we used volcano plots to visualize the number of genes that were significantly differently expressed. For both days, day 4 and day 7, there are only a few differently expressed genes when comparing organoids cultured in large and small droplets (Figure 4.10 b and c). A comparison of the transcriptome within the same condition on day 4 and day 7 shows that there is a moderate amount of significantly differently expressed genes, indicating that the organoids progress in their developmental stage (Figure 4.10 d and g). Eventually, comparing the organoids cultured in droplets to the organoids cultured in bulk gels at a concentration of 1.3 mg/ml shows that the transcriptome on day 4 resembles more closely the transcriptome of bulk organoid culture on day 7, implicating a faster maturation of organoids cultured in droplets which will be assessed in more detail in the next section

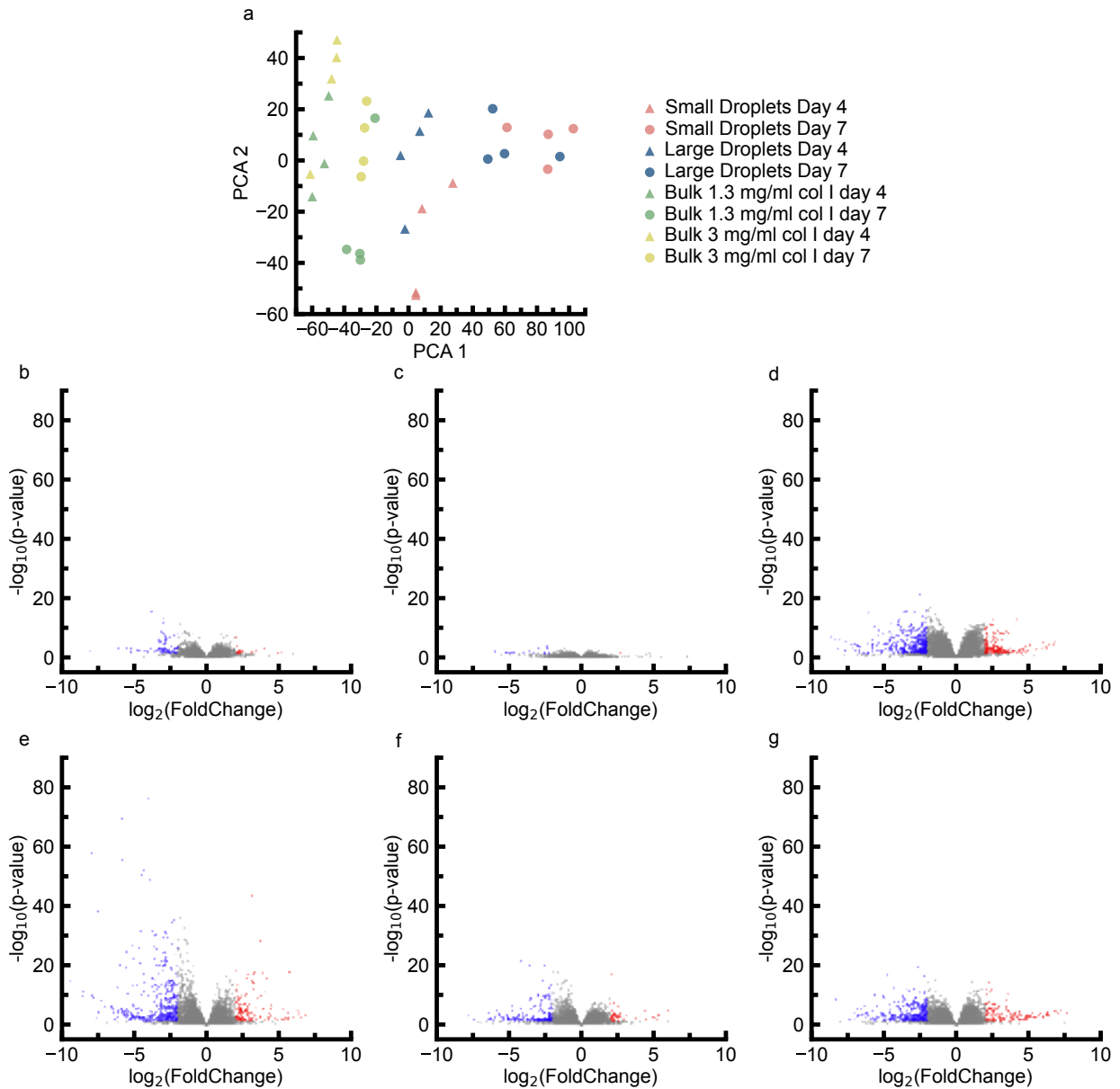


Figure 4.10: Similar transcriptome of organoids cultured collagen droplets of distinct sizes. (a) Principal component analysis carried out for technical replicates of the different culture conditions. (b) The volcano plot comparing organoids grown in small droplets to those grown in large droplets on day 4. (c) Differentially expressed genes represented in a volcano plot for organoids grown in small droplets compared to organoids cultured in large droplets on day 7. (d) The volcano plot compares the gene expression of organoids grown in small droplets on day 4 and day 7. (e) Volcano plot comparing organoids cultured in bulk collagen gels on day 4 to organoids cultured in large collagen droplets on the same day. (f) Volcano plot illustrating the differentially expressed genes grown in bulk collagen gels on day 4 versus organoids grown in large droplets on day 7. (g) Volcano plot comparing organoids cultured in large droplets on day 4 and on day 7.

(Figure 4.10 e and f).

4.5 Faster maturation of droplet-grown organoids

Accelerated lumen formation in droplet-grown organoids: The transcriptomic data presented in the previous section, along with the observation of a collagen cage as early as day 5 for droplet-grown organoids, indicated accelerated maturation in droplet-based organoids compared to conventional bulk organoid culture conditions. To further assess this hypothesis, we compared the timing of specific morphological features between the two culture conditions. Between days 7 and 9, conventional bulk organoid culture transitions through the branch elongation phase. During this phase, the cells contained within the organoids' branches typically display a mesenchymal phenotype, resulting in collagen invasion and branch elongation. The elongated shape of the cells during this invasive phase reflects their mesenchymal character (Figure 4.11 a). In contrast, organoids cultured in droplets already display a mesenchymal phenotype on day 4 (Figure 4.11 b). For conventional bulk organoid culture, during the thickening phase, the epithelial cells start to undergo a mesenchymal to epithelial transition. Therefore, the epithelial cells' morphology changes from a more elongated cell shape towards a cobblestone morphology, characterized by a more pronounced square-like cell shape. Lumen formation is initiated as early as day 11 in the final phase of organoid development. A nascent lumen can be distinguished by the accumulation of F-actin and E-Cadherin at the lumen formation site (Figure 4.12 a). Not only did organoids cultured in droplets already display clear signs of epithelialization on day 6, but, contrary to conventional bulk culture, they also exhibited first lumen nucleation sites as early as day 6 (Figure 4.12 b). On day 7, it was already possible to observe the first fully opened lumen (Figure 4.12 c). Therefore, lumen formation was initiated five days earlier than in bulk organoid culture.

Lumen formation also accelerated for organoids cultured in small droplets: We also investigated if spheroids cultured in small droplets displayed signs of lumen formation. We observed that organoids cultured in small droplets adopted an epithelial phenotype on day 3, much earlier than organoids culture in large droplets or in bulk (Figure 4.13, day 3). More importantly, the adopted morphology closely resembled that of rosettes, which are characteristic of bulk-grown organoids where they play an essential role in mediating lumen formation.² From day 4 to 5, the spheroids' cell mass increased by proliferation (Figure 4.13, day 4). On day 6, fully formed lumens were observed for spheroids cultured in small droplets (Figure 4.13, day 6). Most likely, the accelerated adoption of an epithelial phenotype is caused by the altered mechanical properties of the small collagen droplets. Spheroids growing in small droplets were exposed to a significantly softer supporting matrix than those growing in bulk collagen gels. We hypothesize that the PDACs lacked sufficient support from the surrounding hydrogel, leading to their adhesion to one another

²The mechanisms underlying rosette formation have been described by Marion Raich, whose work is currently under preparation for publication.

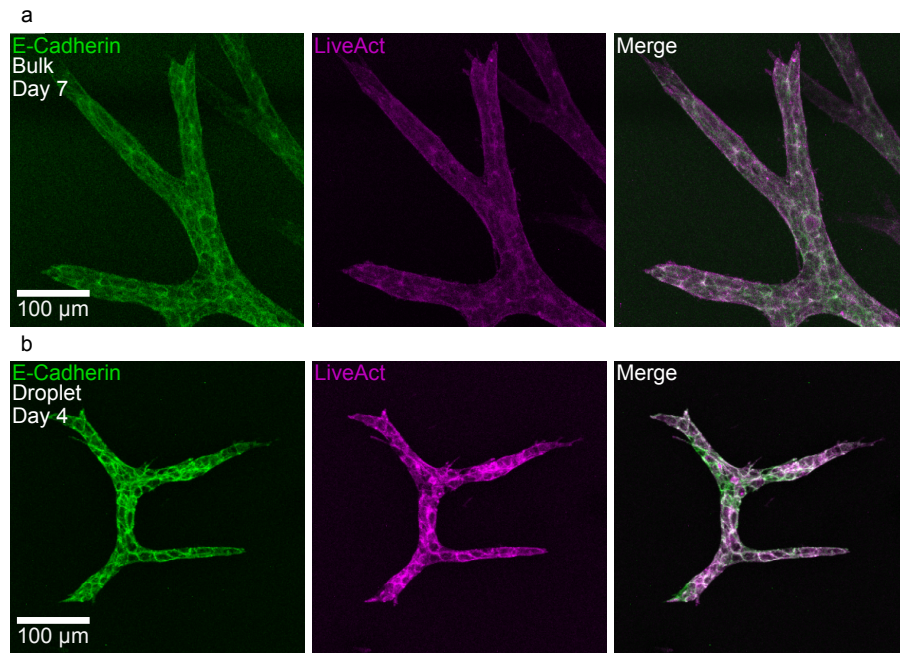


Figure 4.11: Droplet-grown organoids in large droplets display a mesenchymal phenotype during early organoid development. (a) Bulk-grown organoids display a mesenchymal phenotype up to day 9 of organoid culture. (b) Organoids grown in large droplets display a mesenchymal phenotype on day 4 of organoid culture.

and, consequently, a shift toward an epithelial phenotype.

Transcriptomic analysis of organoids grown in large droplets compared to organoids grown in bulk: Our imaging data showed that organoids grown in large droplets developed key morphological features and reached developmental hallmarks five days earlier than those in bulk organoid culture. Given the enhanced morphological similarity between organoids cultured in large droplets and those cultured in bulk, we analyzed their transcriptome, resolving distinct developmental phases to obtain further evidence for an accelerated maturation of organoids cultured in collagen droplets. In a previous study, RNA sequencing of bulk-grown organoids showed that PDAC organoids underwent a mesenchymal to epithelial transition. In the early stages of organoid development, specific genes related to a mesenchymal phenotype were upregulated, whereas as the organoids matured toward a more epithelial phenotype, those genes were downregulated. Similar gene expression patterns were observed for various pathways, such as cell cycle progression and extracellular matrix interactions [164]. To evaluate how geometrical confinement effects influence gene expression dynamics, we analyzed the top 200 most variably expressed genes across all samples, using Euclidean clustering to visualize a gene expression heat map. In bulk culture, we observed the expected oscillatory pattern, where the expression of specific genes switched from day 7 to day 13 as the organoids matured (Figure 4.14 a).

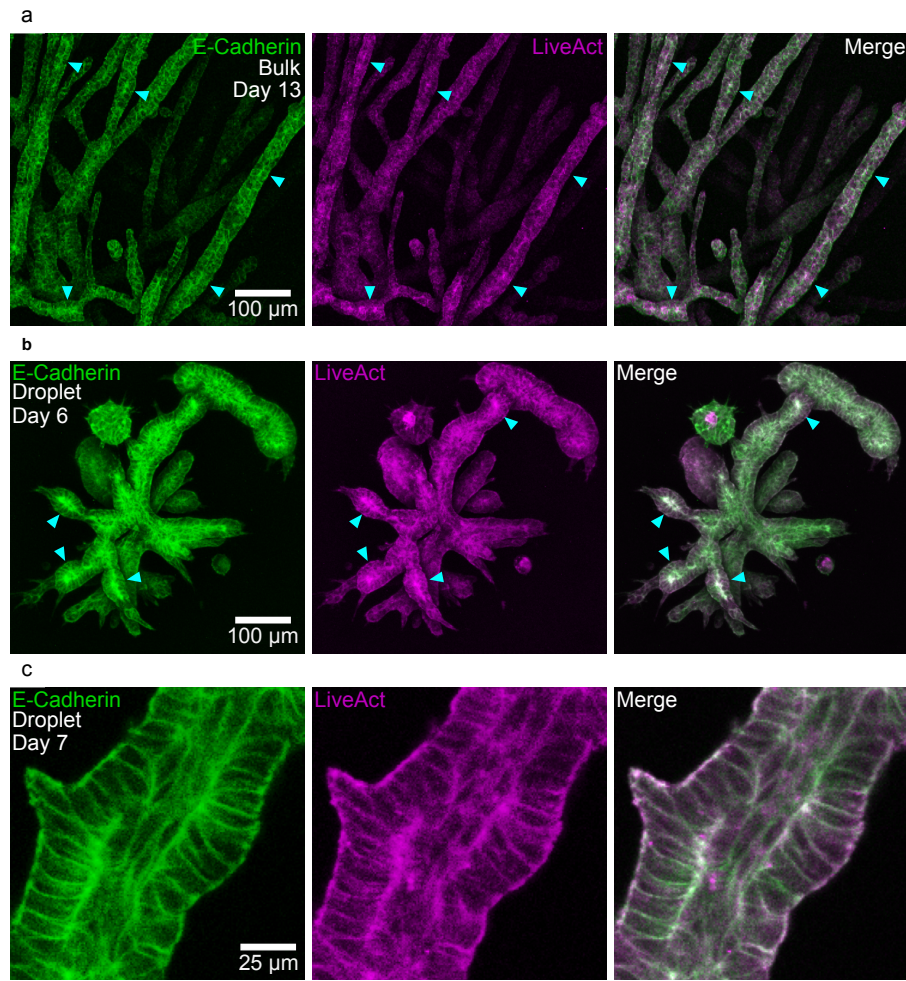


Figure 4.12: Lumen formation is accelerated in droplet-grown organoids. (a) Lumen formation typically starts in bulk-grown organoids between days 11 and 13. The cyan arrows highlight the onset of lumen formation with an upregulation of E-Cadherin and LiveAct. (b) In droplet-grown organoids, early stages of lumen formation can be observed as early as day 6 with enhanced localization of E-Cadherin and LiveAct at the nascent lumen formation sites. The organoid is cultured in a large droplet. (c) On day 7, many organoids cultured in large droplets display a fully formed lumen.

The data set showed the downregulation of specific gene clusters and the upregulation of other clusters as the organoid developed toward a more mature stage. Day 9 represented a transition point, with partially overlapping gene expression profiles between the two states. In droplet-grown organoids, not only did a similar switch in gene expression occur, but the gene expression profile on day 9 also closely resembled the profile on day 13 in bulk culture, consistent with the accelerated maturation timeline observed in their morphology (Figure 4.14 a). Given this shift in the timing of gene regulation, we conducted a deeper analysis of the expression patterns across different gene clusters. We identified a switch

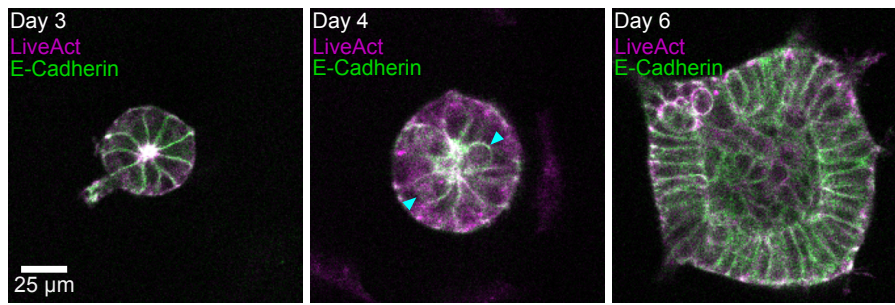


Figure 4.13: Lumen formation in organoids grown in small droplets. PDAC organoids expressing fluorescent E-Cadherin (green) and LifeAct (magenta). The reduced mechanical stiffness experienced by cells cultured in small collagen droplets favors the adoption of an epithelial phenotype early on (day 3). The morphology of the spherical cell assembly, together with cell divisions occurring during organoid growth (cyan arrows), recapitulate closely key hallmarks observed during lumen formation in bulk organoids (day 3 and day 4). At day 6, the spheroids display a well-developed lumen.

in the gene expression pattern involved in critical metabolic processes, such as lumen formation and branching morphogenesis, relevant during PDAC organoid maturation (Figure 4.14 b and c) [164], [170]. For later time points (day 13 bulk and day 9 droplets), we detected a population of overexpressed genes involved in cancer survival and lipid metabolic processes characteristic for enhanced metabolic activity during tumor progression (Figure 4.14 e) [212]. In droplet-based organoid culture on day 9, genes responsible for cancer survival and anti-apoptotic processes follow the characteristic upregulation pattern observed in bulk organoid culture on day 13, indicating faster maturation (Figure 4.14 d).

4.6 Outlook

Impact: Although the underlying mechanism for accelerated maturation remains unclear, our findings match previous observations of reduced differentiation timescales for stem cell aggregates cultured within patterned microcavities [156]. Similarly, faster organoid maturation has been reported for bioprinted organoids contained in microdrops [184]. However, in both studies, the organoids formed through the assembly of multiple cells. Therefore, it remained unclear whether accelerated organoid maturation resulted from a speed-up in cell aggregation due to spatial confinement or from altered culture conditions. If the latter is true, organoids derived from single cells could benefit from reduced culture times. Our experiments confirm that organoids growing from a single cell also exhibit accelerated maturation. These findings hold great potential for high throughput applications in personalized medicine. In a clinical context, faster organoid growth could reduce the time required for component screens, allowing quicker treatment decisions, which may improve patient outcomes and survival. Furthermore, understanding potential shifts in developmental timescales is essential to coordinate drug administration at the correct de-

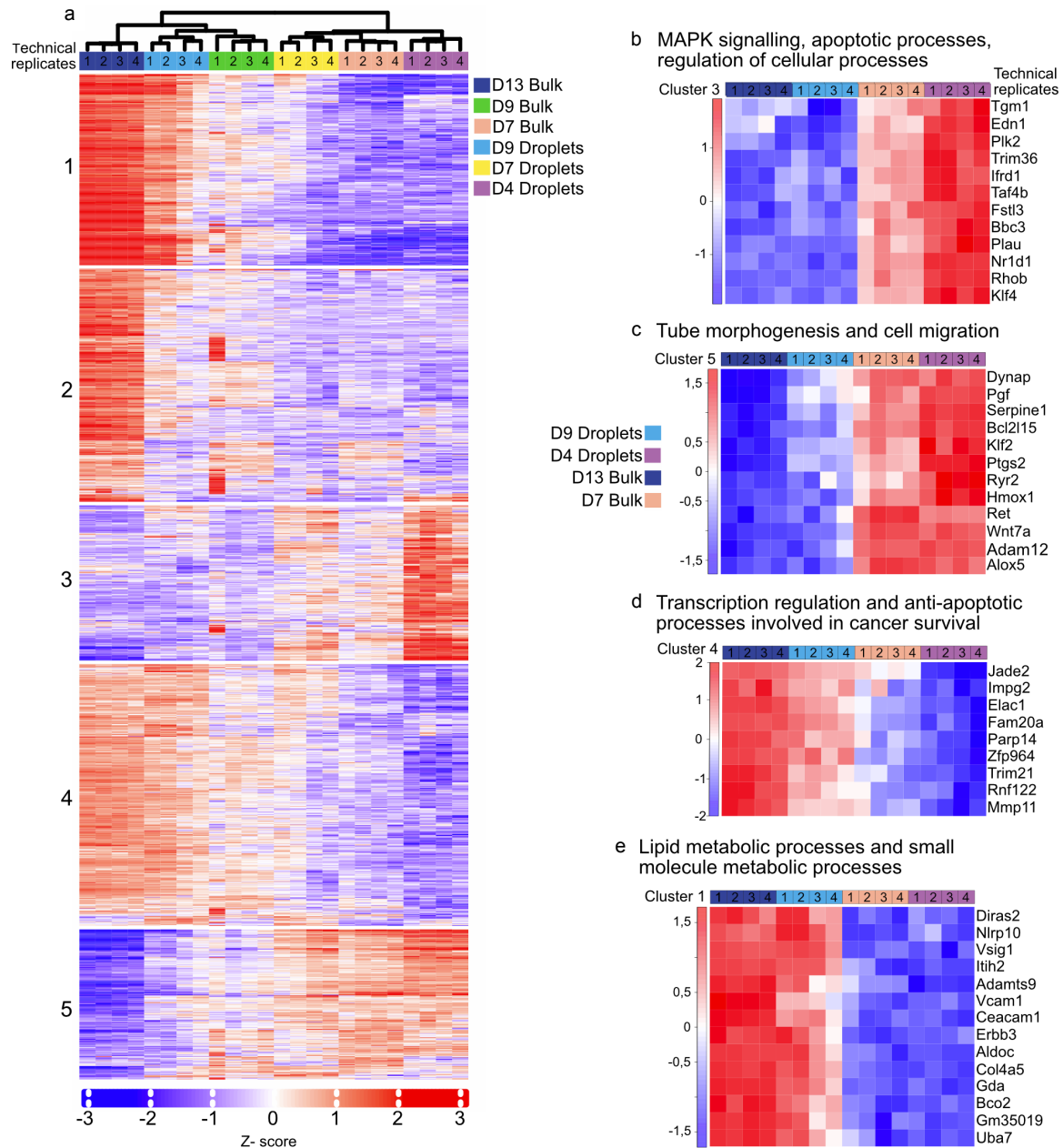


Figure 4.14: RNA gene expression profiles comparing organoids grown in bulk gels to those in droplets. (a) K-Means clustering with five clusters ($k = 5$) using 2000 genes with the most variable expression level between different growth conditions: droplets day 4, 7, 9 and bulk day 7, 9, 13 ($n = 4$ individual experiments), $FDR = 0.05$. (b) GO Biological Processes enrichment analysis from cluster 3 for selected conditions (droplets day 4, 9 and bulk day 7, 13) highlights genes involved in MAPK signaling and apoptosis-related processes. (c) Gene expression heatmap (cluster 5) for selected genes regulating tube morphogenesis and cell migration. (d) Heat map for selected genes (cluster 4) involved in transcription regulation and anti-apoptotic pathways facilitating cancer survival. (e) Gene expression heatmap (cluster 1) of a subset of genes involved in lipid and small molecule metabolism.

velopmental phase. This could improve the generation of robust and reliable data sets from high-throughput screenings.

Potential directions of droplet-based organoid culture: Combining organoid culture with droplet-based microfluidics allows to draw upon an extensive repertoire of already existing microfluidic techniques such as droplet sorting, droplet merging and reagent addition to achieve extensive automation of organoid culture [213]. As those techniques are easily compatible with high throughput screening, this emerging branch of microfluidics is prone to significantly advance organoid culture in the coming years. Besides droplet manipulation, droplet-based organoid culture can leverage already existing analysis tools such as ddElisa to achieve droplet-specific readouts on cytokine secretion [214], [215]. Moreover, employing core-shell structures can significantly increase the throughput of spatially patterned organoid culture and allow the precise control of the spatial organization of cellular layers [216]. Indeed, cerebral organoids still lack a scalable solution enabling control over proportion and spatial organization of distinct brain regions and could significantly benefit from adaptation to droplet-based organoid culture [217].

Beyond viability screening using artificial intelligence: Microfluidics-based organoid culture enables high-throughput screens by enhancing the scalability of organoid seeding. Additionally, compartmentalizing single organoids into small hydrogel droplets facilitates their selective manipulation. These droplets can be easily positioned within microfluidic screening arrays, offering a more precise organoid placement than bulk organoid culture. This enhanced accuracy in positioning and the reduced size of droplet-grown organoids result in a more uniform focal plane, thereby accelerating image acquisition and enabling large throughput screening [218]. The combination of microfluidics and organoid culture significantly enhanced the throughput, which can help turn organoid inhomogeneity, previously considered a drawback, into an asset for drug screening readouts. Conventional drug screens typically rely on dose-response curves to quantify the overall viability of bulk organoid cultures. However, this approach overlooks the possibility that distinct organoid phenotypes might respond differently to the same agent. While classifying organoids into numerous phenotypes and quantifying their response was previously a technically complex challenge, artificial intelligence significantly facilitates the task [180]. As a result, organoid-based screens are expected to shift from simple dose-response analyses to a more comprehensive assessment that captures which cellular populations are affected [180], [219]–[221]. When combined with fluorescent protein labeling, such analyses can provide spatiotemporally resolved data sets offering deeper insights into the action mechanism of specific compounds [180]. In our model, we observed that organoids at different maturity levels exhibit distinct spatiotemporal protein expression patterns. Protein expression levels change over time, and their spatial localization also varies depending on organoid maturity. By leveraging artificial intelligence to analyze those spatiotemporal patterns, it will become possible to capture individual responses of organoids to specific treatments, thereby further enhancing the predictive power of high throughput screens.

Conclusion

Organoids progress through distinct developmental stages during which the cells self-organize to establish an intricate morphology closely resembling that of native tissue. Therefore, 3D cell culture systems hold great potential for pharmaceutical research. However, organoid culture still faces several limitations, such as poor reproducibility, high cost, and scalability issues.

To pave the way toward more reproducible organoid models, understanding the physical mechanisms underlying the self-organization of multicellular systems is crucial. To this end, this thesis established a microfluidic setup to guide collective endothelial cell alignment by applying shear stress. Large-scale, time-resolved imaging revealed that endothelial cell alignment dynamics follow a three-stage process, which includes a temporary dealignment. During this intermediate stage, the active stresses within the endothelial monolayer lead to the nucleation of topological defect pairs, which are stabilized through string excitations. Physical modeling showed that these string excitations may constitute a novel mechanism for long-range force transmission. The physical concepts presented in this thesis may also be relevant in different contexts, such as morphogenesis, where tissue layers moving against one another also give rise to shear stresses. Future studies will aim to translate the physical principles uncovered here to more complex 3D multicellular assemblies. As a first step, the angiogenesis chip developed in Chapter 3 offers a promising model system for investigating how collective cell dynamics drive the formation of 3D vascular networks to engineer more realistic and functional vascularized tissues.

Another approach to enhance organoid reproducibility and scalability was establishing a microfluidic pipeline for automated organoid seeding. The pancreatic ductal adenocarcinoma organoids developed a branched morphology closely resembling that of bulk-grown organoids while simultaneously displaying accelerated maturation compared to bulk culture conditions. The improved scalability achieved through droplet-based microfluidics will allow the accessible adaptation of 3D cell culture systems for high throughput applications.

Chapter 5

Materials and Methods

5.1 Cell culture

Human aortic endothelial cells: Before seeding, 75 cm² cell culture flasks were treated with 0.2% gelatine diluted in MiliQ (7 ml) for 30 min at 37 °C in the incubator. After incubation, the gelatin solution was aspirated, and the flask was washed three times with phosphate-buffered saline (PBS) (7 ml). Typically, 500 000 cells were seeded into one flask and cultured in the incubator at 37 °C, 5% CO₂, and 80% humidity for one week until confluency. After seeding, the cell culture medium (PeloBiotech, PB-MH-100-2190) was exchanged the next day and then every second day. To avoid stressing the cells, all solutions were warmed to 37 °C before being added to the cells. A confluent 75 cm² flask commonly yields $2 \cdot 10^6$ cells/ml. HAOECs were used between passage 5 and passage 9. For freezing, the cells were transferred to cell culture freezing medium (Pelobiotech, PB-6916) and split into 4 equal parts. Custom-made cell culture medium was also used and consisted of 70% FBS, 20 % cell culture medium, and 10 % Dimethyl sulfoxide (DMSO). The custom-made medium was filtered with a 0.22 μ m syringe filter. For freezing, the cells were not counted. To achieve consistent densities in the flow channels, counting the cells before seeding into the flow channels was necessary.

HAOEC cell culture in flow channels: For shear flow experiments, μ -slide I Luer ibi-Treat (Channel height: 0.2 mm) were incubated for 1 h with 40 μ g/ml collagen at 37 °C. After three washing steps with PBS, 50 μ l of HAOECs at a seeding concentration of $3 \cdot 10^6$ up to $6 \cdot 10^6$ cells/ml were seeded into the flow channel and left at 37 °C for 1 h to allow them to form first adhesions. Then, the cell culture medium was added to the reservoirs, and the HAOECs were left overnight to adhere fully to the substrate. The confluent cell layer resulted in 12 000 up to 40 000 cells per a field of view of 40 mm². The cell culture medium, PBS, and the flow channel slides used during this step were kept in the incubator one day before cell seeding to prevent bubble formation in the flow channels.

Perfusion cell culture: To avoid bubble formation, the cell culture medium and perfusion

set were kept in the incubator one day before the flow experiment. The ibidi pump system and perfusion set (ibidi: orange) were prepared according to the manufacturer's instructions [222]. After the pump system was calibrated, the flow channel was connected. To allow the cells to adapt to the shear stress, a flow program slowly increased the flow velocity until reaching the desired shear stress of 15 dyn/cm², respectively 20 dyn/cm² after 1 h.

Perfusion cell culture at low shear stress: To assess the dynamics of the endothelial monolayer in the absence of flow, we stopped the flow during two distinct time points within the steady-state alignment phase. First, we stopped the flow during the early stage of the steady-state alignment phase (III), which corresponded to 36 to 42 hours after flow onset. Secondly, we stopped the flow at very late stages during the steady-state alignment phase, corresponding to 3 days after flow onset. The pump used for generating the flow operates within a pressure range from 5 to 95 mbar. The low shear stress regime lies below the working range of the pump, we estimate that the shear stress during the experiment remained below 5 dyn/cm².

Human umbilical vein endothelial cells: Before seeding, 75 cm² cell culture flasks were treated with 100 µg/ml collagen type I (Corning) diluted in MiliQ for 30 min at 37 °C. After incubation, the collagen solution was aspirated and the flask was washed three times with PBS (7 ml). Typically, 500 000 cells were seeded into one flask and cultured in the incubator at 37 °C, 5% CO₂, and 80% humidity for one week until confluency. After seeding, the cell culture medium (Lonza, Endothelial Cell Growth Medium CC-3162) was exchanged the next day and then every second day. To freeze the cells, we followed the protocol introduced for HAOEC. HUVEC were maintained in culture until passage 5.

Pancreatic ductal adenocarcinoma cells: Primary tumor cells (PDAC) were provided by the laboratories of Prof. Maximilian Reichert and Prof. Dieter Saur (Technical University of Munich). Pancreatic ductal adenocarcinoma cells were cultured in high glucose DEMEM medium with 1% of Penicillin/Streptomycin (Thermo Fisher Scientific) and 10% Fetal Bovine Serum (FBS) (Sigma). Cells were grown at 37 °C with 5% of CO₂ and 80% humidity. Media was exchanged every 48 hours, and cells were passaged by trypsinization upon 80–85% of confluency.

5.2 Microscopy

Phase contrast imaging: Phase contrast imaging was performed using a Leica DMI8 Thunder Imager and a 10x (0.32 NA) air objective (PL FLUOTAR 10x/0.32). We also used Leica Mica with a 10x (0.32 NA) air objective (PL FLUOTAR 10x/0.32).

Large-scale live cell imaging under perfusion: To gather sufficient statistical data, we stitched high-resolution images over a scale of several millimeters for up to 64 hours.

The area of the resulting field of view was 40 mm². The ibidi stage-top incubation system was adapted for live cell imaging with a custom-built lid to accommodate the tubing. The fluidic unit of the pump system was kept in a nearby incubator at 37 °C and 5 % CO₂. Through a small opening in the rear of the incubator, the flow channel was mounted on the microscope stage while remaining connected to the pump system. Images were acquired every 20 minutes over at least 2.5 days.

Epifluorescence imaging: For epifluorescent microscopy, we used Leica Thunder with a 10x (0.32 NA) air objective (PL FLUOTAR 10x/0.32). The speed of the stage was decreased to avoid extensive drifting of the droplets during image acquisition.

Confocal imaging: Samples were analysed using scanning confocal microscope Leica Sp5. Typically, we used a 10x (0.30 NA) air objective for imaging (PL FLUOTAR 10x/0.30). To achieve a better resolution, we also used a 20x (0.70 NA) water immersion objective (HC PL APO 20x/0.70). We also used Leica Mica with a 10x (0.32 NA) air objective (PL FLUOTAR 10x/0.32). To image the droplets on the Mica, we reduced the speed of the microscopy stage to reduce the movement of the droplets.

SiRDNA staining: For nuclei visualization, PDAC organoids were incubated with SiRDNA (Spirochrome SPY650-DNA SC501) for a minimum of 1 h before imaging at 1–2 µg/mL.

5.3 3D culture of PDAC organoids

Preparation of collagen gels for bulk organoid culture: For organoids growing in bulk collagen, we used a previously published protocol [151], [170]. Briefly, PDAC cells (9591) with confluency 80-85% were detached using trypsin and prepared to a final concentration of 500 cells/ml of DEMEM media. Next, the cell suspension was mixed gently with collagen type I solution (rat tail from Corning, NaOH to stabilize pH and neutralizing buffer). The solution was incubated for 1 h at 37 °C to polymerize the collagen fibers, and DMEM media was added after detaching the gels from the dish walls. Media was changed first after 72 hours and then every 48 hours.

Preparation of fluorescently labelled collagen: Collagen was fluorescently labeled as previously described [163]. Briefly, collagen was dialyzed at 4 °C to adjust the pH to 7. Subsequently, collagen was conjugated with Atto 488 through overnight incubation at 4 °C. To remove any non-bound dye, dialysis was performed for 8 hours, followed by an overnight dialysis step with acid to avoid any premature polymerization. The fluorescently labeled collagen was stored at 4 °C until further usage. Before preparing the collagen solution to embed the PDAC cells, the fluorescently labeled collagen was mixed with the collagen stock solution at a volume ratio 1:5.

5.4 Photolithography

Chip design: We used CorelCad to generate the designs for the microfluidic chips.

Deposition of SU8: To deposit the desired thickness of SU8 on the wafers we used a protocol that was established by Perry Ellis in the group of David Weitz at Harvard University. We prepared a mixture of SU8-3005 and Thinner according to the values listed in table 5.1. The wafers are placed on a heating plate that has been calibrated to ensure a horizontal orientation of the hotplate's surface, and that has been placed inside a fume hood. After the required amount of SU8-thinner mixture has been added to the wafers, the heating plate is turned on and heated to 95 °C. The thinner evaporates overnight, and the SU8-coated wafer can be used for photolithography.

Thickness [μm]	SU8 [g]	Thinner [g]	Final volume [ml]	Amount of liquid for 3" wafer [ml]
200	15.80	19.29	35	5
300	23.70	18.96	42	6
500	39.51	18.29	56	8
600	47.41	4.66	49	7

Table 5.1: Dilutions to coat wafer at specific SU8 thickness. The SU8 and thinner are weighted because of the high viscosity of SU8. The last column provides the amount of volume that needs to be added to each wafer.

Photolithography: The SU8-coated wafers were exposed to UV light using a benchtop illumination chamber (ibidi). The exposure energy used for the corresponding thickness of SU8 is listed in table 5.2. After UV exposure, the wafers were baked at 65 °C for 5 minutes and at 95 °C during the post-exposure bake. Again, the times required for the post-exposure bake at 95 °C are provided in table 5.2. The wafers were immersed in SU8 developing solution (PGMEA, Sigma-Aldrich) to develop the structures. The development times required for each thickness are provided in table 5.2. After developing the structures, the samples were baked at 150 °C for 5 minutes during the post-exposure bake.

Fabrication of microfluidic chips from PDMS: After master fabrication, PDMS was mixed with curing agent at a ratio of 10:1 (Sylgard 182 Silicone Elastomer, Dow Corning, United States) and poured on the master. To remove air bubbles, the master was then put under vacuum for 15 minutes. Then, the PDMS was cured by overnight incubation in the oven at 70 °C. The next day, the PDMS replica was peeled off the mold.

For the microfluidic chips used for droplet generation, the channel inlets and outlets were

Thickness [μm]	Exposure energy [mJ/cm ²]	Post-exposure bake [min]		Development time [min]	Hard bake [min]
		65 °C	95 °C		150 °C
200	311	5	13	15	5
300	545	5	21	21	5
500	655	5	27	27	5
600	720	5	33	35	5

Table 5.2: Parameters used for photolithography for different SU8 thicknesses.

generated by using a biopsy puncher of 1 mm diameter. The PDMS was bonded to a glass coverslip after treatment with oxygen plasma. After bonding, the microfluidic chip was incubated at 70 °C for at least 1 hour. Eventually, the microfluidic chips were treated with commercially available Aquapel for 5 minutes at room temperature to obtain a hydrophobic channel surface. The chips were then air dried and incubated at 70 °C for 15 minutes.

For the microfluidic chips used for angiogenesis, the channel inlets and outlets were generated by using biopsy punchers of 1 mm, 4 mm, and 6 mm diameter. To increase the capacity of the reservoirs, we added an additional layer of PDMS that contained holes at the locations of the reservoirs. To ensure convenient access to the channel inlet of the hydrogel chamber, we cut the PDMS accordingly. After plasma treatment of the additional layer and the microfluidic chip, the cutout layer was aligned to the chip and bonded on top of it. Then, the whole setup was bonded to a glass coverslip after treatment with oxygen plasma. After bonding, the microfluidic chip was incubated at 70 °C for at least 1 hour.

5.5 Droplet-based organoid culture

Cell preparation for organoid seeding on the microfluidic chip: The collagen solution for organoid seeding on the microfluidic chip was prepared as previously described for bulk organoid culture [151], [163]. To achieve the incorporation of single cells into collagen droplets, the PDAC cells were mechanically separated after Trypsin treatment by aspiration with a syringe needle (28G, Braun). The remaining doublet cells were removed by using a cell strainer (40 μm , Corning). To generate small droplets with single incorporated cells, the PDAC cells were diluted to a final concentration of 30 000 cells/ml in collagen. To obtain single incorporated cells in large droplets, the cells were diluted to a final concentration of 5 000 cells/ml.

Droplet production for organoid culture on the microfluidic chip: For droplet production, fluorinated oil Novec 7500 (Iolitec, Germany) with 3% (w/w) surfactant RAN008

(RAN Biotechnologies, United States) was used to generate the small droplets. For the production of large droplets, we used fluorinated oil with 5% surfactant. We loaded the oil with surfactant into 5 ml BD plastic syringes to initiate droplet production. The collagen solution containing the cells was loaded into either a 1 ml BD glass syringe or a 5 ml BD plastic syringe, depending on the required droplet amount. The syringes were connected to the microfluidic chip using fluid dispenser tips (PT-050-27, Drifton, Denmark) and polyethylene micro tubing (PE/2, Scientific Commodities, United States). The syringes were operated using programmable or computer-operated syringe pumps (KDE Technologies, Harvard Pumps, or Landgraf HLL). Typical operating speeds for droplet production were 2000 $\mu\text{l}/\text{h}$ and 1000 $\mu\text{l}/\text{h}$ for the oil and collagen to generate small droplets or 1000 $\mu\text{l}/\text{h}$ and 1000 $\mu\text{l}/\text{h}$ for the oil and collagen to generate large droplets. After droplet production, the droplets were collected in a 2 ml Eppendorf tube at 37 °C to initiate collagen polymerization immediately. These tubes were filled with 200 μl 5% RAN dissolved in fluorinated oil. After droplet production, the Eppendorf tubes were transferred to an incubator and kept at 37 °C and 5% CO₂ for 1 hour. After collagen polymerization, the fluorinated oil with surfactant was removed, and the droplets were washed three times with only fluorinated oil. After the last washing step, the fluorinated oil was removed, and fluorinated oil with 4% PFO (v/v) was added to the droplets to break the emulsion. Cell culture medium was added on top of the oil phase and the droplets were left for 5 minutes to transition into the aqueous phase. The droplets were centrifuged using a tabletop centrifuge for 5 minutes to improve droplet recovery. After centrifugation, the pellet formed by the droplets at the interface of the oil and cell culture medium was aspirated with a pipette, and the droplets were transferred into the cell culture medium. The droplets were distributed in 6 well plates. For microscopy imaging, the droplets were transferred to achieve a final concentration of 750 droplets per well, whereas for RNA sample collection, the desired final concentration was 1500 droplets per well.

5.6 RNA sequencing of droplet and bulk-grown organoids

Preparation of RNA samples for mRNA sequencing: 2D PDAC cells were cultivated until 80-85% of confluency, detached, and collected by spinning down (300 g) to obtain cell pellets. Organoids on days 4, 7, 9, and 13 were subjected to collagenase treatment (2.6 mg/ml for 1.3 mg/ml collagen and 6 mg/ml for 3 mg/ml collagen) to remove the organoids from the collagen gel. Next, the solution with organoids was spun down (1200 rpm), and the pellet was gently washed with PBS. Total RNA was extracted from the organoids and 2D samples using RNeasy Mini Kit (Qiagen, Cat. No. 74104) according to the manufacturer's instructions. All steps were performed in RNase free environment. Next, RNA quality was determined using Agilent RNA 6000 Nano Assay. A total of 4 technical replicates was collected for each experimental condition. Library preparation was performed on samples with RIN ≥ 7 and concentration ≥ 30 ng/ μl . mRNA sequenc-

ing was performed by BMKGENE company using Illumina Novaseq X. 6GB of raw data was obtained for each sample. Data was next analyzed using Galaxy Europe server to get normalized read counts [223].

Differential gene expression (DEG) analysis: We used the DESeq2 R package from iDEP for differential gene expression analysis [224].

5.7 Loading of microfluidic angiogenesis chips

Preparation of thrombin stock solution: Thrombin is required to polymerize fibrinogen. To prepare a stock solution, thrombin was dissolved at a concentration of 100 U/ml in 0.1% w/v bovine serum albumin (BSA) in MiliQ and stored at -80 °C.

Preparation of VEGFA stock solution: VEGFA was dissolved in 0.1 ml H₂O to 1 mg/ml. Then, it was diluted to 25 µg/ml in 0.1 % BSA solution and prepared into 10 µl aliquots which were stored at -80 °C.

Preparation of fibrinogen: Fibrinogen was dissolved approximately 3 hours prior to usage in PBS at a concentration of 5 mg/ml. To accelerate its dissolution, it was maintained at 37 ° for 15-30 min. Meanwhile, Thrombin was thawed and diluted to 4 U/ml in Endothelial Basal Medium (Lonza). Fibrinogen immediately gels once mixed with thrombin. To prepare several chips, we aliquoted 10 µl of fibrinogen and thrombin into separate small eppendorf tubes. These tubes were stored on ice until further usage. To load the Fibrinogen solution into the hydrogel chamber, we prepared two pipettes: one for pipetting volumes ranging from 10-100 µl and another one for volumes ranging from 1-10 µl. In the beginning, we found it helpful to fill the hydrogel chambers under the cell culture microscope to avoid leakage of the hydrogel solution into the adjacent channels. To initiate fibrinogen polymerization, thrombin was added to the eppendorf tube containing the fibrinogen. Then, the fibrinogen solution was mixed by pipetting up and down. Afterward, the solution was filled into the hydrogel chamber. The final concentration of the fibrinogen was 2.5 mg/ml. The devices were placed into a humidified Petri dish and allowed to polymerize at room temperature for 15 min.

Preparation of collagen solution: Collagen was prepared similarly to bulk collagen gels used in organoid culture. The collagen was diluted to a concentration of 2.3 mg/ml. After adding the neutralizing buffer, the pH was adjusted to 7 using NaOH. The devices were placed into a humidified Petri dish and allowed to polymerize at 37 ° for 30 min.

Preparation of fibrinogen-collagen mix: Fibrinogen and thrombin were prepared as described in the previous paragraph. Then, 10 µl of collagen type I at a concentration of 2.4 mg/ml and 5 µl of fibrinogen at 5 mg/ml were added into a small Eppendorf tube. Thrombin was aliquoted into a separate Eppendorf tube at a volume of 5 µl. Here, two

pipettes operating at volumes ranging from 1-10 μl were used. To initiate the polymerization of the hydrogel, thrombin was added, and the solution was mixed. Then the fibrinogen-collagen mix was transferred to the hydrogel chamber of the angiogenesis chip. The devices were placed into a humidified Petri dish and allowed to polymerize at room temperature for 15 min.

Fibronectin coating: Fibronectin was diluted to a concentration of 100 $\mu\text{g}/\text{mL}$ in PBS. Then, we injected the fibronectin solution into the channel designated for the endothelial cells. The channel was incubated for 30 minutes with the fibronectin solution to achieve a surface coating to accelerate the adhesion of the seeded endothelial cells. After incubation, the channel was washed with cell culture medium. The endothelial cells were detached by trypsinization and diluted to $3 \cdot 10^6$ cells/mL. The cells were injected into the cell culture channel, and the microfluidic device was flipped by 90° to facilitate the adhesion of endothelial cells at the interface to the hydrogel chamber. The devices were placed into sterile pipette tip boxes. To maximize cell adhesion, we left the device to incubate for 1 h at 37° . After incubation, fresh media was supplemented into the empty media channel. To establish a hydrostatic pressure, we filled the reservoirs opposite the cell culture channel with 100 μl cell culture medium. Medium exchange was carried out every 24 hours. To further promote angiogenesis, the cell culture medium filled into the reservoir was supplemented with 25 ng/ml VEGFA.

5.8 Data analysis

Characterization of nematic director field: We extracted the nematic field from the phase contrast images using a custom Python3 script based on the method from reference [225] as previously described in [226]. We computed the local nematic director $\mathbf{n} = \mathbf{t} \otimes \mathbf{t}$ using the tangent vector $\mathbf{t} = (I_y, -I_x)$ to the gradient of the intensity of the image $\nabla I(x, y) = (I_x, I_y)$ within a box of dimensions $L \times L$. From the local nematic director, we extracted the eigenvalues $n_1 > n_2$ and the corresponding eigenvector $\mathbf{e}_1$ to the biggest eigenvalue, which provided the nematic director associated with the pixel in the center of the box. Here, we used a box of $130 \mu\text{m} \times 130 \mu\text{m}$ to compute the nematic director for a pixel. The nematic director field was computed for each pixel, and the spacing between two pixels is $\approx 5.2 \mu\text{m}$.

Detection of nematic defects: We obtained the $\mathbf{Q}$ tensor from the nematic director field. The defect cores were then identified as the local maxima, respectively minima, of the charge density $q = \frac{1}{4\pi}(\partial_x Q_{xx} \partial_y Q_{yy} - \partial_x Q_{yx} \partial_y Q_{xx})$ where $|q| > 0.1$. Only every 5th pixel in the nematic director field was considered for defect detection.

Extraction of nearest neighbour distance: Defects were considered for kth nearest neighbor (knn) analysis if the defects were present for at least 4 h. We used trackpy to

obtain and filter the defect trajectories.[227] From the defect trajectories, we constructed the knn-distance matrix, which we used to extract the knn distance for $k = 0, 1, 2, 3$.

Correlation functions Taking the projection of the orientation parallel to the preferred axis δn allowed us to identify how far away the director was from the preferred axis. Using this, we observed how regions of similar orientation coarsened by looking at the correlation functions $C\delta n_{\perp}(\Delta r_{\parallel})$ and $C\delta n_{\perp}(\Delta r_{\perp})$, where we split up the correlations along the perpendicular $\Delta r_{\perp}$ and parallel $\Delta r_{\parallel}$ direction to the preferred axis. We extracted a length scale ξ from the correlation functions by determining the length when the correlation function reached the value $1/\epsilon$.

String visualization in experiments: The nematic director $n = [\cos(\alpha), \sin(\alpha)]$ was defined both in orientation region region $0 \leq \alpha_1 \leq \pi$ (first and second quadrant) and $-\pi/2 \leq \alpha_2 \leq \pi/2$ (first and fourth quadrant). The parallel and perpendicular strings were defined to go between two resolution points if the absolute angle difference $|\Delta\alpha_1|$ or $|\Delta\alpha_2|$ was larger than $\pi/2$, respectively.

Length measurement for parallel and orthogonal strings: From the absolute angle difference obtained for string visualization, a binary image was created by setting the points with $|\Delta\alpha_1|$ or $|\Delta\alpha_2|$ larger than $\pi/2$ to one and any other point to zero. Eventually, string length was measured by obtaining the skeletons from the segmented features.

String visualization in simulations: The nematic director $n = [\cos(\alpha), \sin(\alpha)]$ was defined both in orientation region region $0 \leq \alpha_1 \leq \pi$ (first and second quadrant) and $-\pi/2 \leq \alpha_2 \leq \pi/2$ (first and fourth quadrant). The parallel and perpendicular strings were defined to go between two resolution points if the absolute angle difference $|\Delta\alpha_1|$ or $|\Delta\alpha_2|$ was larger than $\pi/2$, respectively.

Characterization of the droplet diameters: Collagen droplets were segmented using Cell Pose 2.0 running on Google Colab [228]. The diameter was extracted from the binary images using the Scikit image package.

Measuring cellular incorporation frequency into droplets: After droplet generation, droplets in 6 well plates were screened for the amount of encapsulated cells.

Counting the amount of spheroids and branched structures present among organoid phenotypes: Four days after droplet generation, droplets in 6 well plates were screened for the number of structures that developed into spheroids and for the number of structures that displayed an invasive phenotype with the ability to form branches.

Droplet and organoid segmentation with feature extraction: Binary masks of 2D projected images of collagen droplets containing PDAC organoids and bulk-grown organoids were analyzed using the selection tool in ImageJ (ver. 1.54f) [229]. Python and the scikit-image package were used to analyze morphological features.

Appendix A

Literature summary of angiogenesis assays

Table A.1: Literature summary of experimental conditions used for angiogenesis.

EC	Co-culture	Matrix	Interstitial Flow	Growth factors	Ref.
HUVEC	-	Collagen and fibrin	Value not available	250 nM S1P 50 ng/ml PMA	[139]
HUVEC	-	Collagen with fibronectin	7-430 $\mu\text{m/s}$	20 ng/ml VEGFA	[230]
HUVEC	-	Collagen and collagen-HA mix	11 $\mu\text{m/s}$	-	[127]
HUVEC	HLF and cerebral organoids	fibrin-collagen mix	-	100 ng/ml CYR61 50 ng/ml HDGG	[111]
HDMEC	HLF and U87MH spheroids	fibrin	-	-	[113]
hiPSC-ECs	hiPSC-CMs hiPSC-CFs hBVPs	fibrin	Value not available	50 ng/ml VEGF	[119]

Continued on next page

Table A.1: Literature summary of experimental conditions used for angiogenesis. (Continued)

EC	Co-culture	Matrix	Interstitial Flow	Growth factors	Ref.
hiPSC-ECs	hiPSC-pericytes and cerebral organoids	matrigel	-	100 ng/ml VEGFA	[120]
HUVEC	HLF	fibrin	2 $\mu\text{m/s}$	-	[121]
HDLMEC	-	fibrin	1-6 $\mu\text{m/s}$	100 ng/ml VEGFC 100 ng/ml ANG-1 100 ng/ml HGF	[124]
HUVEC HMVEC	-	collagen	-	75 ng/ml VEGF 75 ng/ml MCP-1 75 ng/ml HGF 75 ng/ml bFGF 500 nM S1P 75 ng/ml PMA	[125]
HUVEC	HLF	fibrin-collagen mix	-	0-50 ng/ml VEGF	[117]
HUVEC	NHF	fibrin	1 $\mu\text{m/s}$	50 ng/ml VEGFA	[118]
HUVEC	HDF	fibrin	-	-	[128]
HDMEC	-	Fibrin, collagen, and fibrin-collagen mix	-	30 ng/ml VEGF 25 ng/ml bFGF	[137]
HUVEC	HLF	Fibrin-collagen mix	-	-	[138]
HDMVEC	NHLF	fibrin	1 $\mu\text{m/s}$	50 ng/ml VEGFA 100 ng/ml VEGFC 50 ng/ml bFGF 1 μM S1P	[140]
HUVEC	NHLF	fibrin	0.3-1 $\mu\text{m/s}$	100 ng/ml VEGFA 1 μM S1P	[141]

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Acknowledgements

This thesis was made possible by the support of many. In particular, I would like to thank

- My Doktorvater Prof. Andreas Bausch: Thank you so much for giving me these projects, for always being open to developing new ideas, and for providing the supervision needed to guide the projects in the right direction. I would also especially like to thank you for providing me with the opportunity to go abroad. Beyond broadening my knowledge, this research stay impacted me in so many ways for which I will always be very grateful.
- Prof. David Weitz: Thank you for giving me the opportunity to visit your lab during my research stay at Harvard. This was an incredible time, filled with so many interesting conversations and amazing learning opportunities. I am also particularly grateful to the Experimental Soft Condensed Matter group for receiving me so kindly and sharing their knowledge with me.
- Prof. Amin Doostmohammadi and Kristian Thijssen: Thank you for this very pleasant collaboration. I am deeply grateful you always took the time to answer my questions and for helping me begin to understand active nematic systems.
- The E22/E27 chair: Thank you for always providing such a stimulating and welcoming working atmosphere. A special thanks goes to the organid people: Samuel, Marion, Sophie, Caro, Daphné, and Ania, and of course my office mates Timon and Chiao.
- My students: Florian, Tilman, Daniel, Bastian, Joachim, and Clara. Thank you for your contributions and hard work.
- The TAs Gabi, Moni, Karin and Hieu: Thank you for always taking such great care of the lab and making all of this possible.
- The secretaries Nicole, Krisztina, and Katerina: Thank you for always taking the time to answer my questions.
- My friends and family: Thank you for always supporting me, even through difficult times. A special thanks goes to my parents and my sisters, Marie and Veronika, and to Paula, who feels like family to me.

- My partner, Martin: Thank you for always supporting me throughout this thesis and for always having my back. I could not have done this without you.