



From Pattern Formation to Microphysiological Systems

Combining Genetic Engineering with Microfluidics and Bioprinting

Anna Christina Jäkel

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Abstract

Engineering spatial organization in synthetic biological systems is a central challenge in biofabrication applications. Pattern forming processes serve as a fundamental mechanism in biology to organize cells, guide tissue development, and establish spatial structures that define the final form and function of an organism.

In this work, we investigate how biological principles of spatial organization can be applied across multiple length scales, from molecular-level genetic circuits to macroscopic tissue-like structures. First, we demonstrate that non-equilibrium steady-state conditions are essential for pattern formation encoded by genetic circuits, as shown by the emergence of a three-stripe expression pattern in a controlled microfluidic environment. Extending this approach, we embed living organisms in hydrogel matrices to investigate their behavior in a controlled 3D environment. We first focus on *Clostridia* bacteria, to investigate their growth and metabolic activity using fluorescence in situ hybridization (FISH). Additionally, we embed mammalian cells and show that we can successfully induce, transfect or even genetically modify the cells via prime editing in situ. To address the limitations of passive diffusion in larger constructs, we introduce perfusable channel structures mimicking vasculature to locally stimulate embedded cells. Finally, we develop and evaluate 3D bioprinting strategies – including dual extrusion and freeform reversible embedding of suspended hydrogels (FRESH) printing – to generate complex hydrogel architectures with integrated vascular-like features.

Our findings highlight the interplay between genetic control, non-equilibrium dynamics, microfluidics, and biofabrication, offering insights into the design of scalable synthetic biological systems.

Zusammenfassung

Die gezielte räumliche Organisation in synthetisch-biologischen Systemen stellt eine zentrale Herausforderung für Anwendungen in der Biofabrikation dar. Musterbildungsprozesse dienen in der Biologie als grundlegender Mechanismus zur Organisation von Zellen, zur Steuerung der Gewebeentwicklung und zur Etablierung räumlicher Strukturen, die Form und Funktion eines Organismus bestimmen.

In dieser Arbeit untersuchen wir, wie biologische Prinzipien der räumlichen Organisation auf verschiedenen Längenskalen angewendet werden können – von genetischen Schaltkreisen auf molekularer Ebene bis hin zu makroskopischen, gewebeähnlichen Strukturen. Zunächst zeigen wir, dass Nichtgleichgewichtsbedingungen im stationären Zustand essenziell für die Musterbildung genetischer Netzwerke sind. Dies wird anhand eines Drei-Streifen-Expressionsmusters in einer kontrollierten mikrofluidischen Umgebung demonstriert.

Aufbauend darauf betten wir lebende Organismen in Hydrogelmatrizen ein, um ihr Verhalten in einer kontrollierten 3D-Umgebung zu untersuchen. Im ersten Schritt fokussieren wir uns auf Clostridien, deren Wachstum und metabolische Aktivität wir mittels Fluoreszenz-in-situ-Hybridisierung (FISH) analysieren. Zusätzlich betten wir Säugetierzellen in Hydrogel ein und zeigen, dass diese in situ erfolgreich induziert, transfiziert und sogar mittels „Prime Editing“ genetisch modifiziert werden können.

Um die Einschränkungen passiver Diffusion in größeren Konstrukten zu überwinden, integrieren wir perfundierbare Kanalstrukturen, die vaskuläre Systeme nachbilden und eine lokale Stimulation eingebetteter Zellen ermöglichen. Abschließend entwickeln und evaluieren wir 3D-Biodruckstrategien – darunter Dual-Extrusion und FRESH-(Freiform-reversible Einbettung suspendierter Hydrogele) Druck –, um komplexe Hydrogelarchitekturen mit integrierten, gefäßähnlichen Strukturen herzustellen.

Unsere Ergebnisse verdeutlichen das Zusammenspiel von genetischer Steuerung, Nichtgleichgewichtsdynamik, Mikrofluidik und Biofabrikation und liefern neue Erkenntnisse für das Design skalierbarer synthetischer-biologischer Systeme.

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“Programming Diffusion and Localization of DNA Signals in 3D-Printed DNA-Functionalized Hydrogels”, Small 16 (31), 2001815

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Abbreviations

3D three dimensional. 1, 3

aTc anhydrotetracycline. 42

Bcd bicoid. 7

Cad caudal. 7

CDW cell dry weight. 58

CE cell extract. 42

CFFL coherent feed forward loop. 36, 46

CRISPR clustered regularly interspaced short palindromic repeats. 24, 25

dmT2 dmTurquoise2 - (mTurquoise2 with an additional delete for enhanced functionality). 42, 45

DNA deoxyribonucleic acid. 3, 9, 10, 14, 19, 29, 84, 134

DSB double-strand break. 24, 25

EA evolutionary algorithm. 22, 36, 38, 39, 44, 46, 55, 56

ECM extracellular matrix. 2, 30, 32, 91, 98, 105

E. coli *Escherichia coli*. 23, 24

FFF fused filament fabrications. 101

FFL feed forward loop. 13

FISH fluorescence in situ hybridization. 3, 24, 58, 59, 61, 63–66, 68, 105, 135

FRESH freeform reversible embedding of suspended hydrogels. 33, 92, 97, 100, 101, 103

GelMA gelatin methacryloyl. 30, 92, 98

GFP green fluorescent protein. 26

GGA golden gate assembly. 47, 48

gRNA guide RNA. 24, 25

Hb hunchback. 7

HEK293T human embryonic kidney 293T. 71

HPLC high performance liquid chromatography. 66

IFFL incoherent feed forward loop. 13, 54

IFFL-1 incoherent feed forward loop type 1. 36

IFFL-2 incoherent feed forward loop type 2. 14, 36–40, 42, 44, 46, 54

IPTG isopropyl β -D-1-thiogalactopyranoside. 40, 42

MAGE multiplex automated genome engineering. 48

MoClo modular cloning. 47, 48, 134

mRNA messenger RNA. 9

MSC mesenchymal stem cell. 71

mSc mScarlet-I. 45

mVen mVenus. 42

Nos nanos. 7

ODE ordinary differential equation. 39, 46, 55

PBS phosphate-buffered saline. 64

PCR polymerase chain reaction. 48

PDE partial differential equation. 22, 39

PDMS polydimethylsiloxane. 51, 88

PE prime editor. 25

pegRNA prime editing guide RNA. 25

PLA polylactide. 65, 92, 96, 97

pORTMAGE plasmid-based oligo recombineering tool for MAGE. 48

PVA polyvinyl alcohol. 21, 96

RBS ribosomal binding site. 12

RNA ribonucleic acid. 3, 9, 12, 29

RNAP RNA polymerase. 36, 44

ROI region of interest. 53, 65, 68, 69, 93

rRNA ribosomal RNA. 24

RT reverse transcriptase. 25

TF transcription factor. 9

Tor torso. 7

TXTL transcription and translation. 48, 49

Introduction

Synthetic biology aims to reproduce emergent behaviors found in nature or to assemble new synthetic systems from biological components [1]. To achieve this goal, the design of living systems that respond to their environment in programmable ways can be enabled with advances in genetic circuit design, hydrogel scaffold engineering, and three dimensional (3D) bioprinting. These approaches enable the construction of functional biological systems that can replicate or be used to investigate the structure and function of biological tissues.

However, key challenges remain in mimicking the hierarchical organization seen in natural tissues, particularly in reproducing tissue anisotropy, cell polarity, and extracellular matrix composition. Addressing these challenges requires the integration of principles from developmental biology, material science, and engineering to create synthetic tissues with structural and functional fidelity.

For the construction of in vitro models that mimic the structural, functional, and mechanical properties of native tissues, so called microphysiological systems [2], several key aspects must be considered, including the intended function, the choice of materials, and the method of fabrication. Two main strategies exist for tissue construction: In the first, tools such as 3D bioprinting or pre-engineered extracellular matrix (ECM) building blocks are used to define the structure and boundary conditions in advance. Cells can either be incorporated during fabrication or seeded onto the scaffold afterward, allowing them to populate the predefined architecture. In the second approach, cells would be stimulated to self-organize into the desired tissue architecture. Both approaches offer distinct advantages and limitations, depending on the complexity of the tissue and the required functionality. Here, we propose that a combined strategy that integrates cellular engineering, genetic modification, and external boundary conditions is necessary to manufacture microphysiological systems. Cells must not only be genetically programmed and stimulated to differentiate but also provided with a controlled microenvironment that dictates their spatial organization and function.

A fundamental biological inspiration for synthetic tissue formation can be found in developmental biology, where pattern formation plays a critical role in embryogenesis. During embryonic development, cellular differentiation and tissue structuring are directed by morphogen gradients which establish spatial cues. This principle has strongly influenced tissue engineering strategies, where controlled biochemical and biophysical signals are applied to guide cell behavior [3]. Pattern

formation in biological systems is controlled by genetic networks that regulate cellular processes. In nature, these networks exhibit high complexity and are studied within the field of systems biology [4]. Network motifs, such as feed-forward loops, represent fundamental circuit architectures that govern gene expression patterns. Understanding these motifs provides critical insights into how synthetic genetic circuits can be designed to program biological pattern formation. The studies of genetic circuits have advanced our understanding of both natural and synthetic biological systems and are often tested in bacteria as model organisms [5]. Further progress in the field has been driven by computer-aided design and the automated construction of large genetic circuits, leveraging extensive genetic part libraries [6].

A powerful platform for studying genetic networks are cell-free systems, which allow precise biochemical control without cellular constraints. However, sustaining dynamic behaviors in such systems requires non-equilibrium steady-state conditions, which cannot be established in simple batch reactions. Microfluidic systems provide a solution by enabling continuous reaction turnover and controlled energy supply, which are essential for maintaining stable, non-equilibrium conditions [7], [8].

While cell-free systems provide a powerful tool to study genetic circuits in a controlled environment, translating these principles to living cells embedded in engineered matrices introduces additional challenges, such as maintaining cellular viability, spatial organization, and dynamic gene regulation. Additionally, if tissues reach a critical thickness, passive diffusion becomes insufficient to sustain cellular activity. Vascularization strategies become essential for nutrient exchange and waste removal. In nature, vascular networks form through self-organization, guided by biochemical and mechanical cues. However, these processes are slow and not yet fully understood. To overcome this limitation, biofabrication techniques allow for the macroscopic structuring of tissues, which provide initial boundary conditions, while finer vascular structures may emerge later through cellular self-assembly mechanisms. Since its introduction as a tissue fabrication method in 2014 [9], 3D bioprinting has evolved from simple layered constructs to dynamic, four-dimensional biomaterials that respond to environmental stimuli [10]. A key aspect of biofabrication is the choice of scaffold materials, such as extracellular matrix (ECM)-mimicking hydrogels, which offer structural support and biochemical signaling cues [11]. Various 3D-printed constructs have been developed for biomedical applications, including 4D shape-changing materials [12], skin substitutes [13], ECM scaffolds for soft tissue repair [14], printed organ components [15], and even biohybrid soft robots [16]. Despite these advancements, vascularization strategies remain a key challenge. Two primary approaches exist: self-organization of vascular networks, where cells autonomously form capillary-like structures, and engineering pre-defined vascular channels through 3D bioprinting or casting techniques [17], [18].

To further advance the fabrication of complex, functional tissues, bioreactors that incorporate

vascularized structures are explored to support the long-term culture and maturation of engineered constructs [19]. These engineered microenvironments aim to replicate key physiological conditions and try to ensure efficient molecular exchange and structural stability in biofabricated tissues.

Contribution To address these challenges of engineering synthetic biological systems that respond to their environment in programmable ways, in this thesis four aspects are investigated: the design of genetic networks for pattern formation, the monitoring of living organisms in synthetic biofilms, the genetic modification of mammalian cells embedded in 3D matrices consisting of hydrogels, and the 3D-printing of vasculature-like structures to overcome diffusion limitations in larger bio-scaffolds for molecular transport. The first chapter introduces the fundamentals necessary to address these topics. It begins with an overview of the principles of biological pattern formation and regulation, followed by a discussion on artificial tissues, and concludes with an examination of how these artificial environments can be engineered. Chapters two to five present the research findings. Each chapter is structured as follows: the research question is introduced in the context of the broader scope of this thesis, followed by the presentation, discussion, and summary of the results. Each chapter also includes a materials and methods section, presenting the approaches and techniques used to generate the data. The second chapter introduces a synthetic genetic network which was developed to generate a three-stripe pattern in response to a morphogen gradient in a cell-free system. This network, encoded on plasmid deoxyribonucleic acid (DNA), was operated in a cell-free system in a microfluidic environment which provided non-equilibrium steady-state conditions. The parameter space of the circuit was explored using an evolutionary algorithm to find the best parameter set for pattern formation. To expand beyond microfluidic confinement and enable pattern formation in 3D environments, hydrogels were explored as a biocompatible matrix. The third chapter presents a method for monitoring living organisms embedded in a hydrogel matrix. Specifically, a synthetic biofilm composed of two layers containing *Clostridium carboxidivorans* and *Clostridium kluyveri* was analyzed using fluorescence in situ hybridization (FISH). This approach enabled the distinction and spatial analysis of bacterial growth using species-specific FISH probes. Shifting from programming DNA and monitoring bacteria to mammalian cell systems, the fourth chapter investigates the engineering of microphysiological systems. It examines cell growth within hydrogel matrices and explores the feasibility of genetic modification of embedded cells through DNA and ribonucleic acid (RNA) transfection. This facilitates genetic manipulation post-embedding and enables controlled cellular responses within the hydrogel matrix. As diffusion constraints limit the size and functionality of engineered tissues, vascular structures become essential for efficient molecular transport. The final chapter discusses the engineering of vascular-like structures using 3D bioprinting. This approach aims to enhance molecular transport in biofabricated tissues, facilitating the development of more complex and functional artificial environments. The thesis concludes with a summary of the results

and gives an outlook on further improvements.

1. Fundamentals

To engineer synthetic microphysiological systems that exhibit organized behavior, we first need to understand the principles of biological pattern formation, how they are regulated, and how similar behavior can be synthetically engineered. Following this, we will examine strategies used in biofabrication to construct artificial tissues and explore the role of vascular structures. Finally, we will present the methods and concepts used in this thesis to create and control spatiotemporal artificial environments.

1.1 Principles of Biological Pattern Formation and Regulation

Most biological systems in nature are characterized by distinct spatiotemporal dynamics, with many also exhibiting periodic patterns. These patterns are not just aesthetic but play a fundamental role in biological self-organization, guiding the development and function of living systems.

1.1.1 Spatiotemporal Differentiation and Pattern Formation

Spatiotemporal patterns play a crucial role in biological development of bacterial and mammalian systems. In bacterial colonies, gradients of signaling molecules influence the physiology of the population and the characteristics of biofilms [20], whereas in mammalian tissues, they regulate processes like stem cell differentiation, tissue organization, and organ formation [21]. Since organs are composed of diverse cell types with specialized functions, cells must undergo tightly regulated differentiation to establish these varied identities. This process is controlled by spatiotemporal gene expression, which ensures that cells acquire the appropriate fate based on their position and timing [22]. Understanding how these patterns emerge is particularly relevant for tissue engineering, especially for mimicking nature's developmental strategies to construct functional organs.

A well-studied example of pattern formation in embryonic development is the embryogenesis of *Drosophila melanogaster*, where early segmentation is orchestrated by morphogen gradients. These gradients provide positional information that guides gene expression and cellular differentiation,

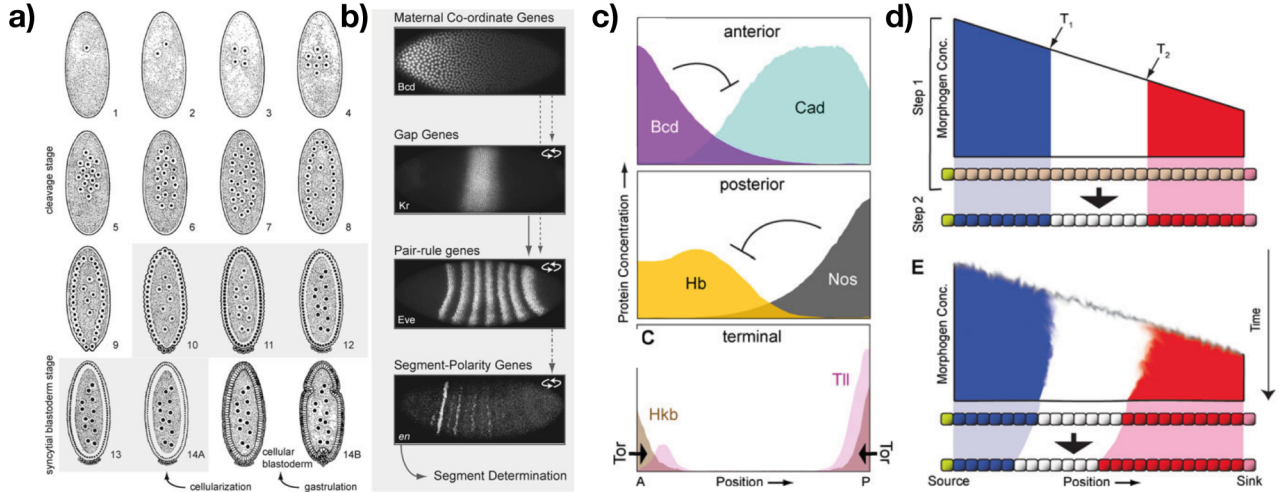


Figure 1.1: Pattern formation during embryogenesis. a) During the early phase of *Drosophila melanogaster* development the blastoderm forms, followed by gastrulation. The numbers indicate the cleavage cycle. The nuclei divide in a common cytoplasm without separation by an additional membrane. b) Parasegmental boundaries in *Drosophila* embryos are determined by an underlying segmentation pattern. This patterning process is encoded on a genetic network by the segmentation genes. It begins with maternal coordinate genes, which are expressed in broad gradients along the anterior-posterior axis and provide positional information to the embryo. These gradients regulate the expression of gap genes and subsequently segmentation genes, which form a molecular pre-pattern. Through activity of these segmentation genes (pair-rule and segment polarity genes) boundaries are defined of the parasegments prior to visible morphological segmentation. The arrows indicate regulatory interactions between classes of segmentation genes, and the circular arrows represent cross-regulation within a class. c) Three maternal systems regulate the gap gene transcription: anterior, posterior, and terminal. The anterior system is based on the Bcd gradient which establishes a posterior gradient of Cad through repression, the posterior system is based on the Nos gradient which establishes an anterior gradient of Hb through repression, and the terminal system is based on the Tor gradient signaling from both ends of the embryo. d) According to Wolpert's French Flag model positional information can be extracted from gradients. If a morphogen is produced on one side, diffuses through the tissue, and is degraded on the other side, a gradient is established. Different concentration thresholds can be detected by cells in the resulting linear gradient (here T_1 and T_2) which switch on alternative target genes. In this model, first, positional information is implemented through a morphogen gradient, and subsequently, the information is passively interpreted by cells. Reproduced from reference [23] under the license CC BY-NC 2.0.

illustrating how genotype specifies phenotype. Figure 1.1a shows the early phase of the development of *Drosophila melanogaster*. It displays in this case the nuclei that divide in a common cytoplasm without separation by an additional membrane. The nuclei are exposed to different concentrations of morphogens according to their position within the segmentation pattern which is determined by a genetic network of segmentation genes. Figure 1.1b shows the different patterns occurring during early development. These patterns drive the segmentation process by defining parasegmental boundaries. They are regulated by maternal co-ordinate genes which are expressed in broad gradients. The morphogen bicoid (Bcd) for example is expressed at one side of the embryo and diffuses along the anterior-posterior axis. It influences then the expression of the gap genes and these in turn regulate the pair-rule genes. These dependencies and cross-regulations eventually determine in the end the information that the cell nuclei obtain. The underlying morphogen gradients are depicted in Figure 1.1c: Three maternal systems in total regulate the gap gene transcription, which are called anterior, posterior and terminal. They are based on the proteins Bcd, caudal (Cad), nanos (Nos), hunchback (Hb), and torso (Tor). These systems self-regulate through repression, so that patterns of high and low concentrations of all these proteins emerge.

But how do the nuclei, or in general cells, extract information from these patterns? According to Wolpert [24], who proposed the French Flag Model (shown in Figure 1.1d), cells can detect different concentration thresholds within a gradient of a morphogen. In his model a gradient is established when a morphogen is produced on one side, but diminished on the other. This results in a linear gradient between source and sink. Depending on whether the cells detect a concentration below or above a certain threshold value, different target genes are activated.

1.1.2 Mathematical Principles of Pattern Formation

Tkačik et al. [25], have further explored how positional information can be extracted from noisy biological signals, providing insights into the robustness of these systems. The concept was illustrated using a simplified case of a gene located at position x within a one-dimensional embryo. The profile $g(x)$ was normalized to 1 at the maximum and 0 at the minimum. The variance of the profile is then given by $\sigma_g^2(x)$. They compared three idealized cases, shown in Figure 1.2a, a step-like profile, a sigmoidal shape and a linear gradient. While for the step function 'on' and 'off' can be perfectly distinguished, this is more complex for the sigmoidal curve, as the transition region can encode more information than the two states 'on' and 'off'. The linear gradient provides further increase in information by being equally sensitive at every position x , if σ_g is low enough, although it has no well-defined boundary. In sum, this means, that the profiles as well as their variances can carry positional information which can be extracted by cells within these gradients.

Diffusion is the underlying mechanism which establishes morphogen gradients and is described

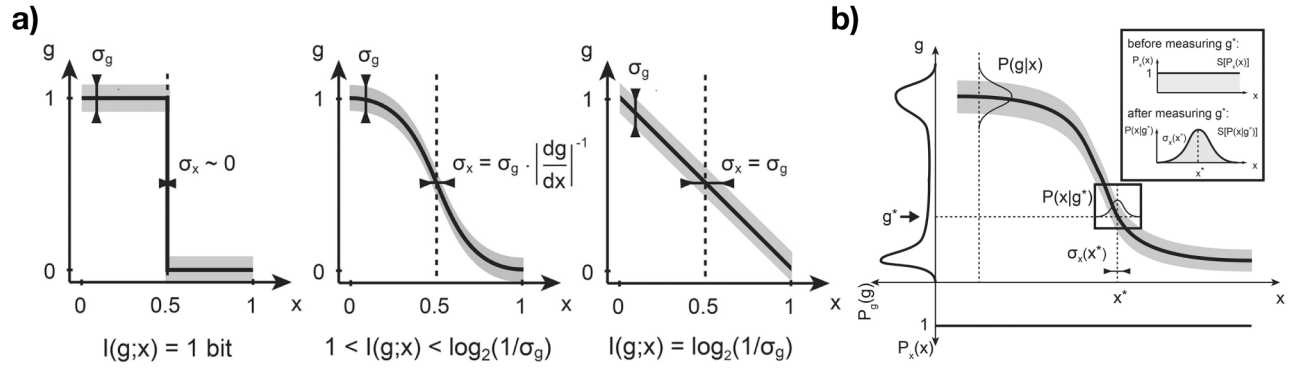


Figure 1.2: Positional information. a) Expression profiles can have different shapes, examples are a step function, a sigmoidal curve, or a linear gradient. While for the step function ‘on’ and ‘off’ can be perfectly distinguished, this is more complex for the sigmoidal curve, as the transition region can encode more information than the two states ‘on’ and ‘off’. The linear gradient provides further increase in information by being equally sensitive at every position x , if σ_g is low enough, although it has no well-defined boundary. b) To determine the position, mathematical analysis can be used: The total distribution of expression levels, $P_g(g)$, is determined by averaging over all positions $P(g|x)$. Before measuring, all positions are equally likely, but after observing the expression level g^* , the positions consistent with this measured values are drawn from $P(x|g^*)$. The best estimate for the true position x^* is the peak of this distribution. Reprinted from reference [25] with permission from Oxford University Press, Copyright © 2014.

by the passive movement of molecules from regions of high concentration to low concentration. This process can be described by Fick’s first law:

$$J = -D\nabla c, \quad (1.1)$$

where J is the diffusive flux, D is the diffusion coefficient, and c represents the concentration of a molecule. The spatial distribution of molecules over time can be described by Fick’s second law:

$$\frac{\partial c}{\partial t} = D\nabla^2 c, \quad (1.2)$$

which governs how concentration evolves due to diffusion alone. While diffusion tends to homogenize concentrations, biological pattern formation requires mechanisms that counteract this process to create stable, non-uniform distributions typically implemented by production sources and degradation sinks. Over large distances (whole tissues) diffusion is a very slow process but over small distances (size of a cell) diffusion is rather fast, as Crick showed [26]. He assumed a 1D arrangement of cells next to each other where one end functions as a source and the other as a sink of a morphogen. After some time the system approaches dynamic equilibrium with the result of a linear gradient of

the morphogen along the cells. The time t for this can be calculated with:

$$t = An^2l^2 \left(\frac{1}{D} + \frac{1}{Pl} \right), \quad (1.3)$$

where A is a numerical constant, n is the number of cells between source and sink, P is the permeability of the cell membrane, and l is the length of each cell. This leads to the conclusion that diffusion is a fast enough mechanism over a length of approximately 100 cells, with assuming that 'fast enough' is defined as a few hours, and estimating the parameters with: $D = 0.8 \times 10^{-6} \text{ cm}^2/\text{s}$, $P = 8 \times 10^4 \text{ cm/s}$ and $A = 0.5$. This is in agreement with Wolpert [24] who states that embryonic fields involve less than 100 cells.

But most dynamical processes in biological systems cannot only be described by diffusion but by also reaction. The dynamics of reaction-diffusion systems can be described in general with the formula:

$$\frac{\partial c_i(\vec{x}, t)}{\partial t} = D \nabla^2 c_i(\vec{x}, t) + r(c_i(\vec{x}, t)) - d(c_i(\vec{x}, t)), \quad (1.4)$$

where c_i is the concentration of species i , D is the diffusion coefficient, $r(c_i)$ is the function representing the reaction term which contains all the reactions of the species i , and $d(c_i)$ describes the degradation of species i which can be described by a linear term $-d \cdot c_i(\vec{x}, t)$. These equations cannot be solved analytically anymore, but have to be studied numerically.

Thus, the formation of patterns depends on both timescales of reaction- and diffusion rates, and length scales (= spatial features of emerging structures). The characteristic diffusion length L is often estimated as:

$$L = \sqrt{\frac{D}{k}}, \quad (1.5)$$

where D is the diffusion coefficient and k is the reaction rate constant, that defines the spatial range over which a molecule can influence its surroundings before being degraded. By understanding these mathematical principles, we gain a framework for analyzing natural pattern formation and designing synthetic systems with controlled spatial organization.

1.1.3 Gene Regulation and Synthetic Genetic Circuits

Precise spatial and temporal responses to morphogen inputs are regulated through genetic networks. Gene regulation controls when and how genes are expressed, primarily through the interaction of transcription factors (TFs), polymerases, and regulatory sequences [4]. The fundamental steps involve transcription, where a gene's DNA sequence is transcribed into messenger RNA (mRNA), and translation, where mRNA is subsequently translated into proteins. The expression of a gene can be activated or repressed by TFs binding to promoter regions, thereby modulating RNA polymerase

activity. A schematic, where the function of a repressor is shown, can be seen in Figure 1.3a. The repressor binds to an operator in the promoter region and blocks the polymerase from transcribing the DNA.

The kinetics of this reaction can be described with:

$$\frac{d[BR]}{dt} = k_{\text{on}}[B][R] - k_{\text{off}}[BR], \quad (1.6)$$

where $[BR]$ is the total concentration of the formed complex, $[B]$ the concentration of empty binding sites, and $[R]$ the concentration of free repressor. The rate k_{on} is usually limited by the rate of collisions of a diffusing repressor hitting DNA, thus a diffusion-limited value of $10^8 - 10^9 \frac{1}{\text{Ms}}$ results for k_{on} [4, p. 241ff]. But because of one-dimensional diffusion effects like sliding of the transcription factor along the DNA the diffusion limit is actually even higher: $k_{\text{on}} \sim 10^{10} - 10^{11} \frac{1}{\text{Ms}}$ [27]). The total concentration of the binding site is given by:

$$[B_T] = [BR] + [B]. \quad (1.7)$$

Solving equation 1.6 in steady state $\left(\frac{d[BR]}{dt} = 0\right)$ leads to

$$K_d[BR] = [B][R], \quad (1.8)$$

where $K_d = \frac{k_{\text{off}}}{k_{\text{on}}}$ is the dissociation constant. To solve for the promoter activity (= mRNA production rate), one can consider the probability of the binding site not being occupied through a repressor which is given by the ratio of free binding site B over the total concentration of the binding site B_T . This leads to a promoter activity of

$$f(P) = \frac{\beta}{1 + \frac{[R]}{K_d}}, \quad (1.9)$$

with β being the maximal transcription rate. For a 50% chance of occupation, the concentration of the repressor must be equal to K_d .

In reality most transcription factors are composed of several sub units and the above formula has to be modified by replacing $[BR]$ with $[nM_R B]$, where n denotes the number of subunits that are denoted with M_R . The promoter activity can then be calculated with:

$$f(P) = \frac{\beta}{1 + \left(\frac{[M_R]}{K_d}\right)^n}. \quad (1.10)$$

In case of an activator, equation 1.8 is solved for the complex $[AB]$ and the promoter activity

can be calculated in this case with:

$$f(P) = \frac{\beta[B_T]}{\frac{K_d}{[A]} + 1} \quad (1.11)$$

The three parameters K_d , β and n describe the activation coefficient, the maximal expression level of the promoter and the Hill coefficient. The unit of the activation coefficient is concentration and it represents the concentration required to achieve half-maximal expression. The activation coefficient has concentration as its unit and defines the concentration needed to reach the half-maximal expression. The Hill coefficient describes the function's steepness. Typically n has a value between 1 and 4 [4, p. 246].

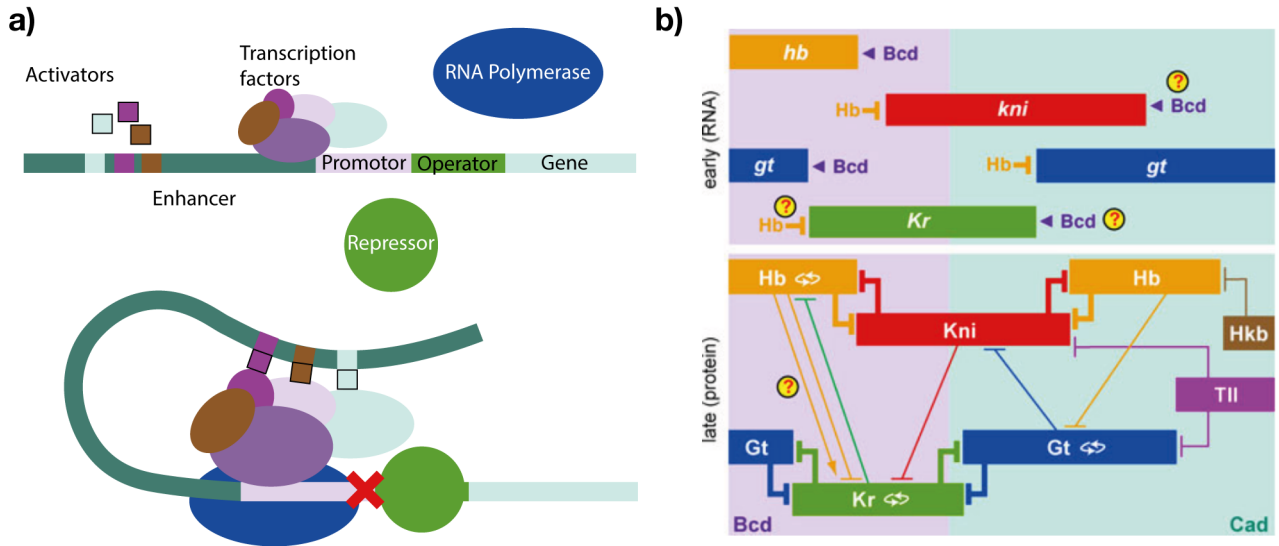


Figure 1.3: Gene regulation. a) Repression is implemented for example through the expression of transcription factors which block the polymerase. b) The gap gene regulation in *Drosophila melanogaster* can be divided in early and late regulation. While the early phase is characterized by regulation of maternal gradients, the late phase features primarily cross-regulatory interactions. There, auto regulation, mutual asymmetric repression lead to positive feedback and incoherent feed-forward loops and thus lead to specific patterns of gene expression. Reproduced from reference [23] under the license CC BY-NC 2.0.

In natural transcriptional networks, genes regulate each other in complex interdependent systems. These networks include motifs like feedback loops, oscillators, and signal amplification mechanisms, that allow cells to process information and respond dynamically to changing conditions. An example of such a genetic network is shown in Figure 1.3b. As discussed previously in Section 1.1.1 the early regulation of the gap gene network of *Drosophila melanogaster* is characterized by maternal gradients but the late regulation features primarily cross-regulatory interactions as auto regulation, mutual asymmetric repression which lead to positive feedback, and incoherent feed-forward loops.

Synthetic biology leverages our understanding of gene regulation to construct artificial gene

networks that perform specific functions. These networks are designed using modular genetic components, including promoters, ribosome binding sites, coding sequences, and terminators. By assembling these components, gene circuits that function analogously to electronic systems can be implemented, capable of executing logic operations such as AND, OR, and NOT. A fundamental concept in synthetic circuits is the use of logic gates, where gene expression is contingent on specific input conditions. For example, an AND gate may require the presence of two transcription factors to activate a downstream gene, whereas a NOT gate suppresses gene expression in the presence of a repressor. Examples of such logic implementations are presented in Figure 1.4a. Through the choice of transcription factors and polymerases the rate and specificity of transcription can be tuned. In Figure 1.4b six different approaches are shown to tune the Hill parameters: The promoter strength can be modified, the target can be degraded, gene dosage or ribosomal binding site (RBS) can be adapted, and small RNAs as well as decoy operators can be used.

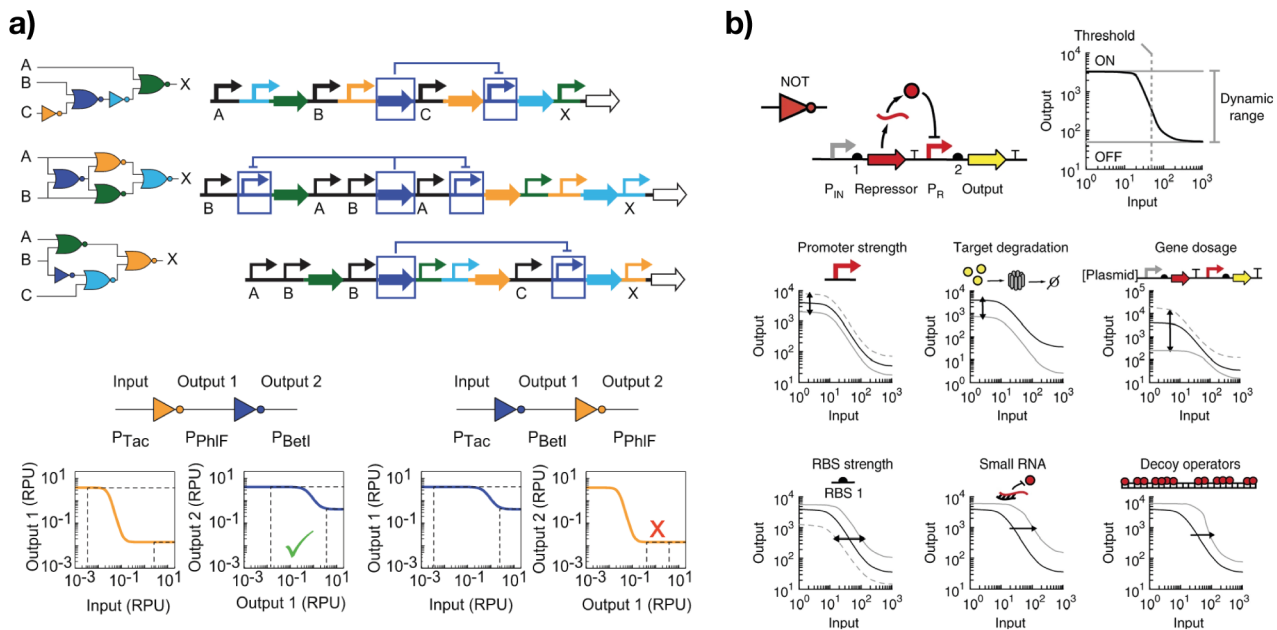


Figure 1.4: Genetic gates and parameter tuning. a) Different gates to perform certain circuit functions can be implemented through combination of different repressors. The inputs are implemented with sensor input promoters and the circuit output promoter controls the actuating gene. The shapes of the gate response functions have to match so that they can be functionally connected. Reprinted from reference [6] with permission from AAAS. b) There are various strategies to tune the Hill coefficient: The promoter strength can be modified for a shift in output strength, the target can also be degraded, or gene dosage can be adapted. To implement a change in K_d , the RBS can be adapted, or small RNAs, as well as decoy operators can be used. Reproduced from reference [28] with permission from Springer Nature, copyright © 2014.

Beyond cellular environments, gene regulatory networks can be studied and implemented in cell-free systems [29], [30], where transcription and translation occur outside living cells. These systems

provide a simplified platform to test synthetic networks without the complexity of cellular metabolism and resource allocation. They offer a great platform for prototyping genetic circuits, enable precise control over reaction conditions and the systematic exploration of regulatory dynamics.

One well-characterized motif in gene regulatory networks is the feed forward loop (FFL), where an upstream regulator controls a downstream gene, both directly and indirectly through an intermediate regulator. Depending on whether activation or repression is involved, different types of FFLs exist, each with distinct dynamical properties such as signal filtering, pulse generation, or response acceleration.

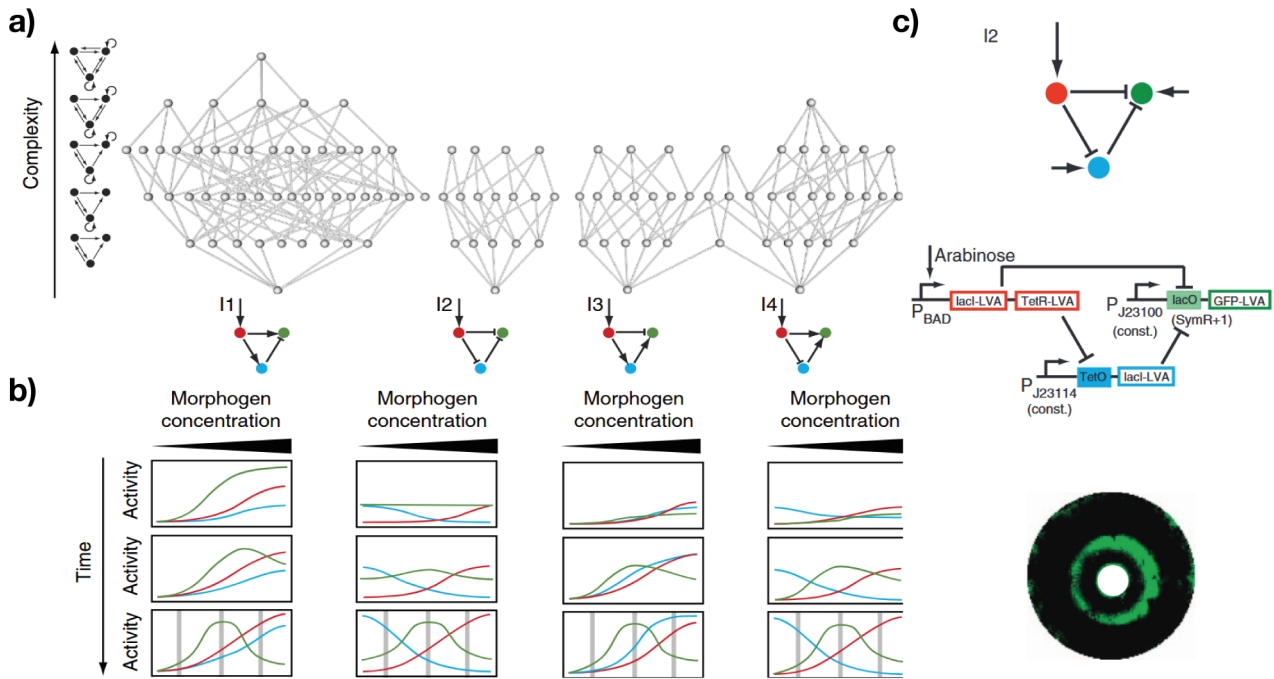


Figure 1.5: Stripe forming networks. a) Gene regulatory networks are depicted as gray circles linked by edges which show a single connectivity change. Below minimal networks are presented which are four incoherent feed forward loops that produce a stripe pattern. b) The temporal concentration output of the three genes which are encoded by the gene circuit are depicted. c) An example implementation for one of the genetic circuits shows a single stripe as a ring around the central application of arabinose on a bacterial lawn. Reproduced from reference [31] under the license CC BY-NC-SA 4.0.

Especially the incoherent feed forward loop (IFFL) plays a crucial role in stripe forming networks. Schaerli et al. [31] explored the design space of possible dynamical mechanisms for 3-node stripe-forming networks computationally and synthetically. They discovered four different dynamical mechanisms, shown in Figure 1.5a, to form a stripe and identified the minimal network of each group. All of them had in common that their minimal network motif could be identified as the IFFL. The corresponding output patterns are shown in Figure 1.5b. In Figure 1.5c one synthetic

implementation of the incoherent feed forward loop type 2 (IFFL-2) is shown. The most upstream node of the network gets activated by an arabinose-dependent promoter. Upon its activation two repressors are expressed which deactivate the other two nodes. Additionally, one of these nodes also represses the most downstream node. This node, upon activation, expresses a fluorescent molecule that can be visualized with a fluorescence microscope. The most downstream node is only active in a specific regime, when the two other nodes are down regulated. An agar plate was cultivated with bacteria containing the genetic network encoded on plasmid DNA. Arabinose was placed in the middle of this plate and a circular stripe forms around the source.

1.2 Biofabrication of Artificial Tissues: Concepts and Challenges

Engineered constructs that aim to replicate the structural and functional properties of native tissues were already envisioned in the 1990s, when opportunities for creating and utilizing artificial tissues were discussed [32]. They serve as essential models to study biological processes, as platforms for drug testing, and as potential solutions for regenerative medicine [33]. The term ‘artificial tissue’ comprises a broad spectrum of structures, ranging from simple cell-laden hydrogels to complex, layered systems composed of multiple cell types. Their fabrication relies on key factors such as scaffold composition, precise cellular organization, and mechanical properties that influence tissue behavior.

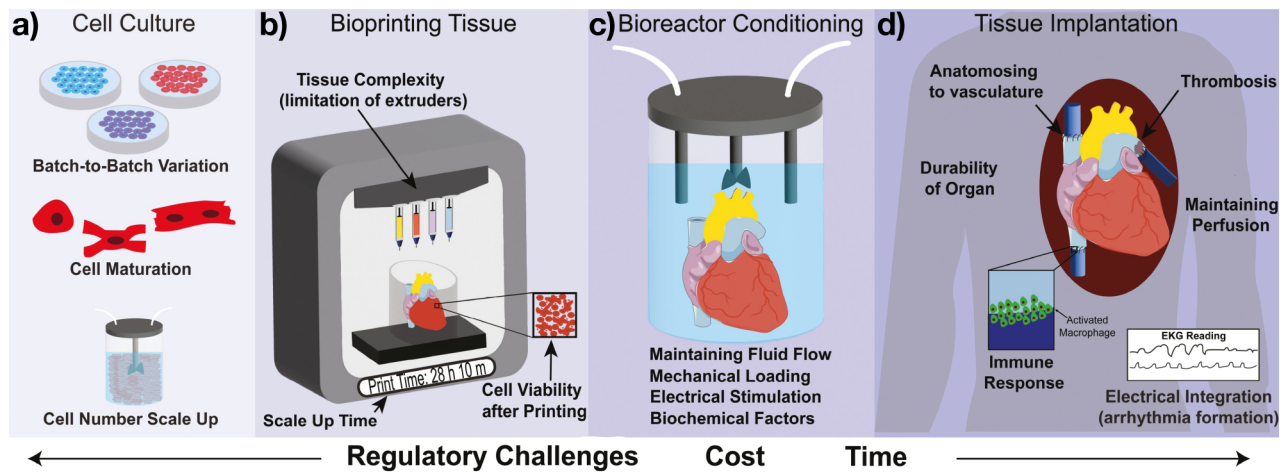


Figure 1.6: Tissue engineering. During tissue engineering multiple challenges arise. a) The quality of cells during cell culture phase affects cell function in the later produced tissues. b) For construction of a tissue 3D bioprinting is a promising strategy but can decrease cell viability due to long printing time and shear stress on the bioink during extrusion. c) After the printing process, the tissues have to be cultured in bioreactors which have to mimic in vivo-like conditions. d) Specialized procedures may have to be implemented for implantation of the engineered construct. Reproduced from reference [34] under the license CC BY.

Despite significant progress in the field, multiple challenges remain in tissue engineering (Figure 1.6). The quality of cells during the culture phase plays a crucial role in determining their functionality in the final tissue construct. For the fabrication process, 3D bioprinting has emerged as a promising approach, but prolonged print times and shear stress during extrusion can compromise cell viability. Following printing, the constructs must be cultured to replicate in vivo-like conditions to support proper tissue maturation. Different types of bioreactors have to be engineered to achieve long culture times. Additionally, specialized procedures may be required in case the engineered

tissues should be implanted.

One of the most critical challenges remains the integration of vascular networks to provide sufficient nutrient and oxygen supply [35]. Strategies such as perfusion bioreactors and engineered micro vasculature have been developed to address these limitations. In the following section key aspects of biofabrication, including morphogenetic differentiation within printed scaffolds, strategies for achieving three-dimensional organization, and approaches to vascularization in synthetic tissues will be discussed.

1.2.1 Morphogenetic Differentiation in Printed Scaffolds

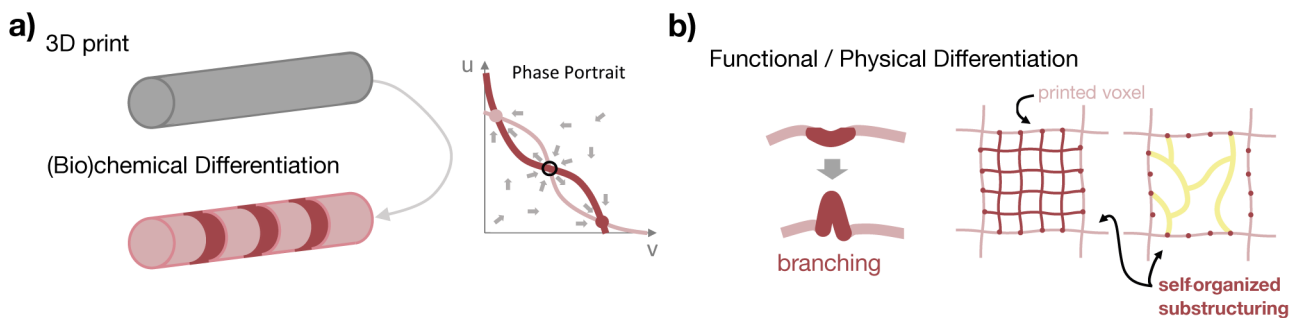


Figure 1.7: Morphogenetic differentiation of printed scaffolds. a) Morphogenetic molecular programs may further sub-pattern low resolution printed scaffolds (gray). Two morphogens u (light red) and v (dark red) act as molecular rulers to mark sectors via a tailored reaction-diffusion mechanism. This requires rationally designed reaction networks (phase portraits). b) Downstream processing then rewrites critical biomaterial properties in the scaffold accordingly. These may involve zonal biomineralization, scaffold rigidity adaptation, or higher resolution tiling through suitable self-organization processes. Reproduced from reference [36] under the license CC BY-NC 4.0.

As discussed in the previous section, morphogen signals play a crucial role in regulating embryonic development. Secreted or membrane-bound signaling molecules drive cell differentiation. The timing and location of their expression is encoded in genetic networks. To construct artificial tissues, we aim to harness these mechanisms. In printed scaffolds, morphogenetic differentiation refers to the process of cells undergoing spatially and temporally controlled differentiation to form organized tissue structures within biofabricated constructs.

This process is influenced by a combination of biochemical and biophysical cues, including growth factor gradients, cell-cell interactions, and the mechanical properties of the scaffold. In 3D bioprinting, the precise deposition of cells and biomaterials enables the recreation of tissue-specific microenvironments that guide differentiation. However, maintaining cell viability and ensuring correct differentiation pathways remains still a challenge.

An important consideration in morphogenetic differentiation is the role of length scales and spatial dimensions. Cellular processes operate at the micrometer scale, where local gradients in signaling molecules influence fate decisions. However, scaffold architectures must also be designed to support tissue formation at the millimeter to centimeter scale, bridging the gap between individual cell behavior and macro scale tissue function. Features such as micro patterned substrates, controlled porosity, and hierarchical structuring allow for the integration of spatial cues across multiple length scales. These concepts align with the multi-scale approach to biofabrication, which combines additive manufacturing with programmable self-assembly strategies that we proposed earlier in this reference [36].

This concept is illustrated in Figure 1.7, which depicts how morphogenetic molecular programs can refine low-resolution printed scaffolds. In this framework, rationally designed reaction networks act as molecular rulers to create distinct tissue sectors (Figure 1.7a). The downstream processing of these biochemical signals then modulates critical biomaterial properties, enabling zonal biomineralization, rigidity adaptation, or finer-scale organization through self-organizing mechanisms (Figure 1.7b). These strategies highlight how biofabrication methods can integrate biochemical cues to direct tissue formation beyond the initial printed structure.

1.2.2 Three Dimensional Organization in Synthetic Tissues

A highly organized 3D architecture offers structural as well as functional complexity to biological tissues. In synthetic tissue engineering, achieving this level of spatial organization is critical to enable the replication of native tissue function. Cells rely on their microenvironment for biochemical and mechanical cues that direct their behavior including adhesion, migration, and differentiation. In engineered tissues, this organization can be guided through scaffold design, biofabrication techniques, and self-assembly processes.

3D bioprinting enables the precise spatial arrangement of multiple cell types within biomaterial matrices. However, beyond direct printing, alternative strategies leverage modular assembly to construct synthetic tissues from predefined biological units. As illustrated in Figure 1.8, tissue engineering can also be approached through a building-block strategy, where fundamental units composed of cells, biomaterials, and bio molecules are first fabricated (Figure 1.8a). These living building blocks range from single cells to multicellular aggregates or scaffold-supported micro tissues, allowing for controlled cellular composition and micro environmental cues (Figure 1.8b). To achieve complex, multidimensional tissue architectures, these building blocks can be further organized through self-assembly, remote manipulation (e.g., magnetic or acoustic fields), or directed assembly using external guidance systems (Figure 1.8c). Integrating these modular approaches with biofabrication techniques offers enhanced control over tissue structuring at multiple length scales,

bridging the gap between engineered constructs and the hierarchical complexity of native tissues.

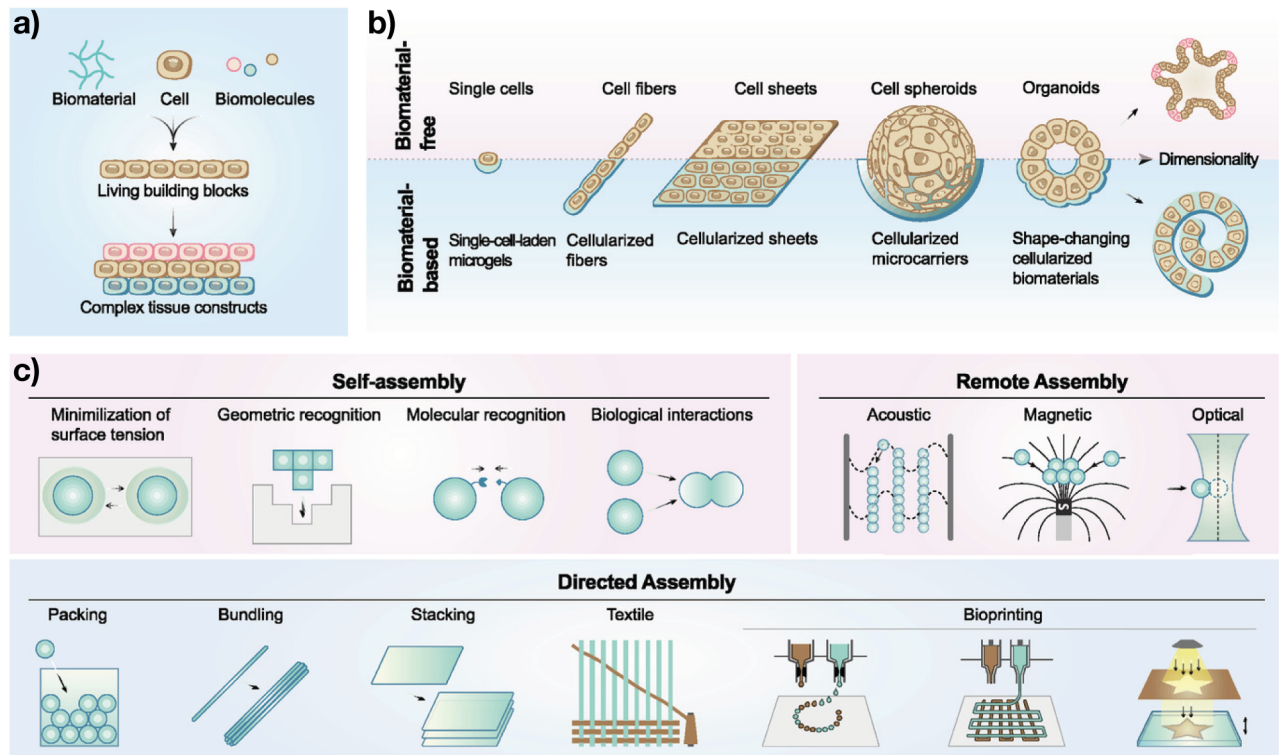


Figure 1.8: Building blocks for tissue engineering. a) Living building blocks are fabricated of cells, biomaterials and bio molecules. b) To achieve multidimensional structures, different building blocks, ranging from single cells to multicellular units can be engineered. c) These building blocks can be assembled via self-assembly, remote or directed assembly. Reprinted from reference [37] with permission from Wiley, copyright © 2020.

An optimal balance between externally guided construction and self-organization has to be found. As illustrated in Figure 1.9, externally guided approaches prestructure tissues by defining boundary conditions and assembling functional constructs from cell sheets, organoids, cell aggregates, or hydrogels (Figure 1.9a). In contrast, self-organization leverages the intrinsic ability of cells to establish structures within a hydrogel matrix that are driven by cellular interactions and biochemical cues. While each strategy has strengths, we propose a hybrid approach that integrates both principles (Figure 1.9b). 3D bioprinting serves as a macroscopic framework, embedding cells, DNA, proteins, and vascular-like structures within a hydrogel matrix to construct millimeter- to centimeter-scale tissues. At finer resolutions, self-organization mechanisms guide micro scale tissue formation, allowing cells to refine their own microenvironments and develop functional architectures.

One of the most pressing challenges in synthetic tissue design is overcoming diffusion limitations in larger constructs (Figure 1.9c). Molecular transport within hydrogels is constrained by slow diffusion, particularly over millimeter-length scales, which impacts nutrient and signal delivery in

centimeter-sized tissues. Incorporating vascular-like features into engineered tissues is essential to mimic physiological signaling and transport processes, which are critical for maintaining function and viability in larger 3D constructs.

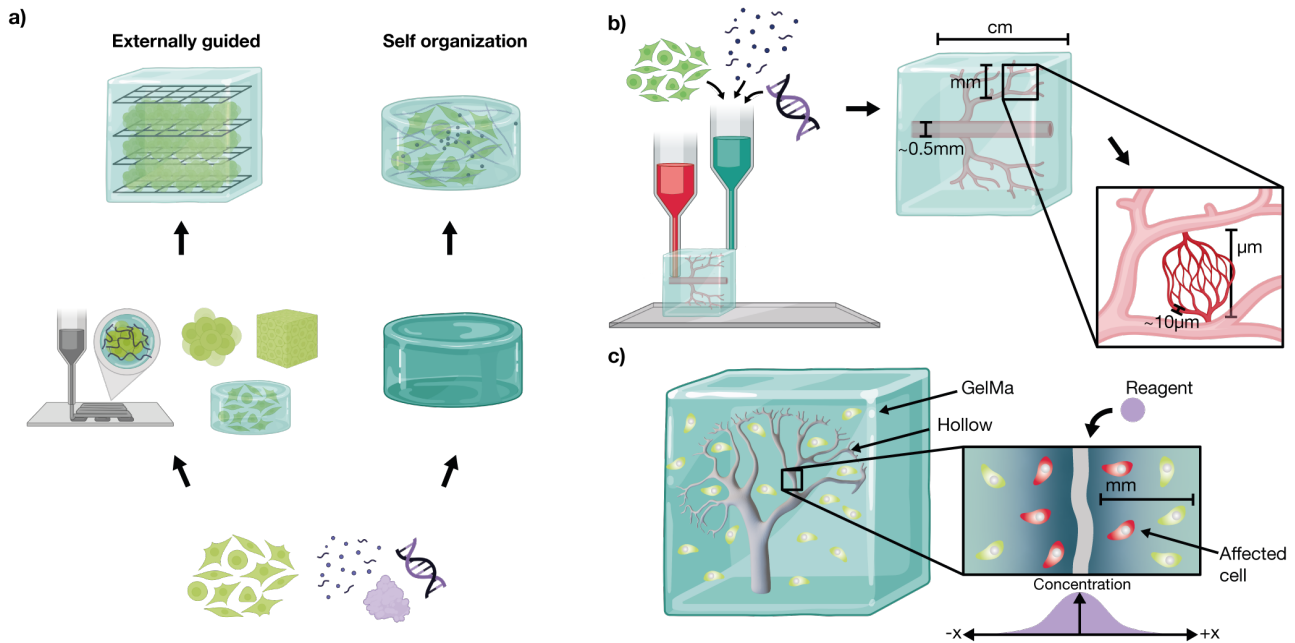


Figure 1.9: Challenges in tissue fabrication. a) Tissue engineering can follow two primary strategies: externally guided construction or self-organization. The externally guided approach utilizes techniques such as 3D bioprinting to prestructure tissues, define boundary conditions, and assemble functional constructs using cell sheets, organoids, cell aggregates, or hydrogels as scaffolds. In contrast, self-organization relies on the intrinsic ability of cells to grow and establish structures within a hydrogel matrix, driven by cellular interactions and biochemical cues. b) A hybrid strategy that integrates both approaches is shown. 3D bioprinting provides a macroscopic framework by embedding cells, DNA, proteins, and vascular-like structures within a hydrogel matrix, allowing for the fabrication of constructs on the millimeter to centimeter scale. At finer resolutions, self-organization mechanisms guide micro scale tissue formation, leveraging the natural behavior of cells to refine the structure. This combination enables precise control over tissue architecture while preserving the ability of cells to develop functional microenvironments. c) Vascular-like structures are essential for overcoming diffusion limitations in engineered tissues. Molecular transport in hydrogels is constrained by slow diffusion, particularly over millimeter-length scales, which affects nutrient and signal delivery in centimeter-sized constructs. Reagents perfused through channel structures selectively affect cells in proximity, enabling controlled stimulation and analysis of diffusion timescales in 3D tissue constructs where cells are embedded in hydrogels. Reproduced from reference [38] under the license CC-BY-NC-ND 4.0.

1.2.3 Approaches to Vascularization in Biofabricated Tissues

Just as synthetic tissue organization can follow externally guided or self-organized strategies, vascularization in biofabricated tissues also relies on two fundamental approaches: prestructuring vascular networks or stimulating cells to form them autonomously. Vascular channels can either be directly fabricated using 3D bioprinting, sacrificial molding, or casting techniques, which enable precise spatial control over vessel architecture (Figure 1.10a and b). Alternatively, self-organization approaches harness the intrinsic angiogenic potential of cells to stimulate endothelial networks to form in response to biochemical and mechanical cues (Figure 1.10c). While prestructured networks provide immediate perfusion pathways, self-organized vascular structures more closely mimic the adaptive and hierarchical nature of native vasculature.

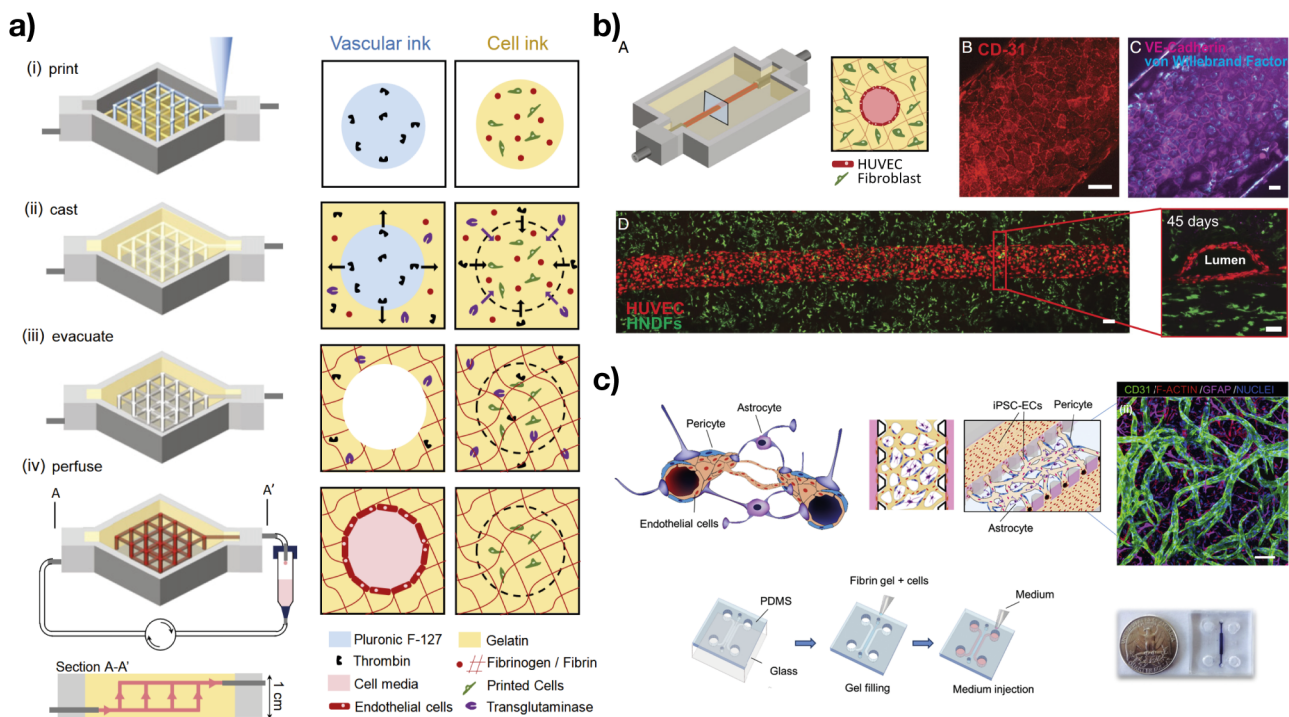


Figure 1.10: Vascularized tissue fabrication. Different approaches exist to engineer vasculature in fabricated tissues. Either vascular channels are built through 3D printing and casting methods or cells are stimulated to self built vascular structures. a) Schematic of a 3D bioprinting and casting approach. Fugitive ink is printed and cell ink is deposited on top. After crosslinking the cell ink, the fugitive ink is liquefied through cooling. Reproduced from reference [18] under the PNAS open access option. b) A perfused HUVEC-lined vascular channel is shown in a 3D perfusion chip. Reproduced from reference [18] under the PNAS open access option. c) Schematic and self-assembled model of the blood-brain barrier is presented. Used with permission of Elsevier Science & Technology Journals, from reference [39], Copyright © 1980; permission conveyed through Copyright Clearance Center, Inc.

Various strategies have already been explored to generate synthetic vasculature for tissue engi-

neering. For instance, Paulsen et al. proposed 3D printing of vascular networks to guide structured tissue formation [40]. This concept was subsequently implemented by multiple groups, including Kolesky et al. [18]. They used for example Pluronic F-127 as a vascular ink which was deposited by a 3D bioprinter and gelatin was used as a cell ink cast around the fabricated channel network. Through the integration of fibrinogen, thrombin, and transglutaminase the casted matrix crosslinks. Pluronic F-127 was chosen as a vascular ink due to its fugitive behavior upon cooling: Pluronic liquefies when being cooled. After evacuation of the vascular ink, the channels could be perfused with cell media and endothelial cells which grow then at the channels walls. Different materials can also serve the purpose, as shown by Pimentel et al. in their study [41] where they used polyvinyl alcohol (PVA) as a sacrificial ink.

A different approach was investigated for example by Griffith et al. who demonstrated that human umbilical vein endothelial cells can form vascular networks by self-organization in hydrogel matrices [42], or by Campisi et al. [39] who tried to model the micro vascular structure of the brain environment through self-assembly of endothelial cells, pericytes, and astrocytes in fibrin gels.

1.3 Principles of Creating and Controlling Spatiotemporal Artificial Environments

As we discussed in the previous sections the creation and regulation of artificial environments involves several challenges. In this section, we present the key methods used in this work to study pattern forming genetic networks, how to monitor living organisms embedded in hydrogels and their genetic modification, as well as the biofabrication of vascular structures for the creation of larger constructs. Together, these techniques enable the systematic study and fabrication of complex, functional biological systems.

1.3.1 Designing Genetic Circuits Using Evolutionary Algorithms

The behavior of genetic circuits can be described by partial differential equation (PDE) systems. In the case of a three node network, as we will present in Chapter 2, the system has a lot of parameters. To find the best parameter set, such that the genetic circuit shows the desired behavior, we implemented an algorithm that optimizes the parameters to match as closely as possible the optimization criterion.

Broadly, optimization algorithms can be divided into gradient-based methods and derivative-free methods. Gradient-based methods, which rely on derivative information are particularly efficient for smooth and convex problems, but they are also successfully applied to certain non-convex problems such as in neural network training. Derivative-free methods are often preferred when dealing with highly non-convex, noisy, or black-box functions where gradient information is unavailable or unreliable. Among those, evolutionary algorithms (EAs) form a prominent class of population-based meta-heuristics inspired by the principles of natural evolution. In nature, systems are optimized through biological evolution, which is driven by mechanisms such as reproduction, mutation, recombination, and selection. The individuals interact with their environment and compete for survival. Those who persist, mutate or recombine. A population of living organisms undergoes the evolutionary process across generations.

Building on these evolutionary principles, an EA does not necessarily converge on the fittest individual in terms of peak performance but often favors parameter sets that are robust under perturbation. This reflects findings from quasi-species theory and digital evolution experiments, which show that under high mutation rates, genotypes occupying flatter regions of the fitness landscape, for example those with higher mutational robustness, can outcompete faster-replicating but more fragile genotypes [43]. However, while natural populations can vary in size, as their individuals are subjected to selection, reproduction, mutation, and recombination, EAs maintain a fixed population size throughout the process. Through an iterative process, these candidates reproduce, undergo se-

lection pressure, and evolve towards an optimized solution. The initial population is typically chosen randomly, and each individual is evaluated based on a predefined fitness function, which determines its suitability to solve the problem. In the context of a mathematical problem, the parameters within an equation form a set of candidates, which evolve over successive generations through cycles of fitness evaluation, selection, mutation, and recombination. A single evolutionary cycle consists of: (1) evaluating each individual's fitness, (2) selecting the best individuals to advance to the next generation, and (3) applying mutation and recombination to generate new candidates. Once the next generation is established, the process repeats, with fitness values recalculated at each iteration (see Pseudo code 1). To ensure that the algorithm stops within a reasonable timeframe a stopping criterion is typically implemented [44, p. 22ff].

Algorithm 1 Evolutionary Algorithm

Require: certain parameters, fitness function

Ensure: population size, stopping criterion

 Create an initial population

while Stopping criterion is not fulfilled **do**

 Evaluate individuals

 Pass best individuals to the next generation

 Recombine individuals

 Mutate individuals

end while

1.3.2 Synthetic Biology Tools for Engineering and Visualizing Artificial Systems

Synthetic biology provides a versatile tool set for engineering genetic systems and visualizing their behavior in vitro as well as in vivo environments through e.g. adapted fluorescent proteins. These methods enable precise control over gene expression, genetic modifications, and molecular interactions. The field of synthetic biology comprises the engineering on all levels of biological structures from biomolecules to whole organisms [1]. A prominent model organism is the gram-negative bacterium *Escherichia coli* (*E. coli*) in which regulatory mechanisms such as the *lac* operon were discovered [45] and later were adapted for engineering. With the discovery of restriction enzymes [46], DNA ligases [47], polymerase chain reaction [48], and advances in DNA sequencing methods [49] the basis for cloning techniques was established. Through the assembly of standard biological parts like promoters, ribosome binding sites, genes and terminators genetic modules could be implemented [50] and transferred to a host cell as plasmid DNA.

Plasmids commonly occur in bacteria as circular double stranded DNA which is extra chromosomal and can replicate independently [51]. They fuel bacterial adaptability, as well as diversity through

plasmid exchange between bacteria and maintain the circular DNA templates over numerous cell cycles. Compatible plasmid DNA is transcribed by the expression machinery of the bacterial cell. Thus, plasmid DNA serves as an additional modular genetic tool, and can be used to express synthetic circuits and encode functional proteins. However, the circuit performance can be restricted due to unpredictable genetic crosstalk or resource sharing effects [52]. This is why plasmids are commonly engineered in *E. coli*, where they can be assembled, replicated, and extracted using commercial plasmid purification kits, but cell-free expression systems provide a much more controllable system to study just the behavior of the engineered synthetic gene circuit [53].

In addition to the bacterial genome and plasmid DNA, bacterial cells also contain ribosomal RNA and various regulatory RNAs, which influence gene expression. These can serve as binding sites for FISH probes in RNA-based imaging studies. The FISH technique has already been invented a long time ago, it was first introduced by Gall and Pardue in 1969 as a molecular technique for detecting and locating specific nucleic acid sequences within intact cells [54]. Initially, radioactive labels were used for visualization, but advancements by Rudkin and Stollar in 1977 introduced fluorescent labels to improve safety and resolution through immunofluorescence [55]. The FISH probes are short single stranded DNAs or RNAs which are labeled with a fluorophore at their 3' end [56]. The probes are designed to have the complementary sequence to the target DNA or RNA and can bind then directly at the target location. Exemplary target locations are the subunits of the prokaryotic ribosomes, 16S and 23S ribosomal RNA (rRNA). The 16S rRNA gene is a highly conserved component of the small subunit and plays a critical role in protein synthesis. The same is true for the 23S rRNA which is an important part of the big subunit. They both make an ideal target for identifying and distinguishing bacteria at the species level, as they contain hypervariable regions that are unique to each bacterial species despite their conserved regions [57].

With genome engineering, organisms or cells can not only be targeted but also modified with high specificity. These techniques are widely applied in areas like human health but also in agriculture [58]. Whereas in bacteria plasmids can be introduced via heat shock, transfection reagents have to be used for introducing DNA or RNA to mammalian cells. Once inside the cell, these nucleic acids can be used to transiently express genes, induce stable genetic modifications, or regulate endogenous pathways. Depending on the approach, cells can be programmed to produce specific proteins, express reporter genes, or even undergo directed differentiation.

One revolutionary system is clustered regularly interspaced short palindromic repeats (CRISPR) / Cas which introduces targeted genetic changes in a wide range of organisms [59]. It further enables precise gene knock-ins or knock-outs, epigenetic editing, or the construction of synthetic gene circuits that allow dynamic control over cellular behavior. Programmable dsDNases like Cas9 are used to induce targeted double-strand breaks (DSBs) in the genome. They can be programmed by guide RNA (gRNA) that includes a handle for the Cas protein to be recognized and target DNA sequence that

can invade the double-strand via a strand displacement mechanism. Upon binding Cas9 introduces a blunt-end dsDNA break which is subsequently repaired by non-homologous end joining, which can introduce indels at the cleavage site.

However, as CRISPR / Cas9 relies on DSBs and cellular repair mechanisms, unintended mutations can occur. To overcome these limitations, prime editing was developed as a more precise alternative by Anzalone et al. [60]. Prime editing does not create DSBs but instead uses a modified Cas9 enzyme and a reverse transcriptase to introduce targeted genetic changes with fewer errors [61]. The modified Cas9 protein (SpCas9) is fused to a reverse transcriptase (RT) domain to form the prime editor (PE). A gRNA that can bind to the Cas protein is extended to include the template with the modified sequence for the edit. This sequence is mostly complementary to the targeted DNA, but includes the modifications. The whole template is then called prime editing guide RNA (pegRNA), and the complex of PE and pegRNA prime editing complex.

For a prime edit to take place the prime editing complex binds to the target DNA: The complementary part on the pegRNA hybridizes to it. The Cas protein can then cut the unbound strand, followed by binding of the primer binding site to the nicked strand. The template is then reverse transcribed into this nicked strand. After the prime editing complex unbinds, a thermodynamical process takes place which can be described as strand migration. Either a stable edit or the original sequence is produced by DNA repair mechanism. In case of the original DNA being the final product, the prime editing complex can bind again and the whole process starts over. Over time this results in high efficiencies of the edit to take place [60].

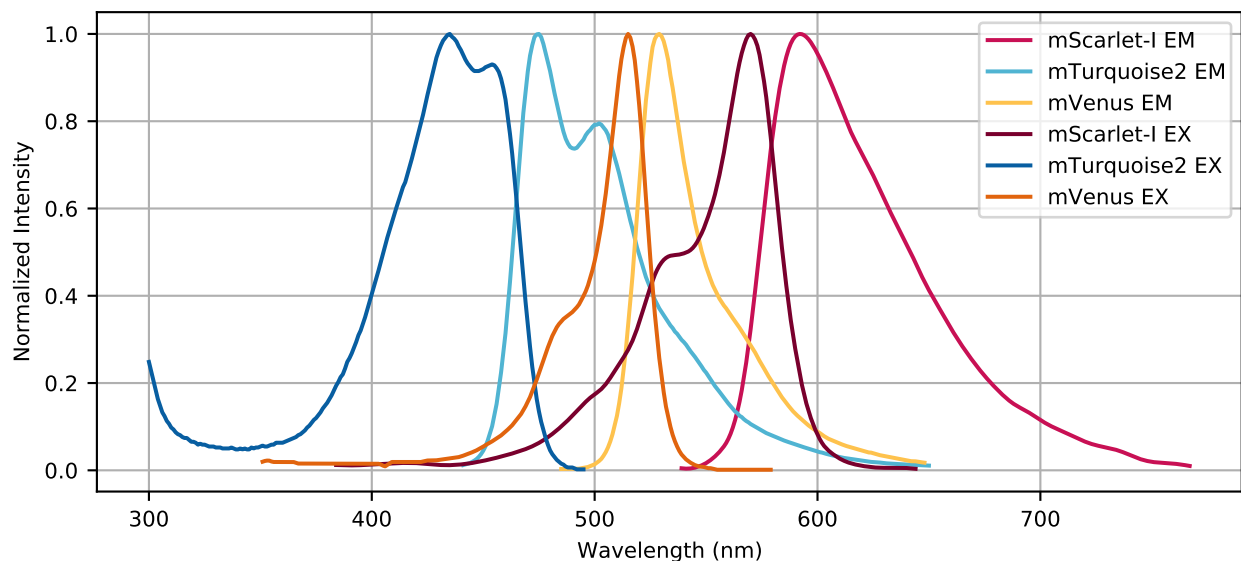


Figure 1.11: Emission and excitation spectra of three different proteins: mTurquoise2, mVenus, and mScarlet-I are shown.

To study the behavior of synthetic circuits and engineered cells, fluorescent proteins and dyes are widely used as visualization tools. When imaging more than one fluorescent protein or dye at the same time it is important that their spectra are orthogonal to each other. With the choice of excitation laser and emission filter it can be ensured that no bleed-through appears. Figure 1.11 shows how excitation and emission spectra of three different proteins can overlap.

Most of the fluorescent proteins used to date are genetically modified to enhance their signal to noise ratio or to shift their spectra so that they better fit the usage. One example is the blue-shift of green fluorescent protein (GFP) as shown by Heim et al. [62]. They screened for variants with altered spectra and found that the spectra of GFP was shifted from green to blue when the amino acid tyrosine was replaced by histidine in the amino acid sequence of GFP at position 66.

1.3.3 Microfluidic Systems for Non-Equilibrium Steady-State Conditions

Biological systems operate far from thermodynamic equilibrium, as they continuously exchange energy and matter to sustain life processes. To study and control such non-equilibrium steady-state conditions, microfluidic systems provide an ideal platform. These systems enable the continuous exchange of reactants and products, mimicking the dynamic conditions found in living tissues. Due to their small volume sizes and precisely controlled fluid manipulation, microfluidic reactors allow for well-regulated biochemical reactions and are a great tool to study pattern formation.

As illustrated in Figure 1.12, microfluidic systems can be implemented with varying degrees of complexity. The simplest designs, such as single observation chambers, allow pre-mixed fluids to be pumped through a reaction area (Figure 1.12a). More advanced systems incorporate multiple layers for flow and control units, which can precisely regulate in- and outflow via microfluidic inlets and pumps (Figure 1.12b). These setups enable dynamic control over biochemical environments, where reactions can be monitored in real-time using microscopy.

While traditional microfluidic systems provide precise control over biochemical reactions, they are inherently limited by their rigid channel walls, which confine reactions to designated reaction chambers. In contrast, in biological tissues, the reactions take place throughout the extracellular space rather than being restricted to predefined fluidic pathways. These channel-structures are mostly used for the transport of nutrients, signaling molecules, and waste products. To better mimic these *in vivo* conditions, several studies have explored the development of biological microfluidic and even macrofluidic systems, where fluid transport occurs within engineered biomaterials rather than enclosed micro channels [19], [41], [65], [66].

As shown in Figure 1.13, various strategies have been implemented to fabricate bioreactors that integrate fluidic systems directly into 3D bio scaffolds. These designs share the common feature that

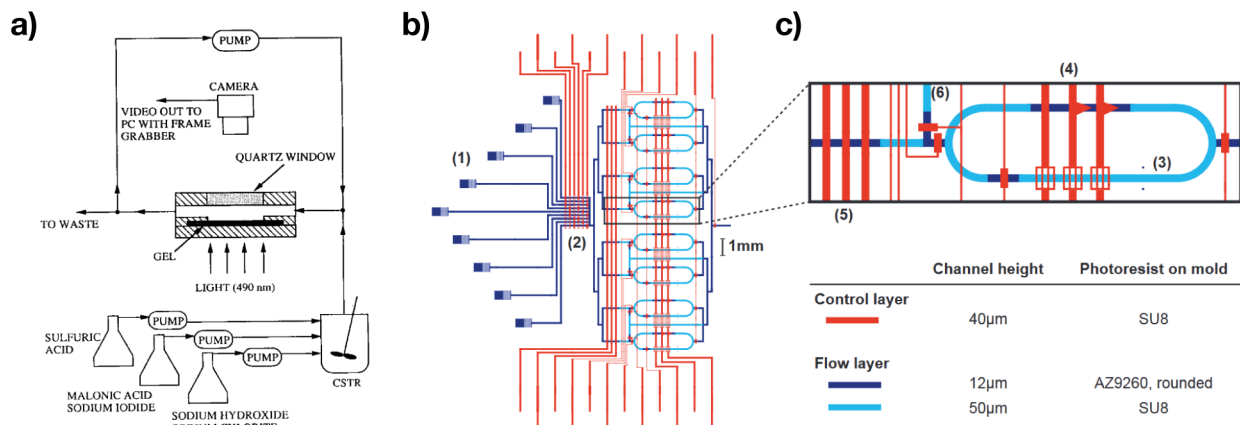


Figure 1.12: Microfluidic reactors can be used to establish non-equilibrium steady-state conditions as they occur in biological tissues. Microfluidic systems can be implemented with increasing complexity: From a) a single observation chamber where premixed fluids can be pumped through (Reprinted from reference [63], Copyright ©1992 with permission from Elsevier.) up to b) systems containing multiple layers for flow and control units. The in- and outflow can be controlled via in- and outlets, the flow itself is regulated via pumps and mixing can be achieved using peristaltic pumps. The reaction can then be viewed with a microscope in these single reaction units. Reproduced from reference [64] under the PNAS open access option.

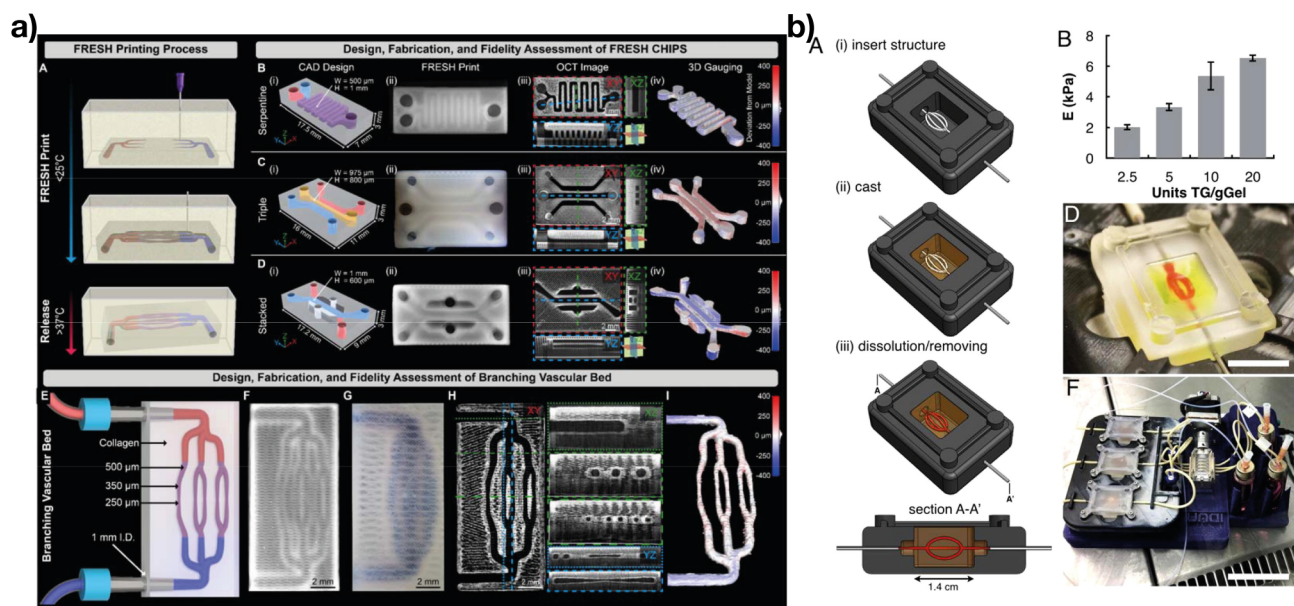


Figure 1.13: Fluidic systems in 3D bioscaffolds. Different strategies have been implemented to fabricate bioreactors which all have in common that the reactions to be observed take place in the bioreactor material itself which is contained in some sort of chamber (mostly fabricated through some sort of plastic). Nutrients, stimulating molecules and other reagents are then flushed to fabricated channel structures which perfuse the bioreactor material itself. a) Reproduced from reference [19] under the license CC BY 4.0. b) Reprinted from reference [41], copyright © 2018 with permission from Elsevier.

the biochemical reactions take place within the scaffold material itself, rather than being confined to the channel structures (Figure 1.13a). To sustain cellular activity, nutrients, stimulatory molecules, and other reagents are perfused through fabricated channel networks, that allow for controlled transport and diffusion into the tissue-like material (Figure 1.13b). Such perfusion bioreactors provide a more physiologically relevant environment for studying cell behavior, tissue development, and biochemical processes, bridging the gap between conventional microfluidic systems and complex biological tissues.

1.3.4 Bioprinting and Material Considerations for 3D Tissue Fabrication

Bioprinting enables the precise deposition of cells, biomaterials, and bioactive molecules to create complex, three-dimensional tissue constructs. This technique provides spatial control over both cellular organization and scaffold architecture, which makes it a powerful tool for engineering functional tissues. Several bioprinting approaches are available, as illustrated in Figure 1.14, including extrusion-based, inkjet-based, and laser-assisted bioprinting. Each method offers distinct advantages in terms of resolution, material compatibility, and printing speed. In extrusion-based and inkjet-based printing, the bioink is deposited through a nozzle onto a substrate, forming the structure layer by layer. In contrast, laser-assisted bioprinting and stereolithography rely on laser or UV light to crosslink the material during the printing process. While laser-based methods and stereolithography achieve high resolution on the micrometer to sub-micrometer scale [67], [68], extrusion and inkjet printing typically yield coarser features. However, a key advantage of extrusion-based bioprinting is their independence from photo-crosslinking. This is particularly relevant when working with sensitive biological components such as genetic material or living cells which can be affected by exposure to certain wavelengths.

To construct functional materials (Figure 1.14e and f), the bioink can be functionalized with genetic material and living organisms prior to printing, or these biological components can be introduced after the printing process. The choice depends largely on the sensitivity of the embedded cells or genetic elements. In particular, the mechanical stress and shear forces during printing can significantly affect cell viability. As a result, it is common practice, especially in tissue engineering applications, to seed cells onto pre-printed scaffolds rather than incorporating them into the bioink directly.

In extrusion-based bioprinting, various methods are used to deposit the bioink. These include pneumatic extrusion, piston-driven systems, and screw-based mechanisms, all of them offering different shear profiles and levels of control. Pneumatic systems, where pressure is used to push the material through the nozzle, are currently the most common in commercial bioprinters, likely due

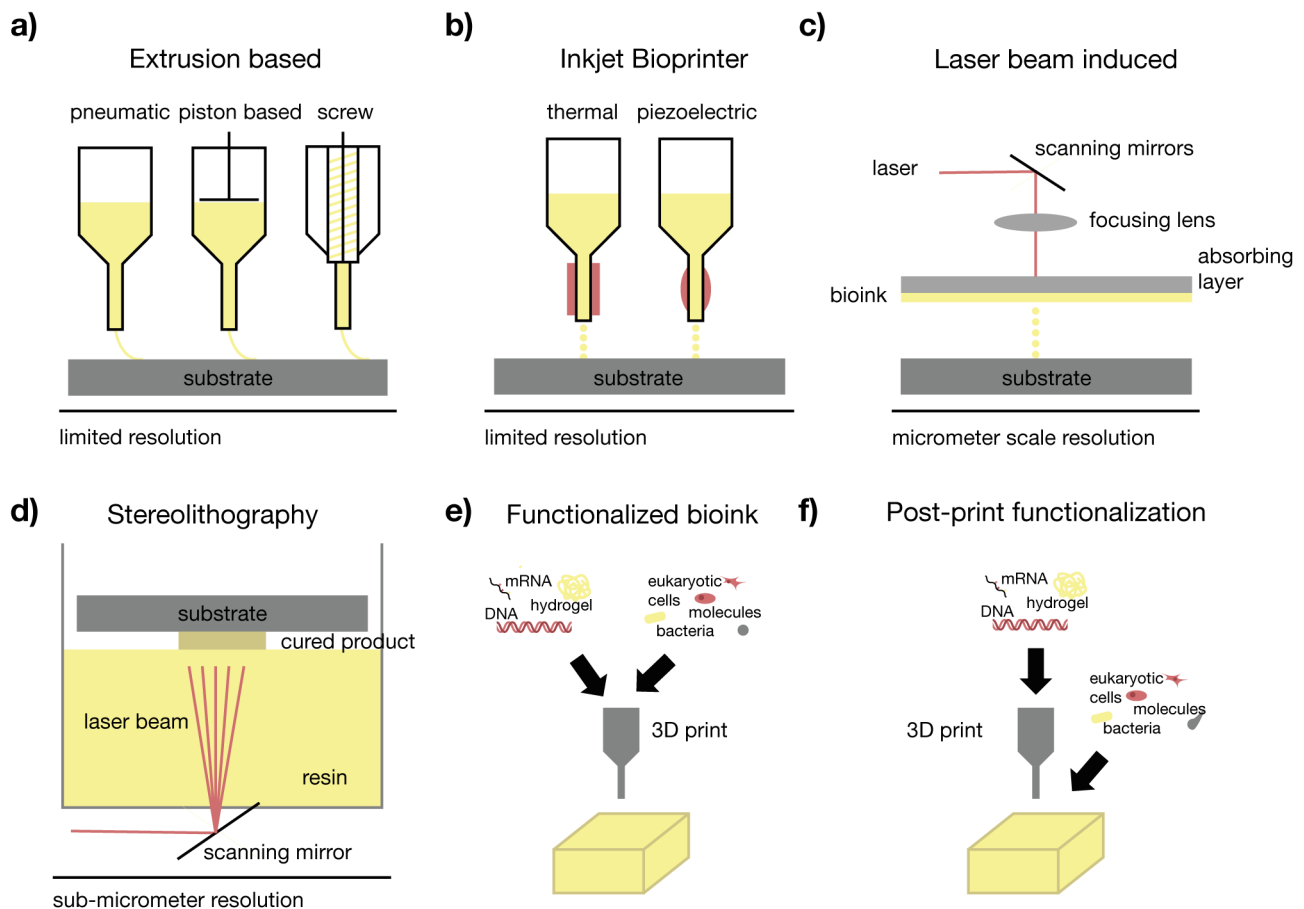


Figure 1.14: Schematic representation of different 3D printing techniques. a) Three major extrusion-based techniques exist, in which printed material is extruded either via application of pneumatic pressure, with a piston or with a screw. These techniques are particularly suited for printing of lines. b) In order to create structures out of small droplets, inkjet techniques can be used, which either use thermal or piezoelectric actuation. c) An alternative 3D printing method is laser-induced 3D printing, for which small droplets are released from a bioink layer with a laser. Here, the resolution is much higher compared to extrusion-based 3D and inkjet printing. d) Even higher resolutions can be achieved with stereolithography where a laser scanner is used to define structures by curing layer by layer from a resin bath. Two different approaches are used for 3D bioprinting: e) functional components – such as DNA, RNA, or proteins for structure formation or control, bacterial or eukaryotic cells, function-enhancing molecules such as growth factors, or inducers – and the bioink itself are mixed before printing of the object; or f) some of the functional components are added after printing and allowed to diffuse or grow into the object. Reproduced from reference [36] under the license CC BY-NC 4.0.

to their simple design and ease of implementation. However, pneumatic extrusion has notable limitations, such as the inability to retract material and the restricted control over minimum extrusion volume [69]. Piston-driven systems, on the other hand, typically use a stepper motor to control the piston movement, allowing for precise calculation of the extruded volume and the ability to retract material as needed. The material behavior during deposition also plays a crucial role in defining the final structure and resolution. Figure 1.15 illustrates typical deformation effects observed in extrusion-based bioprinting. In particular, it shows in Figure 1.15a the interaction between the first and second printed layers, and in Figure 1.15b the fusion process that occurs between adjacent hydrogel layers. These effects influence the precision of printed geometries and must be considered when optimizing print parameters and material formulations.

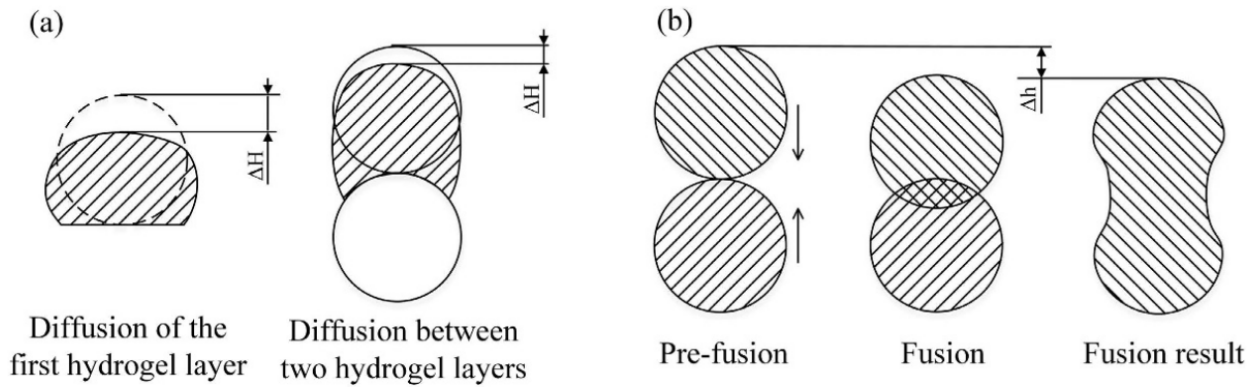


Figure 1.15: Deformation of hydrogel after deposition by a 3D extrusion bioprinter. a) Change of the first and second layer. b) Fusion process of two hydrogel layers. Reproduced from reference [70] under the license CC BY 4.0.

Once deposited, the bioink can be crosslinked either during or after the printing process. In addition to photo-crosslinking, thermal crosslinking and physical crosslinking via ionic or non-covalent interactions are also possible [71]. These diverse crosslinking mechanisms expand the range of usable materials and enable researchers to choose a method suited for the specific application – particularly important when printing sensitive biological content such as living cells or genetic material.

A key factor that influences the choice of extrusion method and crosslinking strategy is the choice of hydrogel, as it directly impacts the printability, mechanical properties, and biocompatibility of the final construct. Hydrogels used in bioprinting can be broadly categorized into natural and synthetic. Natural hydrogels, such as gelatin and collagen, are widely favored due to their inherent cell-adhesive properties, biodegradability, and close resemblance to the native ECM. In contrast, synthetic hydrogels offer superior tunability in mechanical properties and greater structural stability but often lack intrinsic bioactivity [72]. Especially gelatin methacryloyl (GelMA), a semi-synthetic hydrogel, is widely applied in bioprinting applications. It consists of gelatin that is modified with

methacrylamide and methacrylate groups. This functionalization introduces reactive double bonds, allowing GelMA to be crosslinked into a stable hydrogel network via photopolymerization. [73]

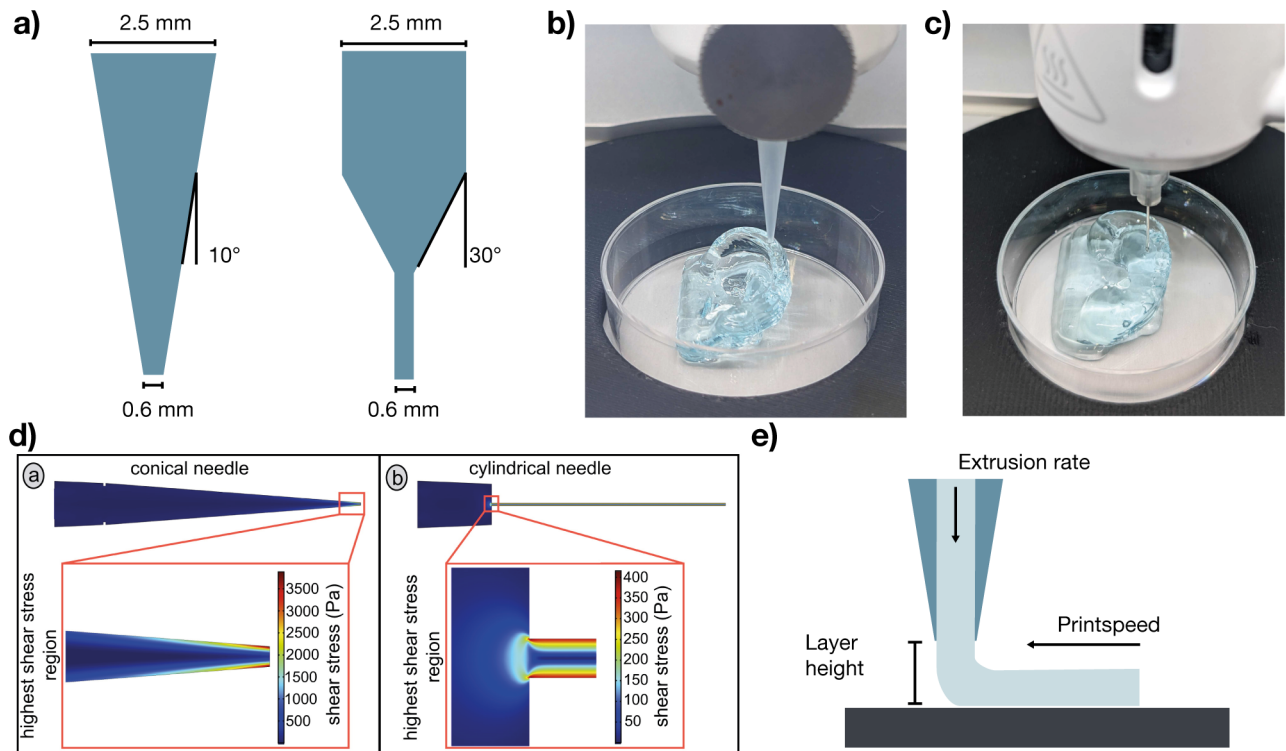


Figure 1.16: Hydrogels show shear thinning behavior a) Two different types of tips are mostly used in bioprinting approaches: conical (left) and cylindrical (right) needles. The main difference is the angle between the bioink reservoir and the tip, which leads to differences in the shear stress profile. With a constant extrusion rate the layer height of the released bioink is also dependant on the printspeed. b) Human ear printed with a light blue bioink (Cellink, Cellink Start) with a conical tip. c) The same print when using a cylindrical needle. d) Shear stress profile of two types of needle tips. Reprinted from reference [74], copyright © 2014 with permission from Elsevier.

For successful bioprinting, the rheological properties of the bioink must be carefully optimized to ensure both structural fidelity and cell viability [75]. Key factors include viscosity and shear-thinning behavior, which facilitate smooth extrusion while minimizing mechanical stress on embedded cells. Notably, the shear stress on the bioink during printing also depends on the geometry of the extrusion needle. Typically, either conical or cylindrical nozzles are used (Figure 1.16a). Even with identical printing parameters, like extrusion rate and travel speed, the bioink behaves differently depending on the nozzle shape, due to variations in shear stress distribution. As shown in Figure 1.16b and c, the print outcomes differ between nozzle types. The stress within a cylindrical nozzle is more uniformly confined, resulting in increased shear thinning of the bioink during extrusion. This leads to improved interlayer adhesion and a smoother surface appearance in the printed constructs. The corresponding shear stress profiles are illustrated in Figure 1.16d. In particular, cylindrical tips generate higher

and more localized shear stress, which can compromise cell viability. Therefore, conical nozzles are typically preferred for cell-laden bioinks.

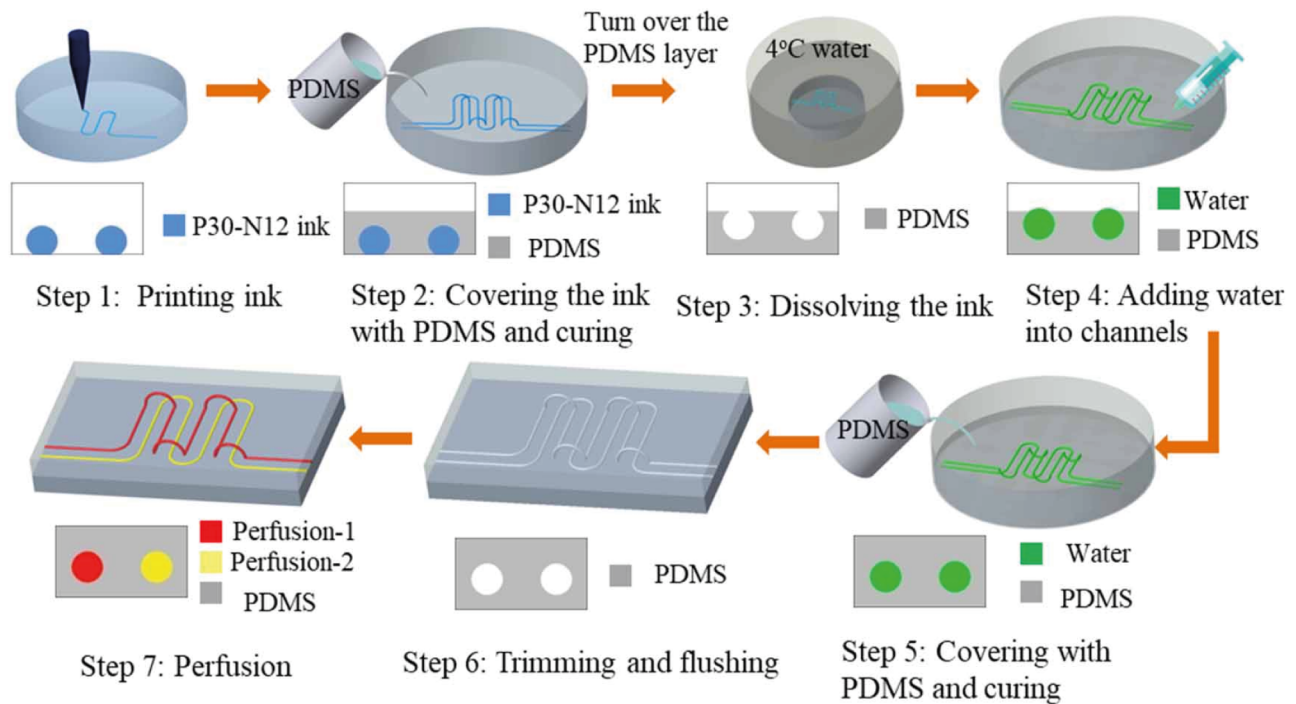


Figure 1.17: Evacuation based production of vascularization. Used with permission of IOP Publishing, Ltd, from reference [76], Copyright © 2006; permission conveyed through Copyright Clearance Center, Inc.

In addition to nozzle geometry, printing parameters – especially travel speed – strongly influence the quality of the printed construct. For instance, the line width is directly affected by the print speed and must be tuned to match the nozzle diameter (Figure 1.16e).

Beyond scaffold fabrication, bioprinting can also be leveraged in vascularization strategies, that enable the integration of perfusable networks within engineered tissues. The integration of vasculature-like structures is of importance to overcome diffusion limitations in thick tissue constructs. Several techniques exist to create vascular-like channels, among them the use of sacrificial bioinks and support-bath printing. When using sacrificial bioinks the vascular structures are printed with a bio-printer and the ECM material can be either printed or just cast on top, which would be beneficial for the inclusion of cells, as they do not have to experience the stress during printing. The disadvantage of the casting method is, that structures are rather limited in complexity as the printed structure can only be designed in one layer. This casting method is shown in Figure 1.17. Pluronic F-127, also known as Poloxamer 407, is often used as a sacrificial ink when applying this approach. It belongs to the group of Poloxamers (registered by BASF) that are non ionic triblock copolymers [77]. A central hydrophobic chain of ≈ 56 units of polypropylene glycol is flanked by two hydrophilic chains

of polyethylene glycol with ≈ 101 repeat units. It is well suited as sacrificial ink for bioprinting approaches due to its thermo-reversible behavior [78]. The Pluronic can be removed after printing and casting another material on top by dissolution to create open channels, as it liquefies upon cooling, whereas the other material shows the opposite behavior and gels upon lower temperatures.

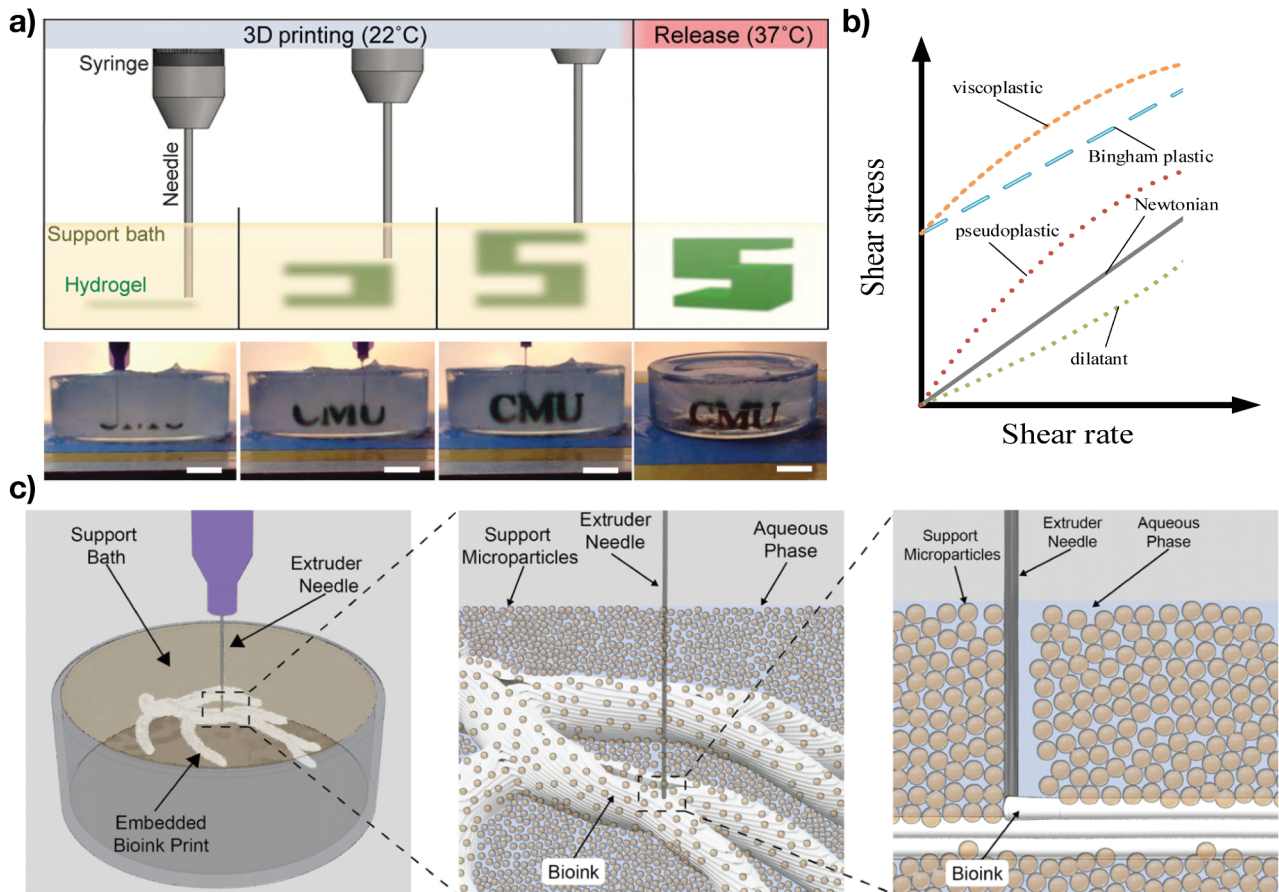


Figure 1.18: FRESH printing. a) Adapted with permission from reference [79] under the license CC BY 4.0. b) Adapted from reference [80] under the license CC BY. c) Adapted from reference [81] under the license CC BY.

Although casting techniques and fugitive ink strategies enable the formation of predefined architectures, they inherently lack true three-dimensional patterning capabilities. In these approaches, structures are typically created in a two-dimensional plane, followed by casting a secondary material, which restricts spatial freedom and complexity. A seemingly straightforward alternative is the direct extrusion of multiple bioinks into air using a bioprinter; however, this method is fundamentally constrained by the low mechanical integrity of hydrogels, which are prone to deformation or collapse when printed without support.

To mitigate this issue, Adam Feinberg's lab introduced a technique known as freeform reversible embedding of suspended hydrogels (FRESH), which enables bioinks to be printed into a support bath

that provides structural stabilization during deposition (Figure 1.18). This method allows printing at room temperature and overcomes the limitations of unsupported hydrogel extrusion. The support bath consists of a microparticle slurry that behaves like a Bingham plastic [79], exhibiting solid-like behavior under low stress to hold the bioink in place, but flowing under higher shear. Importantly, the support material can be liquefied at physiological temperatures (37°C), allowing gentle release of the printed construct for subsequent culture or analysis. Well-known examples of Bingham plastics include toothpaste and ketchup. A Bingham plastic is a type of non-Newtonian fluid that behaves like a solid at low stress and flows like a viscous liquid once a certain yield stress is exceeded [82]. During printing, this yield stress is applied locally when the needle tip moves relatively quickly through the material. The microparticles that make up the slurry are temporarily pushed aside by the moving needle but provide support again once the tip moves away. This effect is demonstrated in Figure 1.18c. After printing, the support bath containing the construct is heated to 37°C , causing the slurry to liquefy. The printed structure can then be carefully extracted by hand or by aspirating the support material.

2. Programming Genetic Circuits for Spatial Control

This chapter is based on the content of the publication:

Steady-State Operation of a Cell-free Genetic Band-Detection Circuit

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2.1 Introduction

Biological growth and development provide examples for a wide range of pattern formation processes, in which molecular programs are executed in the context of the physical and chemical boundary conditions set by their environment as we have discussed in Section 1.1. Inspired by such biological pattern formation mechanisms, there have been various attempts to establish synthetic gene networks ('gene circuits') that result in gradient-based spatial differentiation, and thus explore principles of biological pattern formation under well-controlled model settings [5], [28], [31].

Emulation of biological pattern-formation processes is also of great interest for the development of bio-inspired active materials and systems that are able to detect chemical or physical cues from their environment and adapt to them in response. Gaining insights into the function, regulation, and design flexibility of genetic circuits is therefore essential. One potential route towards this goal is the implementation of pattern-forming gene circuits in cell-free expression systems [84], [85], which would allow, e.g., the spatially differentiated production of proteins of interest. Among many advantages of cell-free systems [86] one is the possibility to combine components of different biological origin with completely synthetic components [87], which is important for potential not purely biological applications. Several attempts have been made to implement bio-inspired pattern formation and

differentiation processes in cell-free model systems. Isalan et al. [88] emulated *Drosophila* embryonic pattern formation in a 1D gel slab containing a cell-free expression system and genes coding for transcriptional repressors that were controlled by T7 and SP6 RNA polymerase (RNAP) as activators. Positional information was provided by gradients of T7 RNAP diffusing into the gel from its two ends. Using a simpler *in vitro* system requiring only a DNA polymerase, a nicking enzyme, and a nuclease, Zadorin et al. [89] were able to generate a French-Flag pattern and even utilize it to control the aggregation of DNA-linked microparticles. Guo & Murray demonstrated gene expression pulses generated by incoherent feed forward loop type 1 (IFFL-1), by realizing circuits composed of two activators and one repressor [90]. Dupin et al. implemented simple forms of symmetry-breaking [91] or stripe-formation [92] within assemblies of emulsion droplets that contained feedback gene circuits operated in bacterial cell extract. Notably, reaction-diffusion systems were also realized in enzyme-free systems exclusively based on DNA strand displacement reactions [93], [94], [95], [96].

Major challenges for cell-free systems operated in closed reactors, however, are the absence of a fully functional metabolism [30], [97] and the inability to fully self-regenerate [98], [99], which limits their lifetime to several hours [100]. Dynamic circuits that require continuous production and degradation of their constituents are therefore difficult to realize. For instance, Dupin et al. found the performance of a simple pattern-forming feedback circuit to be severely limited inside of a droplet network as the feedback cycle could not be completed within the finite lifetime of the encapsulated cell extract [92]. In order to address this problem, cell-free circuits have been previously operated in microfluidic flow reactors [7], [64], [101], [102], [103], [104] which offer the possibility to continuously provide chemical energy and remove waste products, and therefore maintain the systems out of equilibrium for extended periods of time. Microfluidic systems were used to demonstrate cell-free oscillating gene circuits [7], [101], [105], [106], which are difficult to implement in closed reactors. More recently, Pieters et al. [104] investigated coherent feed forward loop (CFFL) architectures involving post-transcriptional regulation by toehold switches [107] in a microfluidic flow reactor.

In the present work we focused on a cell-free implementation of a gene circuit with IFFL-2 topology [5], [31]. We computationally explored the parameter space for our system using an EA, which allowed us to identify critical design parameters for the realization of a band-detect function, or, in a spatial context, stripe-formation. One of the major challenges posed by the IFFL-2 topology is the establishment of repression and double repression between the nodes of the network, which requires extended run-times and removal or degradation of some of the gene products. Our simulations suggested that double repression and stripe-formation can be achieved, in principle, with our circuit design, but would require continuous production and degradation of its components. We therefore studied the system in microfluidic ring reactors [64] that allowed a semi-continuous operation of the system for at least 24 hours. We demonstrate that our cell-free

IFFL-2 performs as designed when operated under non-equilibrium steady state conditions, while it fails to show the desired behavior in a batch system. Our results underline the importance of maintaining cell-free synthetic biological systems out of equilibrium in order to realize non-trivial biological self-organization phenomena.

2.2 Results

2.2.1 Experimental Design

A schematic representation of our implementation of a stripe-forming incoherent feed forward loop is shown in Fig. 2.1a. The IFFL-2 is composed of three nodes, of which one node (the input node) exerts a direct inhibitory effect on a downstream node (node 2 in our notation) as well as a stimulatory effect achieved via double repression involving an intermediate node (node 3). With this design a NOR logic is implemented at node 2. In the context of pattern formation, an externally provided morphogen activates gene expression from input node 1. In its fully active state, node 1 will produce enough repressor to repress the two downstream nodes 2 and 3. For lower morphogen concentrations, the repressive action of node 1 will be reduced. Conversely, node 3 will produce larger amounts of repressor, when the morphogen is present at lower concentrations. In order to generate a stripe pattern, the system has to be tuned such that node 2 generates its maximum output at an intermediate concentration of morphogen, i.e., repression via node 1 must be low enough, and repression via node 3 not yet too strong (we have chosen the order of the numbers for the nodes to reflect their spatial position in the pattern - node 1 is most active on the left, 2 in the middle, and 3 on the right).

At the genetic level, the three nodes of our circuit were implemented through the use of six plasmids, which are indicated in Fig. 2.1a. Node 1 comprises three plasmids containing genes for the repressors TetR and LacI and the fluorescent protein dmTurquoise2, derived from mTurquoise2 [108] truncated at N-terminus to optimize for cell-free gene expression [30], [109], respectively, which are each put under the control of an E.coli σ^{28} -promoter. Sigma factor σ^{28} activates transcription of these genes and is used as the external input to the circuit - hence, σ^{28} takes the role of a 'morphogen'. Node 2 is realized with a single plasmid coding for the fluorescent reporter mScarlet-I [110] under the control of a $P_{\text{LtetO-1}}$ promoter, while node 3 comprises two plasmids coding for TetR and mVenus [111], each under the control of the promoter $P_{\text{LlacO-1}}$. Both promoters had been previously demonstrated to allow for independent and tight regulation in E.coli [112]. When subjected to a gradient of σ^{28} , the circuit is thus expected to generate dmTurquoise2 (from node 1) at high morphogen concentrations, mScarlet-I (node 2) at intermediate, and mVenus (node 3) at low concentrations (cf. Fig. 2.1c).

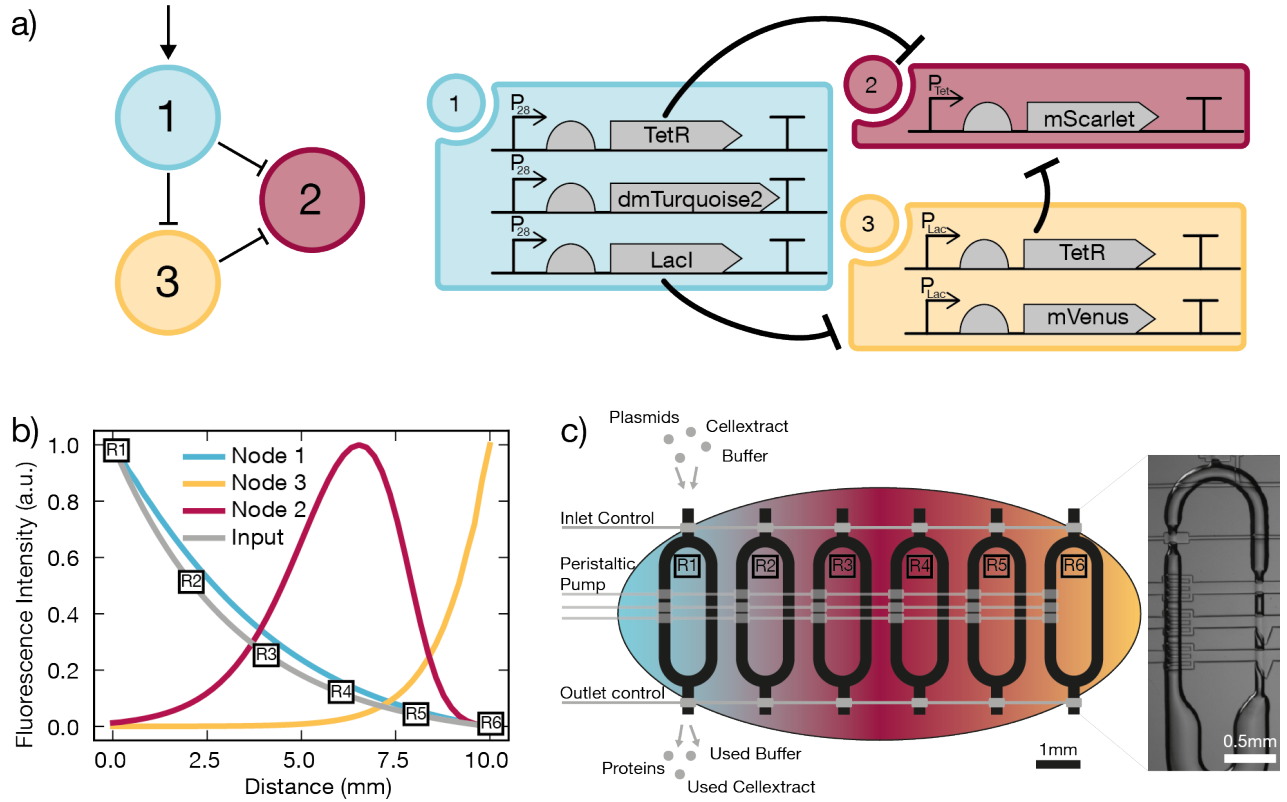


Figure 2.1: a) Schematic representation of our implementation of a stripe-forming incoherent feed forward loop. In contrast to the usual notation with node 1 being the input node, node 2 being the intermediate node, and node 3 being the downstream node; we used a notation that reflects the spatial order of the nodes. The IFFL-2 is composed of three nodes, of which node 1 acts as the input node responding to the morphogen. Node 1 has a direct inhibitory effect on node 2, but also an indirect stimulatory effect that is exerted via double repression involving the intermediate node 3. With this design a NOR logic is implemented for node 2. The nodes of the genetic circuit are implemented with six different plasmids (indicated as transcription units). The three plasmids representing node 1 contain genes for the repressors *TetR* and *LacI*, as well as a plasmid for the fluorescent protein *dmTurquoise*, all under the control of an *E.coli* σ^{28} -promoter (P_{28}). The plasmid of node 2 contains a gene for the fluorescent protein *mScarlet-I* under the control of a $P_{LtetO-1}$ promoter (P_{Tet}) and the plasmids realizing node 3 contain genes for the repressor *TetR* and the fluorescent protein *mVenus* under the control of a $P_{LlacO-1}$ promoter (P_{Lac}). b) Simulated spatially varying output of the circuit in response to an exponential σ^{28} gradient after 100 generations of optimization with an EA. The boxes (R1-R6) indicate the positions, whose input concentrations were sampled in the experiments. c) In order to emulate the situation of a developing embryo (schematically indicated in the back) in a cell-free system, the IFFL-2 circuit was operated in microfluidic ring reactors [64] that were supplied with different concentrations of morphogen corresponding to their position in the (virtual) input gradient shown in b). The distance between neighboring reactors is 2 mm (center-to-center), but they are not directly connected to each other.

In order to assess whether our IFFL-2 would be capable of generating a three-stripe pattern in a spatial context, and to further guide our experiments, we simulated the corresponding system of differential equations using an ordinary differential equation (ODE) solver, and optimized the parameter settings using an EA (cf. Section 2.4.12). For the simulation, we assumed a one-dimensional system spatially extended in half-space with a morphogen source with fixed concentration at $x = 0$. Assuming a constant, spatially homogeneous degradation of the morphogen, the source generates an exponentially decaying morphogen concentration profile with inverse decay length $\lambda = 1/(0.3 \text{ mm})$, as indicated in Fig. 1b. Accordingly, copies of the IFFL-2 gene circuit at different positions are exposed to different morphogen inputs, depending on their distance from the source. We further neglected diffusion of the gene circuit components and products themselves (see discussion below). The resulting expected expression levels as a function of distance from the morphogen source using optimized circuit parameters - expression strengths, induction thresholds, Hill coefficients, and degradation rates (cf. Table 2.4) - obtained after 100 iterations of the EA (cf. Supplementary Information of reference [83] Figure S13) are shown in Fig. 2.1b. As desired, the signal produced by node 1 decreases with decreasing amounts of morphogen, while the signal of node 3 increases with higher amounts. At intermediate input concentrations, node 2 has its maximum activity.

The simulations show that a three-stripe pattern can be generated for experimentally realistic production rates and Hill parameters. In addition, the analysis of the parameters (cf. Supplementary Information of reference [83] Figure S12) suggests that a higher amount of LacI repressor compared to the amount of TetR repressor under the control of σ^{28} would lead to a more distinct pattern. Importantly, the simulations show that the middle stripe only emerges for a sufficiently large degradation/dilution rate d (cf. Supplementary Information of reference [83] Figure S14). Further, a pronounced middle peak requires a sufficiently high reaction rate b (cf. Supplementary Information of reference [83] Figure S15).

We tested whether diffusion would change the expression pattern appreciably and numerically solved the corresponding PDE system for varying values of the diffusion coefficient (cf. Supplementary Information of reference [83] Figure S16). The simulations suggest that the pattern would not be affected for diffusion coefficients below $\approx 100 \mu\text{m}^2/\text{s}$, which has to be compared with typical diffusion coefficients for proteins that lie - depending on their environment - in the range from $1\text{-}10 \mu\text{m}^2/\text{s}$ [113], [114]. As a caveat, one has to consider that the simulation was performed based on the physical dimensions of our microfluidic system - extending over a length of 10 mm. A physical implementation of the same circuit at a much smaller scale would lead to noticeable diffusive broadening of the pattern.

As alluded to in Fig. 2.1c, our model system is roughly inspired by the situation in a *Drosophila* embryo, where the gap gene circuit is exposed to a spatially continuous gradient of the maternal morphogen. There are numerous differences between the systems, however. For our experimental

realization using a cell-free expression system, we ‘discretized’ the system using six microfluidic ring reactors [64], which is justified for the comparatively large length of our ‘virtual embryo’. As indicated in Figure 2.1b we probed the output of the genetic circuit at six discrete positions in the morphogen gradient. Rather than generating a physical morphogen gradient by diffusion, we emulated the gradient by simply supplying the rings with concentrations of the σ^{28} morphogen that corresponds to specific positions within the exponential gradient, ranging from 1000 nM close to the (virtual) source (reactor R1) to almost 0 nM for the most remote reactor R6.

In a *Drosophila* embryo, which has a length of only about 500 μm , an initial gradient of the morphogen Bcd is formed along the anterior-posterior axis [114], [115]. The morphogen decay length is about 100 μm and thus the ratio of total length to decay length is comparable to our system. However, the Bcd gradient is stable for only ≈ 1 hour, during which it activates expression of the next layer of morphogens, which in turn affect each other within the gap gene network. [114], [116] By contrast, our ‘virtual gradient’ of σ^{28} is held stable during the whole duration of the experiment (24h), and the IFFL-2 circuit - which is much simpler than the gap gene network - is allowed to reach steady state after 5-8 h, without involving additional morphogens.

2.2.2 Cell Extract Engineering

Our circuit components were initially tested in an S30 cell extract prepared in-house from *E. coli* strain BL21-Rosetta 2 (DE3) following the protocol described in Ref. [117]. As mentioned, TetR and LacI were chosen as repressors for our implementation of the IFFL-2 due to their good orthogonality and tight regulation. However, efficient use of LacI is compromised by the fact that LacI repressor is already present in the cell extract. As shown in Fig. 2.2a, expression of mVenus from the $P_{\text{LacO-1}}$ promoter in standard cell extract is strongly affected by the addition of the inducer isopropyl β -D-1-thiogalactopyranoside (IPTG), indicating that the promoter is appreciably repressed by spurious LacI in the extract.

As the execution of the IFFL-2 circuit, and thus formation of a stripe pattern, relies on the accurate regulation of the promoters involved, any background LacI in the cell extract would interfere with the circuit. In order to overcome this issue, we engineered a LacI-deficient variant of BL21-Rosetta2 (non-DE3) using the pORTMAGE genome engineering method [118], and then used the engineered strain for the production of cell extract (cf. Section 2.4.3). As desired, IPTG does not have any effect on the expression of mVenus from the lac promoter in the engineered extract and its overall expression level is higher (Fig. 2.2a).

We next separately studied the performance of the three nodes of the IFFL-2 circuit in the LacI-deficient extract. As shown in Fig. 2.2b, node 1 is properly activated when supplied with the morphogen sigma factor σ^{28} . In order to characterize node 3, we studied the influence of the

node 1 plasmids on the expression of mVenus under the control of P_{LacO-1} . As can be seen in Fig. 2.2c, mVenus expression is reduced in the presence of the upstream plasmids, which is likely caused by leaky expression of LacI from the σ^{28} -promoter as well as resource sharing effects within the cell extract (full activation of dmTurquoise production leads to a roughly two-fold reduction in the expression of mVenus) [119]. Most importantly, however, in the presence of σ^{28} , the expression of LacI from a fully activated node 1 results in a strong repression of the promoter P_{LacO-1} , as desired. We also tested multiple combinations of plasmids to explore the influence of the upstream nodes 1 and 2 on node 3 (Fig. 2.2d). In addition to this, we also tested varying amounts of σ^{28} and the plasmids P_{28} expressing the repressors TetR and LacI to find their optimal concentrations (cf. Supplementary Information of reference [83] Figure S2-3).

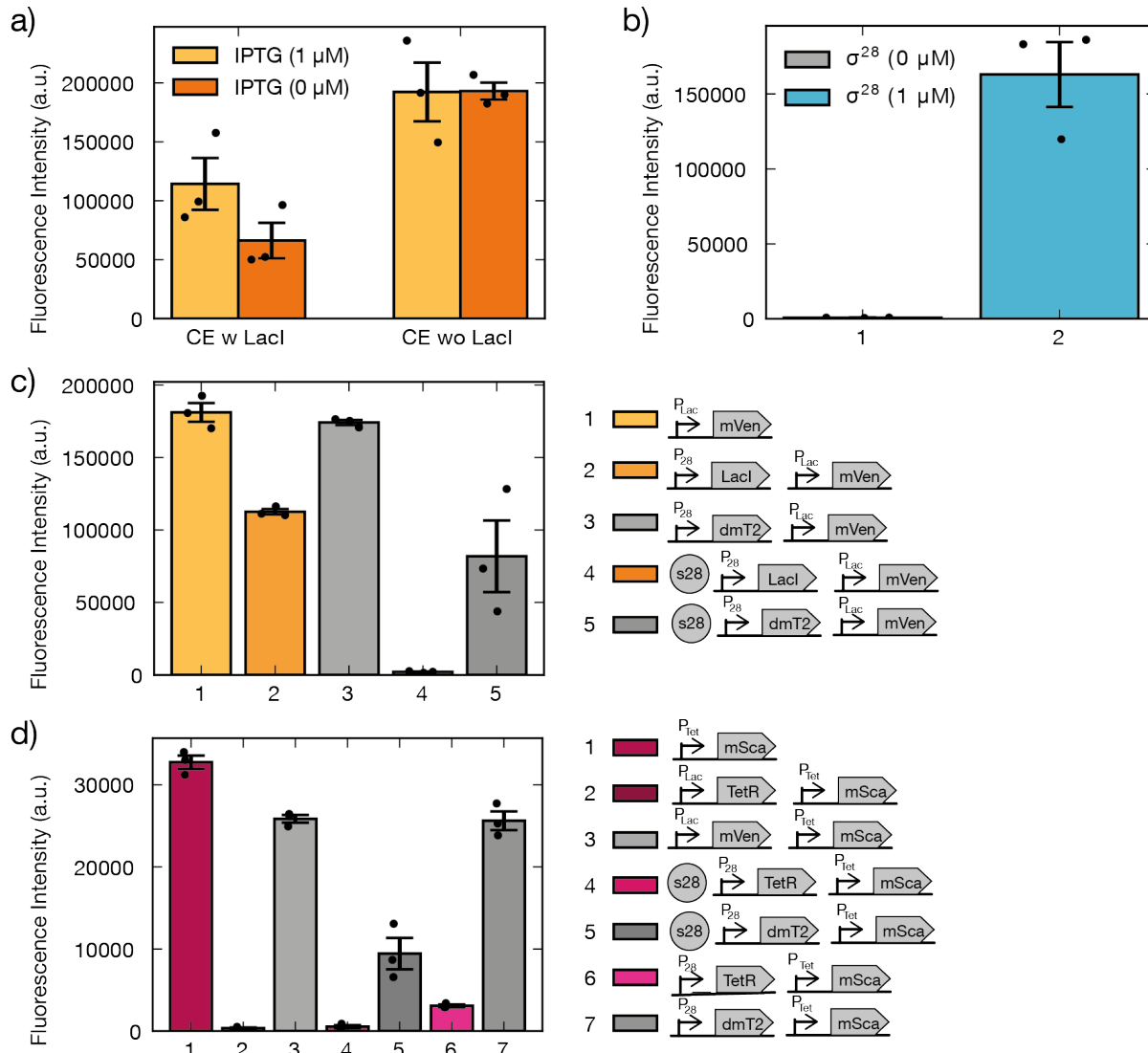


Figure 2.2: Analysis of sub-circuits of the IFFL-2 shown in Fig. 2.1a. a) Left: Expression of the fluorescent protein mVenus (mVen) under the P_{LacO-1} (P_{Lac}) promoter is affected by LacI repressors already present in the cell extract. Hence, in the absence of the inducer IPTG the expression level is reduced. Right: When using an engineered cell extract deficient of LacI, gene expression levels are higher, and the addition of IPTG does not have an effect. b) Performance of node 1 in the LacI-deficient cell extract (CE). Addition of σ^{28} (s28) activates expression of dmTurquoise2 - (mTurquoise2 with an additional delete for enhanced functionality) (dmT2) from the σ^{28} -promoter (P_{28}). c) Analysis of the effect of node 1 on node 3. Expression of mVen from P_{Lac} is affected by the presence of the plasmid coding for LacI under the control of P_{28} even in the absence of σ^{28} (#2), indicating some background expression of the repressor. Upon addition of σ^{28} , production of mVen is fully repressed as desired (#4). Also the presence of the node 1 plasmid coding for dmTurquoise 2 has an effect on the mVen expression which is lowered (#5). d) Effect of plasmids contained in node 1 or node 3 on node 2. TetR produced from node 1 or node 3 represses expression of mScarlet-I (mSca) as expected (bars #2 and #4). When P_{28} is not activated by σ^{28} , the mScarlet-I expression is increased (compare #6 and #4). As in c) the presence of additional plasmids generally reduces the expression level due to resource sharing effects. All fluorescence values were determined in a plate reader after 16 h of cell-free expression of the respective plasmids. Error bars show the standard error of the mean of biological triplicates. Grey colored bars indicate control experiments. Raw values are shown, as only the relative fluorescence levels were of interest (corresponding time traces are shown in Figures S4–S6 in the Supplementary Information of reference [83]). For conversion of arbitrary plate reader units to protein concentration, a calibration curve is provided in Figure S1 of the Supplementary Information of reference [83].

The results indicate that $P_{LtetO-1}$ is properly repressed by TetR produced from node 3 (via P_{LacO-1}) as well as from node 1 (through σ^{28} -promoter). Again, the signal levels for the measurements with two plasmids appear to be reduced due to resource sharing effects. Rather than adjusting the plasmid concentrations, the system could potentially also be tuned by changing the concentrations of the inducers IPTG and anhydrotetracycline (aTc).

2.2.3 Closed vs. Open Reactors

While our control experiments demonstrated the direct repression of node 2 by its upstream nodes, operation of the double repression cascade from node 1 along node 3 to node 2 failed when it was studied in a closed reactor (i.e., in a microtiter well) (Fig. 2.3a). In the initial phase of the experiment, enough TetR is produced to repress expression of mScarlet-I from node 2. Even when the concentration of LacI ramps up over time, repression of TetR production from node 3 does not lead to a de-repression of node 2, as the concentration of TetR does not decrease. Furthermore, the feed forward cascade results in a time delay during which the activity of the cell extract diminishes [92] - so even if there was de-repression, the exhausted extract would not be able to produce mScarlet-I at this later point.

We surmised that these issues - lack of protein degradation and exhaustion of the reaction - could be circumvented by operating the system in a microfluidic reactor that could refresh the cell extract and provide a regular turnover of circuit components. We therefore studied the double repression cascade in a ring reactor (cf. Fig. 2.1d), which allowed exchanging a defined fraction R (the 'refresh ratio') of the reaction volume with fresh medium every $t_R = 15$ min. The refresh ratio can be related to a dilution rate via the expression $d = -\ln(1 - R)/t_R$. In the experiments, we used $R = 0.2$, which corresponds to $d = 0.00025 \text{ s}^{-1}$, about half the rate assumed in the simulations. In such a system, initially produced TetR is flushed out over time, while its further production is repressed by the production of LacI. By emulating a simple 'metabolism' in this way, the double repression cascade effectively results in activation of mScarlet-I production, as shown in Fig. 2.3b.

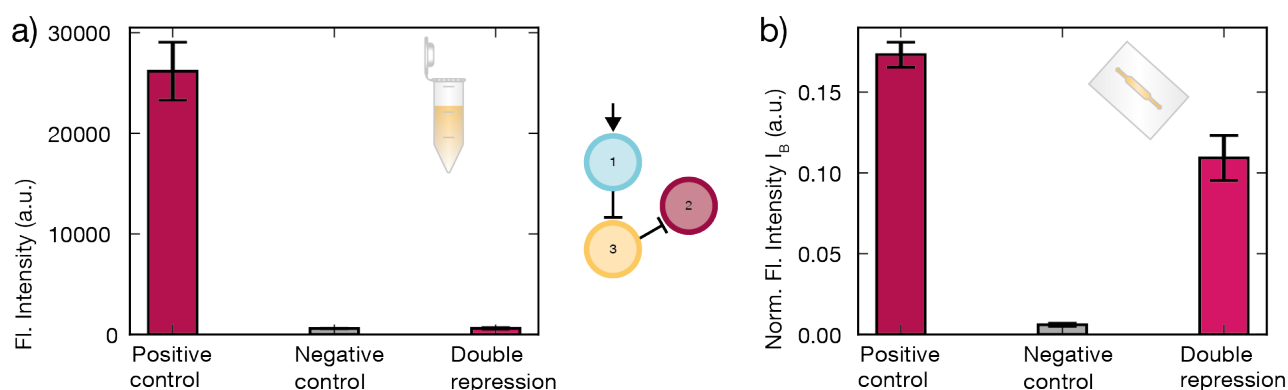


Figure 2.3: Operation of the double repression cascade $1 \rightarrow 3 \rightarrow 2$ in a) a closed reactor (data are taken after 16 h of measurement in a plate reader, raw values are shown. Plasmids expressing a repressor are added at a concentration of 1 nM and the plasmid expression the reporter is added at a concentration of 2 nM.) and b) a microfluidic device that maintains non-equilibrium steady-state conditions (measured in a ring reactor for 20h - values are normalized according to Eq. (2) to account for variability in the reactor geometry using a fluorescent reference dye as described in the Section 2.4.10. Plasmids expressing a repressor are added at a concentration of 0.5 nM and the plasmid expressing the reporter is added at a concentration of 1 nM). For the corresponding time traces cf. Figure S7 of the Supplementary Information of reference [83]. As a positive control the expression of mScarlet-I under a $P_{\text{LtetO-1}}$ promoter in the absence of TetR is used. In the negative control, a node 3 plasmid expressing the TetR repressor from a $P_{\text{LlacO-1}}$ promoter was added. For the double repression, a third plasmid (from node 1) was added, which expresses LacI under a P_{28} promoter in the presence of 200 nM of σ^{28} , and thus represses TetR production from the second plasmid. Compared to the scheme in Fig. 1, the plasmid connecting node 1 with node 2 is left out. Only in the microfluidic reactor steady state protein levels can establish that result in an activation of mScarlet-I production by double repression.

2.2.4 Generation of a ‘Virtual’ Stripe Pattern

Following the successful execution of the double repression cascade, we finally operated the whole IFFL-2 circuit in the ring reactors (Fig. 2.4), where each reactor was fed with a different concentration of σ^{28} supplied with the plasmid and cell extract mix (cf. Section 2.4.4), corresponding to a different position in a hypothetical morphogen gradient. The concentrations of the plasmids expressing the fluorescent proteins were chosen to be 1 nM, while the concentrations of the plasmids expressing the repressors were chosen to be 0.5 nM. As suggested by our simulations with the EA, the concentration of the plasmid expressing the TetR repressor under P_{28} was reduced by 20% compared to the plasmid expressing LacI under P_{28} (cf. Section 2.4.12). Experimentally we find that with these settings indeed the amount of expressed dmTurquoise2 decreases with lower amounts of the input σ^{28} , whereas in contrast the level of mVenus expression increases. Only at intermediate concentrations of the σ^{28} ‘morphogen’, mScarlet-I is found highly expressed (Fig. 2.4a). While there is considerable variability from experiment to experiment (cf. individual data points and error bars in the Figure), we find overall good qualitative agreement with the predicted stripe pattern. Note that a slightly reduced expression strength (b in the model) leads to a shift of the middle peak towards the front (cf. Supplementary Information of reference [83] Figure S15).

We can also visualize the establishment of quasi-steady state execution of the IFFL-2 in the ring reactors by assessing the temporal evolution of the expression levels. Taking reactor 4 as an example (Fig. 2.4a), one can see that in the initial phase of the reaction, in the absence of the repressors TetR and LacI, both mScarlet-I and mVenus signals rise, while dmTurquoise2 lags behind (Fig. 2.4b). Upon expression of the repressors by node 1, the signal caused by node 3 is repressed, and only the mScarlet-I stays at a high level as desired for the ‘middle stripe’. In the experiment, the initial transient lasts for about 8 hours, after which the signals stay approximately constant for the following 16 hours (i.e., until the end of the experiment). Notably, a typical cell-free gene expression reaction carried out in a closed reactor would only run for a few hours and thus would not survive long enough to generate the desired pattern (as can be seen in Figures S4-S7 of the Supplementary Information of reference [83], the output of our cell-free reactions saturates after ≈ 5 h in a closed system, which is consistent with other cell-free studies [100]). The observed time lag between dmTurquoise2 and the other fluorescent proteins can be interpreted as a resource-sharing effect: in node 1, dmTurquoise2 is expressed from the σ^{28} promoter, which is slightly weaker than the σ^{70} promoter controlling the other nodes. When initially nodes 2 and 3 are active they recruit most of the available polymerases. Over time, node 1 generates repressors that reduce transcriptional activity of nodes 2 and 3, and thus more RNAPs become available for node 1, effectively creating a positive feedback on node 1 that results in the observed time lag. This behavior is also apparent in Figure S8 in the Supplementary Information of reference [83].

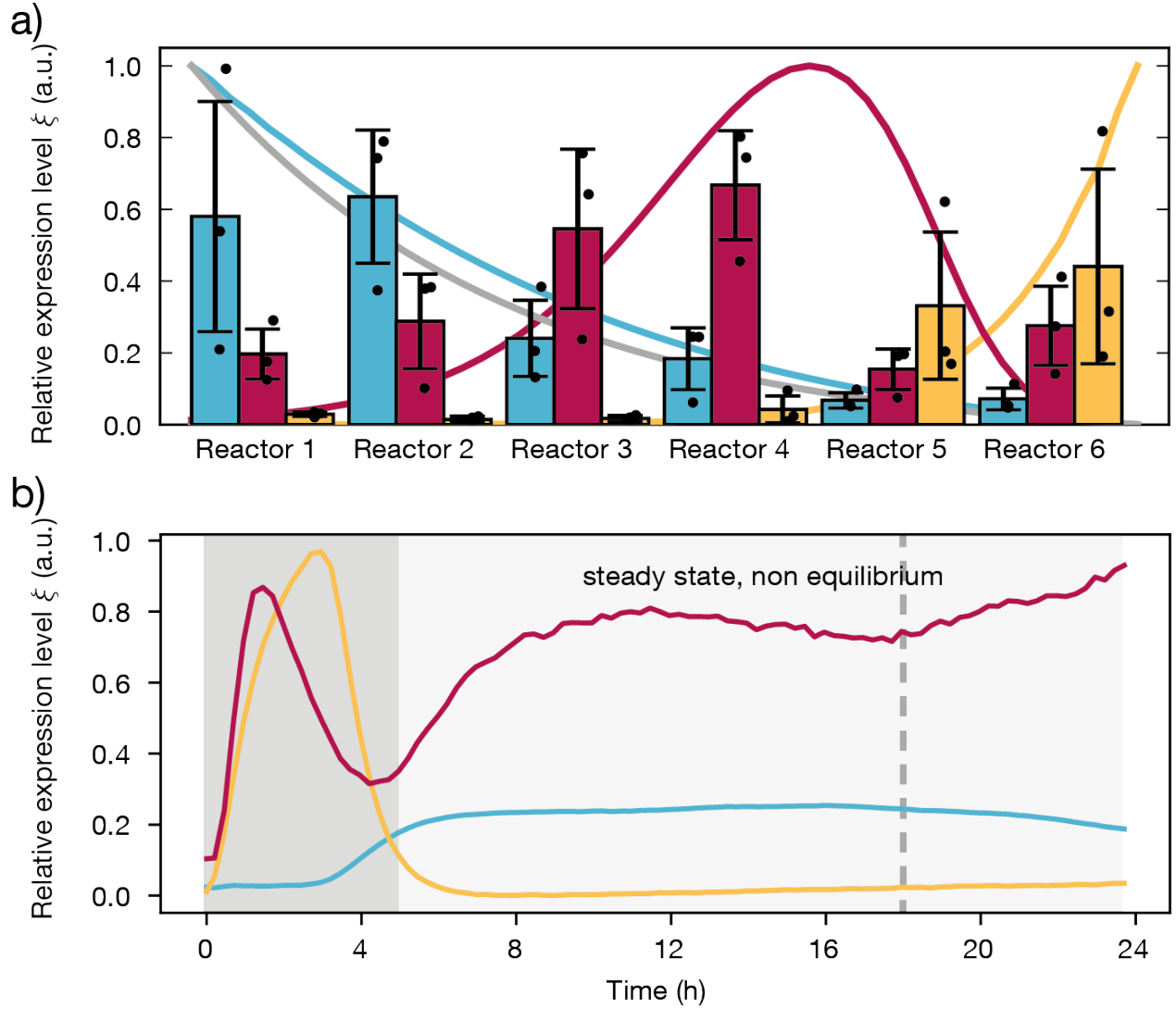


Figure 2.4: Band detection in microfluidic ring reactors. In a) gene expression data normalized to the range $[0, 1]$ is shown as a bar plot together with the corresponding simulation results for the stripe pattern (continuous lines) after 18 h (blue: dmT2, red: mScarlet-I (mSc), yellow: mVenus (mVen)). Expression data is normalized to account for differences in gene expression levels of the fluorescent proteins and differences among the ring reactors (for details cf. Section 2.4.10, Eq. (3)). Error bars show the standard error of the mean of biological triplicates (shown as individual points). The concentrations of the plasmids were $P_{28}\text{-LacI}$: 0.47 nM, $P_{28}\text{-TetR}$: 0.35 nM, $P_{28}\text{-dmT2}$: 1 nM, $P_{\text{Lac}}\text{-mVen}$: 1.14 nM, $P_{\text{Tet}}\text{-mSca}$: 1 nM, $P_{\text{Lac}}\text{-TetR}$: 0.57 nM; and the concentrations of σ^{28} in the reactors were 1: 1000 nM, 2: 500 nM, 3: 200 nM, 4: 100 nM, 5: 50 nM, 6: 0 nM. In b) an example of the temporal progress of gene expression measured in reactor 4 ($[\sigma^{28}] = 100$ nM) is shown for 24 h. The first 5 hours (highlighted in grey) show an initial transient, after which the system settles into a non-equilibrium steady-state phase. The dashed line indicates the time point at which data was collected for Fig. 4a. For all time curves cf. Figure S8 of the Supplementary Information of reference [83].

2.3 Discussion and Conclusion

In this work, we designed a genetic circuit comprising three expression units (nodes) arranged in the topology of an incoherent feed forward loop of type 2, which can act as a genetic band detector. In a spatial context, when exposed to a gradient of activating molecules ('morphogens'), the circuit can generate a spatially differentiated gene expression response in form of a three-stripe pattern. We simulated the behavior of the circuit in a exponential morphogen gradient by solving the corresponding system of ODEs for its three nodes and determined the optimal parameters for the formation of a three-stripe pattern with an EA.

Experimentally, we implemented the circuit on six plasmids expressed in a cell-free gene expression system, where we made use of the widely employed promoters P_{28} , P_{LacO-1} , and P_{TetO-1} . We used sigma factor σ^{28} as the externally supplied morphogen, while the internal wiring of the circuit was achieved with the LacI and TetR repressors. In order to be able to improve the performance of the promoter P_{LacO-1} in the context of our circuit, we engineered a LacI-deficient cell extract produced from a genome-engineered bacterial strain without LacI. Genes put under the control of P_{LacO-1} thus only respond to LacI generated by the circuit itself, and not to residual LacI in the extract.

One of the main challenges for the implementation of an IFFL-2 in a cell-extract with finite lifetime is the execution of a double repression cascade such as *node 1* $\dashv$ *node 3* $\dashv$ *node 2*, which should lead to an effective activation of node 2 by node 1. In the initial phase of a gene expression reaction, node 3 represses node 2, and by the time node 3 is sufficiently repressed by node 1, the cell extract is exhausted and high repressor concentrations have accumulated. This problem can be circumvented by operating the system in a non-equilibrium steady state with a continuous supply of cell extract and controlled removal of products, which we achieved using a microfluidic ring reactor [64]. Indeed, the double repression cascade is shown to work in the microfluidic reactor, while it fails in a bulk reaction.

Based on this insight, we exposed our IFFL-2 to a 'virtual' morphogen gradient by supplying six microfluidic ring reactors with different concentrations of σ^{28} , corresponding to different positions within an exponential morphogen gradient. As desired, the leftmost reactors (R1, R2 - high σ^{28}) highly express from node 1, reactors exposed to intermediate morphogen concentrations respond with a high expression from node 2, while the rightmost reactors (R5, R6 - low σ^{28}) show high expression from node 3.

Our results are in line with other previous studies on dynamic cell-free gene circuits such as genetic oscillators [7], [101], [105], [106] or a CFFL [104], which required microfluidic systems to run over long enough periods of time. Our work on the IFFL-2 shows that also pattern-forming gene circuits can be operated in a cell-free context using microfluidics, and would generate the desired spatial behavior when exposed to a morphogen gradient.

Potential applications of pattern-forming circuits are found in the realization of biomaterials that can differentiate autonomously in response to externally supplied chemical cues. Obviously, for such applications microfluidic ring reactors would be impractical to use, and out of equilibrium conditions would have to be maintained using different methods. One of the major challenges for the future development of this line of research will thus be the realization of materials with simple and efficient supply lines (or 'vasculature'), and the implementation of cell-free metabolic processes and self-regeneration that enable operation of the systems over long enough periods of time.

2.4 Materials and Methods

2.4.1 Circuit Design

The circuit containing three nodes is implemented through the use of six different plasmids, which were cloned using the modular cloning (MoClo) strategy [120]. Node 1 comprises three plasmids that either express the LacI repressor, the TetR repressor, or dmTurquoise2 (modified version of mTurquoise2). The N-terminal sequence of mTurquoise2 was truncated and modified by silent mutations to remove ribosome binding site-like sequences. Same procedure used as in [30], [84]) under the control of a P_{28} promoter, node 3 contains two plasmids that express either the TetR repressor or mVenus controlled by a P_{LacO-1} promoter, while the node-2 plasmid expresses mScarlet-I under a $P_{LtetO-1}$ promoter (cf. Fig. 1). The good orthogonality of the P_{LacO-1} and $P_{LtetO-1}$ promoters was shown previously by Lutz et. al [112]. Details of the cloning procedure, gene and oligonucleotide sequences can be found in the supplementary information of reference [83] (Tables S1 - S3).

2.4.2 Cloning of Plasmids

For engineering of the plasmid DNA golden gate assembly (GGA) was used. A library of level 0 basic modules according to the MoClo strategy was created with promoters, RBS', reporters/repressors and terminators as displayed in Figure 2.5. The special ability of type IIS restriction enzymes (here Esp3I) was used to cleave outside of their recognition side and same overhangs were used for each module category for flexible combination of the different parts. The plasmid sequences can be found in the appendix in Section A1.

2.4.3 Cell Extract Preparation

Cell-free gene expression experiments were performed in crude E.coli S30 cell extract, which was prepared following the protocol of Sun and Noireaux [121] from a BL21-Rosetta 2 (DE3) culture. To use the P_{LacO-1} promoter we used a cell extract that lacks the lac repressor which was engineered

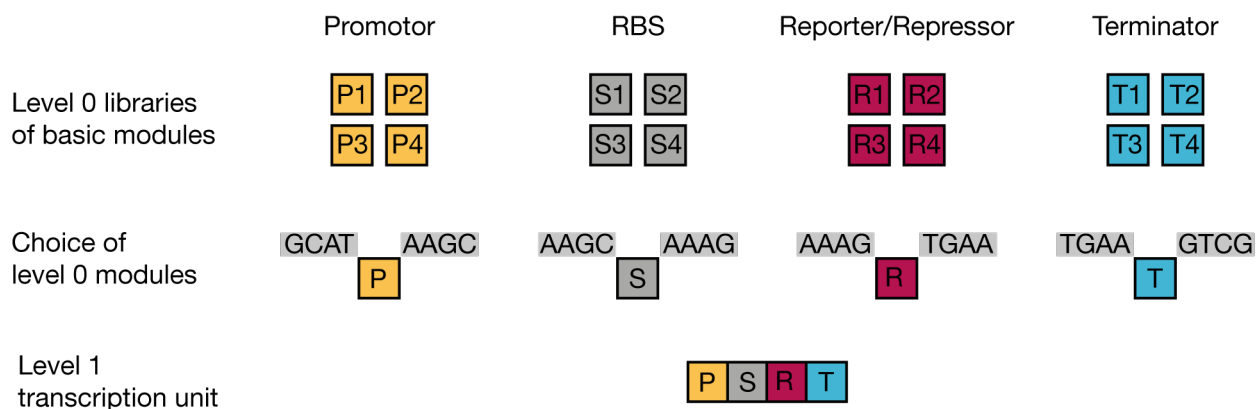


Figure 2.5: MoClo strategy used for engineering of plasmid DNA. A level 0 library was created which was then used for GGA to engineer the nodes of the genetic circuit.

by Lukas Aufinger. The details are described in the materials and methods section of the publication [83]. In short, the genome of BL21 (NEB) was engineered via pORTMAGE [118], which is based on multiplex automated genome engineering (MAGE) [122]. The target gene *lacI* was mutated to contain three stop codons within the first 100 bases of the respective coding sequence. BL21 was transformed with the pORTMAGE-2 plasmid, followed by 22 MAGE cycles following the original protocol [122], [123] with masc-polymerase chain reaction (PCR) screenings after cycles 3, 10 and 22. masc-PCR was performed according to [124] using KAPA2G fast multiplex mix (Merck, #KK5801) with optimized annealing temperatures and elongation times. The final strain (BL21-Rosetta 2(DE3) *lacI*₋ *araC*₋ *endA*₋ *lacZ*₋) was cured of the plasmid-based oligo recombineering tool for MAGE (pORTMAGE)-2 plasmid by growing overnight at 37°C, followed by plating and transformation with the pRARE2 plasmid, obtained from the Rosetta2 strain. LacI-deficient cell extract was produced from this strain and stored at -80°C until further use. In principle, it should be possible to conduct the experiments also with commercially available cell extract such as myTXTL® and using other repressor choices to circumvent the problem of residual LacI. In a previous study, we found that gene expression in our self-made extract differs from the commercial extract depending on experimental details, but shows similar and even higher expression levels [117]. Using commercial extract would therefore likely require a different choice of experimental parameters.

2.4.4 Bulk Experiments

For cell-free gene expression experiments, cell extract and transcription and translation (TXTL) buffer (cf. Table 2.1) were stored on ice after thawing. Reaction tubes were prepared with proteins and/or plasmid DNA for the different experiments. The total reaction volume was usually 15 µL, which was composed of 33 % cell extract and 42 % buffer containing plasmid DNA and/or proteins. The

volume was filled up with nuclease-free dH₂O. The plasmid concentrations used for the experiments are given in the captions of the corresponding figures. Reporter plasmids were used at higher concentrations than repressor plasmids, as in a previous study by Garamella et al. [30]. To obtain optimal concentrations of σ^{28} and the plasmids expressing repressors under the control of σ^{28} a dilution series of the corresponding plasmids and σ^{28} was conducted (Supplementary Information of reference [83] Figure S2-S3). The TXTL reaction mixtures were distributed on a 384-well plate (Brand, #781687) either manually or using a liquid handler (Dispendix I.DOT) with an I.DOT pure plate90 and analyzed at 29 °C. Fluorescence measurements were conducted using a platereader (CLARIO-star® from BMG labtech) for 16 h every 2 min, using the corresponding filter settings for mScarlet-l, mVenus, and dmTurquoise2 (cf. Supplementary Information of reference [83] - Experimental methods).

Table 2.1: Buffer for cell-free reactions, prepared according to reference [121]. All amino acids have a final concentration of 6 mM in the stock solution, except of leucine which has a final concentration of 5 mM. Amino acids are thawed at room temperature and kept on ice until diluted in sterile water, except Asn, Phe, and Cys which are kept at room temperature. The energy solution was prepared according to Table 2.2

Component	Concentration
Mg-glutamate (mM)	4 (mM)
K-glutamate (mM)	60 (mM)
Amino acids (mM)	1.5 (mM)
Energy solution (x)	1 (x)
DTT (mM)	0 (mM)
PEG-8000 (%)	2.5 (mM)
Water	fill up volume

2.4.5 Microfluidic Experiments

For microfluidic experiments, we used a modified version of the ring reactors designed by Niederholtmeyer et. al [64] with a smaller feed increment to operate cell-free expression reactions under (approximate) steady-state conditions. Details can be found in the dissertation of Lukas Aufinger [125]. Each device contained eight ring reactors which can be filled individually, and a peristaltic microfluidic pump was used to mix the contents in each of the reactors (cf. Supplementary Information of reference [83] Figure S10 for details). Approximately three minutes are required to generate a homogeneous mixture inside the reactors (cf. Supplementary Information of reference [83] Figure S10). Our refresh cycle (see below) includes mixing for five minutes. The microfluidic reactors were produced by bonding a PDMS device onto a glass slide. For the production of the PDMS device, a silicon wafer was used as described in Ref. [102] as a master mold for the structures (details can

Table 2.2: Energy solution for cell-free reactions, prepared according to reference [121].

Component	Concentration
HEPES (pH 8)	700 mM
ATP	21 mM
GTP	21 mM
CTP	12.6 mM
UTP	12.6 mM
tRNA	2.8 mg/ml
CoA	3.64 mM
NAD	4.62 mM
cAMP	10.5 mM
folinic acid	0.95 mM
Spermidine	14 mM
3-PGA	420 mM

be found in the SI). The microfluidic device was operated in a cage incubator to keep the reaction temperature constant at 29 °C. Images were taken with an inverted epifluorescence microscope (IX 81, Olympus). An example image can be found in Figure S9 of the Supplementary Information of reference [83]. The microscope was controlled by custom LabView software and equipped with a EMCCD camera (iXon3, Andor UK, pixel size 13 μm), a motorized stage (Märzhäuser, Wetzlar), and a lumencor SOLA 360 light source. Time-lapse microscopy measurements were conducted with a 10x objective (Olympus 10x UPlan FL N). Using an Arduino Mega and a custom built valve controller based on 24 solenoid valves (Festo, #MH1), the control lines of the microfluidic device were operated with 1.5 – 2.5 bar. Various functions were implemented with a custom LabView program, which allowed to control feeding and mixing of the reactors and also the data acquisition cycles during time lapse experiments. Two of the eight reactors (R7, R8) were used in each experiment to measure the output of the circuit to the lowest and highest amount of σ^{28} twice to make sure that the chip is functioning correctly. So only six reactors (R1-R6) were left to measure the response of the circuit to the different amounts of σ^{28} .

2.4.6 Photolithography

The photolithography steps were performed in a cleanroom. The design of the ring reactors is shown in Figure 2.6 a). The control layer with 24 inlets and the flow layer with 9 inlets and 1 outlet are displayed in two different colors. Three devices were placed on one 3-inch silicon wafer (Siegert Wafer). The control layer shown in 2.6 b) was prepared only with one layer of SU-8 3050 negative thick-film photoresist (micro resist technology), and the flow layer which is shown in 2.6 c) is prepared with two layers of different photoresists: AZ 40XT (MicroChemicals) which is a positive

photoresist (the designs were inverted by surrounding the structures with an appropriate box and using XOR logic) used for the red structures and again SU-8 3050 used for the gray structures. The photoresists were diluted according to the manufactures specifications to achieve a final layer height of 50 μm .

For production of the control layer the the wavers were first cleaned with isopropanol and heated up to 200 °C for 5 min. Then 1 mL of Ti Prime (MicroChemicals) was spin coated on the waver for 60s with 300 rpm/s to 6000 rpm. After baking it for 2 min at 120 °C, 2 mL of SU8 3050 photoresist were spin coated for 10s with 100 rpm/s to 500rpm and then for 30s with 300 rpm/s to 3000rpm. The waver was then softbaked with a ramp from 65 °C to 95 °C in 15 min. With a maskless aligner (MLA, Heidelberg Instruments) the wavers were exposed with 200 mJ/cm². After exposure it was postbaked for 1 min at 65 °C and for 5 min at 95 °C. It was then developed in PGMEA (Merck), rinsed with isopropanol and blow dried with nitrogen. The construct was finalized with a hardbake for 1 h at 120 °C. The resist thickness was measured with a Dektak 150 surface profiler (Veeco Instruments).

For the production of the double layered flow layer no Ti prime was applied. First, the wavers were prepared the same way as for a single layered wafer, then the second layer was produced on top of it and 2 mL of AZ 40XT resist were spin-coated for 15 s with 100 rps/s to 500 rpm, followed by 45 s with 300 rpm/s to 4000 rpm. The waver was softbaked ramping from 70 °C holding it for 20 s up to 120 °C and holding it for 3.5 min back to 70 °C for 20 s. The resist was exposed with 200 mJ/cm² using the MLA. After exposure the device was postbaked at 70 °C for 20 s up to 105 °C for 40 s and again at 70 °C for 20 s. It was developed in 2.38% aqueous tetramethylammonium hydroxide (TMAH, Merck) for 2 min and rinsed with dH₂O, as AZ 40XT is sensitive to isopropanol. The waver were hardbaked at 120 °C for 30 min before the second layer was prepared with SU8 the same way as for the production of the control layer.

2.4.7 PDMS Lithography

The flow and control layers were prepared by mixing polydimethylsiloxane (PDMS) with the curing agent in a 5:1 and 20:1 ratio respectively. For 3 inch wafers 27 g in total were prepared for the flow layer. For the control layer less was needed as only 2.25 g were applied to the surface, and the layer was spun for 45 s at 1500-1800 rpm to achieve a thickness of approximately 50 μm . Both mixtures were placed in a desiccator for 20 minutes to remove air bubbles, and subsequently left on the bench for 1 h for the surface to self-level and achieve uniform thickness before further processing. The flow layer was baked at 80 °C for 18 minutes, while the control layer was baked at 80 °C for 23 minutes. After baking, the layers were aligned using a microscope, and both layers were baked again at 80 °C for 1 hour. Once cooled, the layers were cut to the desired shape, and holes were punched using a

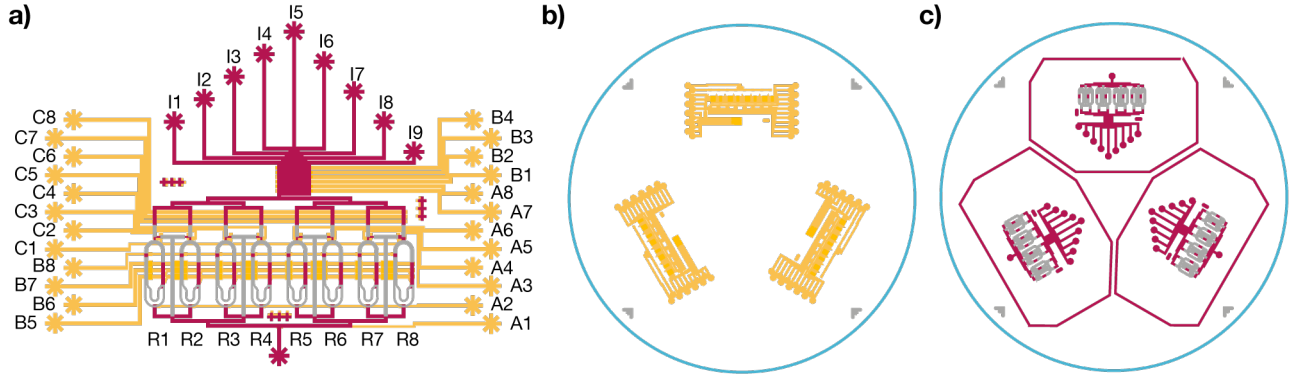


Figure 2.6: Design of the ring reactors. a) One device contains 8 ring reactors that can be filled from 9 different inlets and have only one outlet. The control layer can be connected with a diy quake valve controller [125] via 24 inlets. For production the b) control layer and c) flow layer are fabricated on two different 3 inch wafers with 3 devices on each.

biopsy puncher with a diameter of 0.5 mm. Glass substrates were cleaned and bonded to the device using O_2 plasma.

2.4.8 Calibration of the Reactors and Refresh Cycles

To determine the ‘refresh ratio’ R (the fraction of the reactor volume that is replaced at each feeding step, which involves opening of the reactor inlet and outlet) the reactors were individually calibrated. The reactors were initially filled with a solution containing $25 \mu\text{M}$ fluorescein and a dilution series was generated with $1\times$ PBS, which was provided via the bypass channels. A full intensity I_0 image was taken in the beginning, followed by a dilution of the reactor content for n times (we used $n = 11$) every 15 min. Each dilution step is composed of a feeding step and a mixing step. Feeding was conducted by opening the inlet and outlet of the respective reactor 45 times at 3 Hz and mixing was performed by opening the control valves of the peristaltic pump 500 times, also at 3 Hz. After each dilution step the intensity I_n was measured. We then determined the refresh ratio per feed R by fitting the relation

$$I_n = I_0(1 - R)^n. \quad (2.1)$$

to the data.

2.4.9 Cell-Free Gene Expression in the Ring Reactors

For cell-free expression experiments, the plasmids coding for the gene circuit were mixed with buffer and connected to the flow line of the reactors. Two reservoirs (1.5 ml Eppendorf tubes) containing cell extract were also connected with the flow lines - one with and one without σ^{28} . A custom-built cooling unit was used to keep the extract reservoirs constant at 4°C and the flow line pressures were

regulated to 300 mbar using a pressure controller (OB1, Elveflow). To fill the reactors with a mixture containing 50% buffer and plasmids and 50% cell extract with the respective concentration of σ^{28} , they were initially filled with 100% of the buffer-plasmid mix. Afterwards 50% of the total reactor volume was exchanged in each reactor by flushing in cell extract with different ratios of the two cell extract samples, one with σ^{28} and the other without. The ratio of the two cell extract samples was chosen such that the concentration of σ^{28} varied from 1000 nM to 0 nM when going from reactor 1 to reactor 8 (Table 2.3). Every $t_R = 15$ min, 20% of the reactor volume (10% buffer and 10% cell extract portioned with and without σ^{28}) were exchanged with new medium, corresponding to a dilution rate of $d = -\ln(1 - R)/t_R = 0.00025 \text{ s}^{-1}$. The periodic variation in the fluorescence signals caused by the refresh cycle is not visible in the time traces, as the data are always taken at a specific time point during the cycle. [64].

Table 2.3: Concentrations of σ^{28} in the ring reactors.

reactor 1	1000 nM	reactor 3	200 nM	reactor 5	50 nM	reactor 7	1000 nM
reactor 2	500 nM	reactor 4	100 nM	reactor 6	0 nM	reactor 8	0 nM

2.4.10 Data Analysis

Bulk fluorescence data acquired with the plate reader were used without further normalization, as we were only interested in relative intensities or signal changes. Calibration curves that convert fluorescence intensity into fluorescent protein concentrations are given in Figure S1 of the Supplementary Information of reference [83].

By contrast, fluorescence values recorded from the ring reactors first had to be normalized to allow comparison among different ring reactors, as their sizes and the corresponding excitation volumes varied. This was accomplished by using the fluorescence intensity I_0 of a 25 μM solution of the reference dye fluorescein that was recorded during the calibration of each reactor as described above. Custom Matlab scripts were used to extract information on the reporter gene expression levels from the microscopy images. To correct the images for background and normalization of the values, a region of interest (ROI) and a background ROI were manually selected for each ring (cf. Fig. S9 of the Supplementary Information of reference [83]). After subtraction of the background B , the measured intensity I was normalized to the background corrected intensity $I_0 - B_0$ from the calibration experiment, i.e.,

$$I_B = \frac{I - B}{I_0 - B_0} \quad (2.2)$$

This first normalization step results in fluorescence levels given in units of fluorescence of the reference dye, and was used for the data shown in Fig. 3b.

In order to allow comparison between different reactors and reporter proteins in Fig. 4, an additional normalization step was required, as in this case we were also interested in whether the execution of the IFFL-2 resulted in different expression levels in the reactors. Each fluorescent protein yields a different maximum fluorescence value (in ‘units of fluorescein’) and also varies over time. The intensities I_B for the different reporter proteins were therefore normalized to the range $[0; 1]$ by first determining the absolute minimum and maximum values $I_{B,\min}$ and $I_{B,\max}$ recorded for the expression of the protein in any of the reactors over the whole acquisition period. A second normalization step is then performed to yield the relative expression level ξ :

$$\xi = \frac{I_B - I_{B,\min}}{I_{B,\max} - I_{B,\min}} \quad (2.3)$$

Thus, ξ gives the expression of a fluorescent protein as the fraction of its maximum observed expression level among all reactors. This allows to determine, e.g., whether mScarlet-I is expressed comparatively higher in the middle channels, and allows comparison with our numerical simulations. For data presentation in the figures, the mean of the measured biological triplicates and the standard error of the mean were calculated.

2.4.11 Simulation

Gene circuits with an IFFL topology are capable of generating a stripe pattern in response to a morphogen gradient. In order to gain a better understanding of the requirements for stripe formation, we numerically studied the dynamics of our implementation of the IFFL-2 (Fig. 1) in an exponential gradient of σ^{28} factor, acting as our morphogen. To reduce the number of equations and simplify the simulation, we did not calculate the production rates for each expressed protein but used the total protein output of each node (each controlled by a different promoter). We therefore simulated the following set of three ordinary differential equations using the Matlab *pdepe* solver [126] (which is equivalent to the Matlab *ode15s* solver [127] in the absence of diffusion), which model the expression dynamics of the three nodes of the circuit:

$$\begin{aligned} \dot{c}_1 &= l_1 + b_1 \frac{1}{1 + \left(\frac{K_1}{c_4}\right)^{n_1}} - d_1 c_1 \\ \dot{c}_2 &= l_2 + b_2 \frac{1}{1 + \left(\frac{(1-f)c_1 + c_3}{K_2}\right)^{n_2}} - d_2 c_2 \\ \dot{c}_3 &= l_3 + b_3 \frac{1}{1 + \left(\frac{fc_1}{K_3}\right)^{n_3}} - d_3 c_3 \end{aligned} \quad (2.4)$$

Here, the l_i parameters are the baseline expression rates of the promoters. Activation/inhibition are modeled by the Hill terms, where K_i and n_i are the induction thresholds and Hill exponents, and b_i are the corresponding prefactors with units of a reaction rate. d_i is the degradation/dilution rate. A table of parameters and equations used for the simulation can be found in Table S6 of the Supplementary Information of reference [83].

Parameter c_1 represents the concentrations of the repressor proteins expressed under the control of σ^{28} , c_3 summarizes the repressor proteins expressed under the control of the $P_{\text{LacO-1}}$ promoter and c_2 represents the mScarlet-I concentration. c_4 corresponds to the concentration of the morphogen σ^{28} , which acts as an activator. The factor f represents the $[\text{LacI}]$ fraction of the repressors expressed by node 1, i.e., $[\text{LacI}]_{P_{28}} = f c_1$, and thus $[\text{TetR}]_{P_{28}} = (1 - f) c_1$. This can be summarized as follows:

$$\begin{aligned} c_1 &= [\text{LacI}]_{P_{28}} + [\text{TetR}]_{P_{28}} \\ c_2 &= [\text{mScarlet-I}] \\ c_3 &= [\text{TetR}]_{P_{\text{Lac}}} \\ f c_1 &= [\text{LacI}]_{P_{28}} = [\text{LacI}]_{\text{tot}} \\ (1 - f) c_1 + c_3 &= [\text{TetR}]_{\text{tot}} \end{aligned} \tag{2.5}$$

In order to simulate a spatial response, we assumed that a gradient of the morphogen had already been established by some process. In one dimension, diffusion of the morphogen from a constant source (concentration $c_4(x = 0) = \text{const.}$) with diffusion coefficient D and constant, spatially uniform degradation with rate α results in an exponential concentration gradient $c_4(x) = c_4(0) \cdot \exp(-\lambda|x|)$, where x is the distance from the source and the decay length is given by $\lambda^{-1} = \sqrt{\frac{D}{\alpha}}$. In order to connect the simulation to our microfluidic experiments, we assumed a morphogen concentration $c_4(0) = 100 \text{ nM}$ at the origin. The decay length λ^{-1} corresponds to the typical distance that a morphogen travels into the field before being degraded. In the model, $\lambda = 1/(0.3 \text{ mm})$, the leakage terms were set to $l_i = 0.01 \text{ nM s}^{-1}$ and the decay rates of the reactants were set to $d_i = \alpha = 0.0005 \text{ s}^{-1}$. This corresponds to an assumed diffusion coefficient of $\approx 45 \mu\text{m}^2/\text{s}$.

2.4.12 Evolutionary Algorithm

We used an EA to search for ODE parameters that would result in the formation of a three-stripe pattern. EAs do not necessarily provide optimal, but extremely stable solutions [128]. The ODE system (Eq. 2.4) has 16 parameters in total. After setting the l_i and d_i parameters to fixed values (see previous paragraph), the number of parameters was reduced to 10. The value limits for the parameters that were used can be found in Table S7 of the Supplementary Information of reference [83]. Matlab's function *ga* [129] was used with a population size of 100, 80 % crossover

fraction, a fraction of 5 % elites, and a 15 % mutation rate for 100 generations. To optimize the middle stripe (which is the most difficult feature to generate) the fitness function

$$f = - \frac{0.5 \cdot \Delta_{\text{left}} + 0.5 \cdot \Delta_{\text{right}}}{y_{\text{max}}} \quad (2.6)$$

and an additional constraint

$$\frac{1}{3} \cdot (x_{\text{max}} - x_{\text{min}}) < x_{y_{\text{max}}} < \frac{2}{3} \cdot (x_{\text{max}} - x_{\text{min}}) \quad (2.7)$$

was chosen. The parameter y_{max} is the y-value of the maximum and x_{min} (x_{max}) stand for the minimal (maximal) x-value of the middle stripe, i.e., the position of its left (right) border. The parameter Δ_{left} is defined as the difference between the y-value of the maximum and the y-value at the left border, parameter Δ_{right} is defined accordingly. The fitness function is defined to maximize the middle stripe (cf. Supplementary Information of reference [83] Figure S11). The additional constraint is chosen to ensure that the peak lies in the middle third of the whole width of the stripe.

Reassuringly, the predicted parameters (Table 2.4) do not vary widely among each other and have experimentally reasonable values for expression rates, induction thresholds and Hill coefficients. A simple sensitivity analysis to define the parameter range was also conducted by varying only one parameter at a time, for this cf. Supplementary Information of reference [83] Figure S12. An important input for the experiments is provided by the parameter f , which was used to adjust the ratio of the plasmids coding for TetR and LacI in node 1.

Table 2.4: Parameters of the differential equation system 2.4 found by the EA after 100 iterations. The parameter b represents the expression strength, K and n are the Hill parameters, and f describes the ratio of the two plasmids expressing the repressors under the P_{28} promoter.

$$\begin{array}{lll} b_1 = 1.87 \text{ nMs}^{-1} & K_1 = 62.82 \text{ nM} & n_1 = 1.29 \\ b_2 = 1.64 \text{ nMs}^{-1} & K_2 = 44.5 \text{ nM} & n_2 = 3.98 \quad f = 0.85 \\ b_3 = 1.94 \text{ nMs}^{-1} & K_3 = 56.06 \text{ nM} & n_3 = 4.00 \end{array}$$

3. Fluorescence-Based Monitoring of Living Organisms in Synthetic Biofilms

This chapter is based on the content of the publication:

Immobilization and monitoring of *Clostridium carboxidivorans* and *Clostridium kluyveri* in synthetic biofilms

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3.1 Introduction

Previous studies have demonstrated the successful embedding of synthetic systems into hydrogels, including DNA-based constructs and living bacteria. For example, in our previous work by Müller et al. [95] we embedded DNA into hydrogels for programmable pattern formation, while later work focused on embedding genetically modified bacteria capable of expressing fluorescent proteins for visualization [131]. These approaches relied on either fluorescently labeled DNA or engineered fluorescence in bacteria to enable monitoring within the hydrogel matrix. Expanding visualization strategies to include non-genetically modified bacteria would significantly broaden the range of microbial and eukaryotic systems that can be incorporated into hydrogel-based bioengineering platforms. In this

context, we focus on Clostridia as a representative microbial system for demonstrating the underlying principle. Rather than optimizing for a specific organism, our aim is to investigate a broadly applicable strategy.

A growing area of interest in biotechnology involves the biological conversion of synthesis gas (syngas), derived from organic waste or industrial processes, as a feedstock for producing valuable chemicals [132], [133]. Acetogenic bacteria like *Clostridium carboxidivorans* can convert syngas components (CO, CO₂, H₂) into short-chain compounds such as acetate and ethanol [134], which can then be elongated by other anaerobic microbes like *Clostridium kluyveri* into more valuable medium-chain products like 1-butyrate and 1-hexanoate [135]. Co-cultures of these bacteria can expand the range of products but face challenges due to differing optimal growth conditions. Immobilization strategies – such as biofilm formation or synthetic hydrogels – offer promising solutions to spatially separate and stabilize microbial populations, improving productivity [136]. Various studies demonstrate the benefits of immobilization for enhancing biofuel and biochemical production, indicating a strong potential for further development in microbial syngas fermentation systems [137], [138], [139].

To optimize biological processes, it is essential to have extensive knowledge of as many process variables as possible. The conventional method for determining cell dry weight concentrations over time, based on photometric measurement of optical density, is only applicable to suspended cells. For immobilized cells, the most common non-invasive methods are bacterial cell morphometry, Raman microscopy, confocal laser scanning microscopy, optical coherence tomography, and combined methods [140], [141]. However, in co-cultures, it is necessary to differentiate between the individual cells, which are visually indistinguishable. In suspension, this issue was addressed by Bäumler et al. [142] and Schneider et al. [143] for *Clostridium carboxidivorans* and *Clostridium kluyveri* in co-culture using FISH and analysis with flow cytometry.

To date, there has been no research involving the combination of *C. carboxidivorans* and *C. kluyveri* in a synthetic biofilm. This study aims to determine whether these anaerobic microorganisms remain metabolically active and capable of growth when immobilized in space in an agar hydrogel matrix. Building on the established FISH techniques for suspended *C. carboxidivorans* and *C. kluyveri* [143], our research will adapt FISH to identify immobilized cells within a synthetic biofilm. This adaptation is essential for precise quantification of varying species, enabling a more detailed understanding of the co-culture's metabolic interactions and biotechnological potential. Building on this, this study aimed to develop a method for quantitative determination of individual cell dry weight (CDW) concentrations in synthetic biofilms over the course of a batch process, using FISH labeling and subsequent analysis through fluorescence microscopy.

To evaluate this approach, we designed a dual-layer hydrogel system and studied batch conversion of ethanol and acetate under anaerobic conditions. *C. kluyveri* was immobilized in the first layer of

the hydrogel (average thickness of 4 mm), and *C. carboxidivorans* in the second layer which had roughly the same thickness. The heterotrophic substrates ethanol and acetate were supplied with the anaerobic fermentation medium above the dual-layered synthetic biofilm. The gas phase in the anaerobic flask had a N₂:CO₂ ratio of 1:9 with a total pressure of 1.5 bar. Ethanol, acetate, and CO₂ can be consumed by *C. kluyveri* for growth and chain elongation. No growth substrate is available for the immobilized *C. carboxidivorans* cells. Within a batch process time of 7 days, we monitored the pH and analyzed the organic acids and alcohols produced. No suspended cells were detected, as confirmed by the absence of measurable optical density (OD) in the supernatant. This ensures that the metabolic activity observed is solely due to the immobilized cells. To visualize the changes in cell density in the synthetic biofilms of both bacteria, we employed FISH after taking biofilm samples.

3.2 Results

3.2.1 Fluorescence In Situ Hybridization (FISH) for Characterizing Immobilized Bacteria

To be able to track cell growth and distribution of both bacteria, *C. carboxidivorans* and *C. kluyveri*, in our batch processes, we used the FISH labeling method. Characterization and monitoring of the immobilized cells within the synthetic biofilm was achieved with fluorescently labeled probes to bind specific bacterial ribosomal RNA sequences enabling species-specific visualization of the cells. In our study, we employed three probes: 'EUB338', labeled with Alexa488, which targets a broad range of bacteria to confirm overall cell presence; 'ClosCarb_1516', labeled with Cy3, designed specifically to identify *Clostridium carboxidivorans*; and 'ClosKluy_1516', labeled with Cy5, to detect *C. kluyveri*. This combination of orthogonal fluorescence markers allowed us to distinguish between the bacterial species within the biofilm and assess their spatial distribution accurately. The probe EUB338 demonstrated the highest staining efficiency, with approximately 52% of cells successfully stained. Comparatively, the probe 'ClosCarb_1516' exhibited a staining efficiency of approximately 38%, which was slightly higher than that of ClosKluy, measured at 23%. Staining efficiency was determined by calculating the ratio of fluorescently stained bacteria to the total number of bacteria observed under bright field microscopy, in separate gel samples for the two bacterial species (Supplementary Information of reference [130] - Figures S7 and S8). All probes exhibited high specificity for their respective target bacterial species, with no cross-reactivity observed for non-target bacteria.

3.2.2 Growth Dynamics in Synthetic Biofilms at Low Initial Cell Concentrations

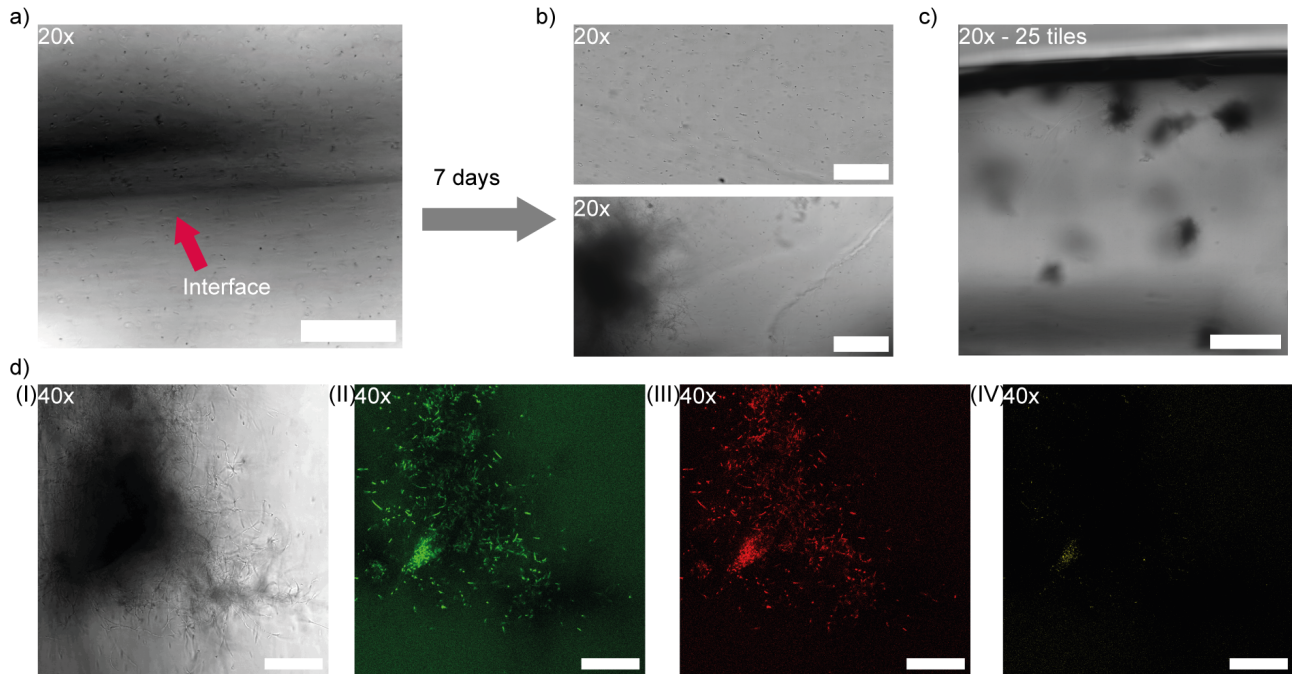


Figure 3.1: Growth of bacteria immobilized in hydrogel to form a synthetic two-layered biofilm. (a) An image of the interface at $t = 0$ between the two hydrogel slices with the two types of bacteria is shown. Bacteria are homogeneously distributed in the gel with a low initial cell concentration; the bacteria cannot be differentiated. Scale bar: $100\ \mu\text{m}$. (b) After 7 days of cultivation, *C. carboxidivorans* (top) did not grow, and the bacteria are still homogeneously distributed in the gel. *C. kluyveri*, (bottom) on the other hand, grew and formed clusters. Scale bar: $100\ \mu\text{m}$. (c) Clusters of *C. kluyveri* can be observed homogeneously distributed inside the hydrogel. Scale bar: $1000\ \mu\text{m}$. (d) Zoom of one bacteria cluster in the different fluorescent channels for (I) brightfield, (II) Alexa488, (III) Cy5, and (IV) Cy3. *C. kluyveri* can be stained with the general FISH probe 'EUB338-Alexa488' (green) and the specific 'ClosKluy_1516-Cy5' probe (red) but not with 'ClosCarb_1516-Cy3'. Scale bar: $50\ \mu\text{m}$.

Bacterial cells were initially uniformly distributed throughout the hydrogel matrix. This is illustrated in Figure 3.1a, which shows the interface between the two layers of the synthetic biofilm. Here, bacteria are dispersed consistently across the gel structure, though it is not possible to visually distinguish between the two bacterial species in this image. The initial homogeneous distribution was intended to represent the starting conditions for subsequent growth and metabolic interaction. After 7 days of cultivation under anaerobic conditions in flasks, notable differences were observed between the two layers. The upper layer, containing *C. carboxidivorans*, retained its original appearance, with bacterial cells remaining evenly distributed and at a similar concentration to the start

of the experiment. The consistent distribution in the upper layer suggests that *C. carboxidivorans* showed no lysis under the conditions provided, without significant cluster formation or migration. In contrast, the lower layer, which contained *C. kluyveri*, displayed a markedly different morphology after the 7-day period, as shown in Figure 3.1b. Instead of the original homogeneous distribution, *C. kluyveri* cells form aggregates, visible as distinct clusters that were, nonetheless, evenly distributed throughout the hydrogel layer (Figure 3.1c). The cells that survived the gel casting process began to grow and form clusters, suggesting that *C. kluyveri*'s growth may be influenced by its immobilization and limited mobility within the synthetic biofilm or by localized environmental conditions within the gel. To confirm the identity of the bacterial cells forming these clusters and to ensure that they corresponded to *C. kluyveri*, FISH probes were used as described in Section 3.4.3. Figure 3.1d presents a close-up view of one such bacterial cluster. Here, intensity signals are displayed across different channels, including the brightfield image (I) and fluorescence channels for Alexa488 (II), Cy3 (III), and Cy5 (IV). The intensity signals generated by the FISH probes EUB338 (green) and 'ClosKluy_1516' (red) were clearly detectable, affirming the presence of *C. kluyveri* within the clusters in the hydrogel. This result verifies both the identity of the bacteria forming these clusters and the specificity of the probes used for this experiment.

3.2.3 Growth Dynamics in Synthetic Biofilms at High Initial Cell Concentrations

In the experiment with higher initial cell concentrations of 3.0 g/l in the hydrogel, we applied our FISH labeling method within our synthetic biofilms to characterize growth patterns. The experiment was conducted in eight anaerobic flasks in parallel, with the first flask stopped after 24 h, the second after 48 h, and so on, to collect a sample of the synthetic biofilm for FISH analysis. The corresponding results are displayed in Figure 3.2, allowing for a comparison of bacterial growth dynamics at different starting concentrations. Interestingly, with this higher initial concentration, we observed that no bacterial clusters formed within the hydrogel matrix, indicating a distinct growth behavior compared to the low-concentration experiment. Figure 3.2 provides a detailed analysis of bacterial growth over time in samples with this high initial cell concentration. In Figure 3.2a, cell counts for both bacterial species over the 4-day cultivation period are displayed. *C. carboxidivorans* (yellow) maintains a stable population throughout, whereas *C. kluyveri* (red) exhibits a significant increase in cell numbers after the initial 48 h, suggesting a delayed but accelerated growth phase for this species. This timeframe corresponds to the period during which an increase in metabolic activity was observed. Starting from 72 h, the cell count progressively declines, with no cells detectable in the fluorescent channel after 96 h. This is likely due to cell death and subsequent degradation of rRNA in the bacteria, which prevents the binding of FISH probes to their target rRNA sequences.

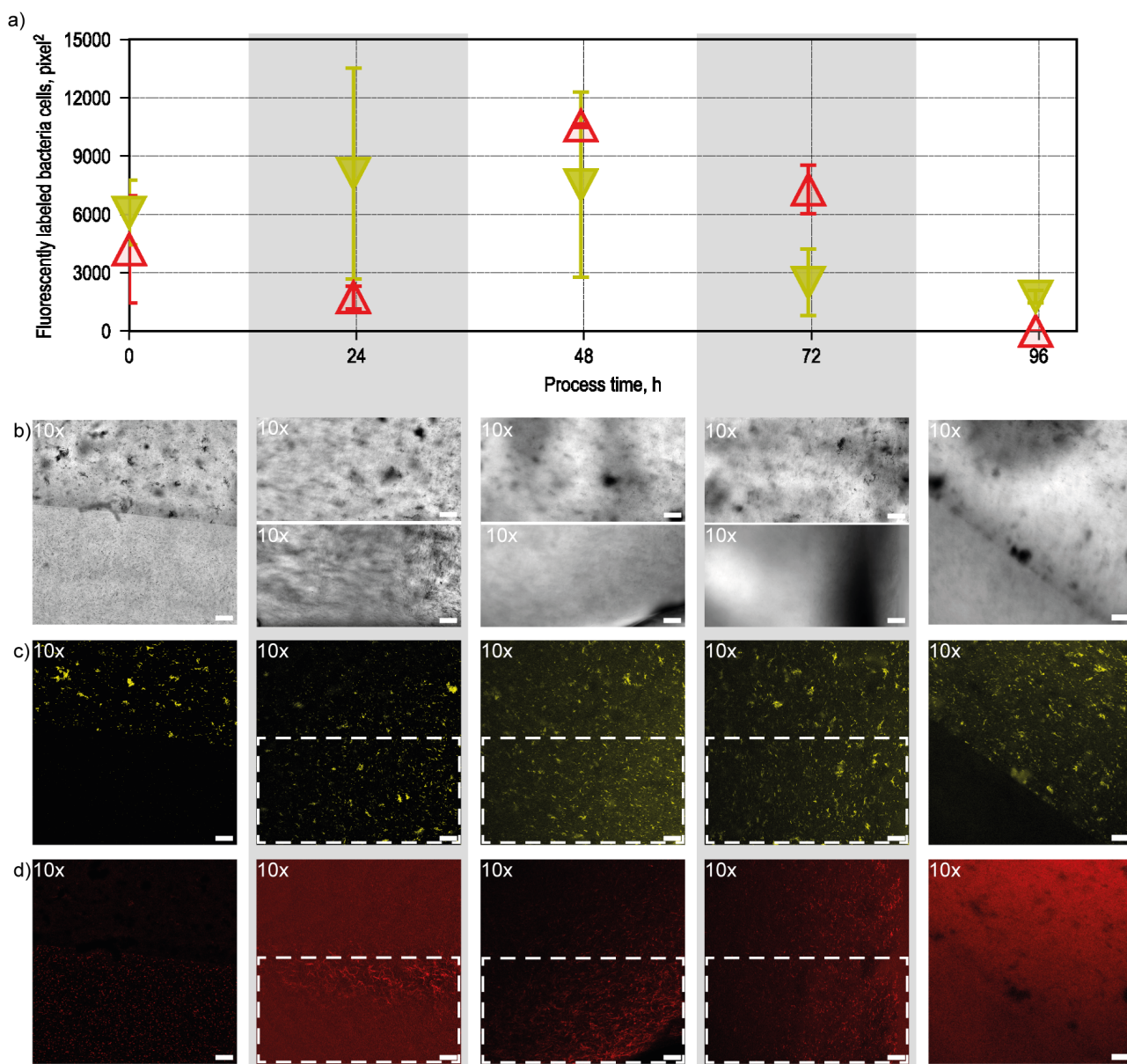


Figure 3.2: Monitoring changes in cell numbers over time for bacteria with high initial cell concentration (3.0 g L⁻¹) in the hydrogel. (a) Fluorescently labeled bacteria cells were measured over time. Areas of 400 by 400 pixels were analyzed, and fluorescent areas were summed up to show the amount of bacteria. For each day, triplicates of gel slices were extracted and measured. *C. carboxidivorans* are depicted in yellow, and *C. kluyveri* are depicted in red. After 5 days, *C. kluyveri* cannot be stained anymore, which is probably due to cell death caused by too low pH levels. (b) Brightfield showing the *C. carboxidivorans* hydrogel above the *C. kluyveri* hydrogel. (c) Cy3 and (d) Cy5 images of the gel slices of each layer for days 1 to 5 (from left to right). The dashed rectangles depicts the area which is shown in row (b). The interface between the two gel phases can be observed on images for days 1 and 5 as gel slices stayed intact. For days 1–3, the gel phases separated after extraction. Scale bar: 100 μ m.

Figure 3.2b presents brightfield images of gel slices extracted daily over a 4-day period (further images of the triplicates can be found in the Supplementary Information of reference [130] Figures S9–S11). The gel slices from days 0 and 4 remained intact, with the interface between the two biofilm layers clearly visible. However, on days 1 through 3, the two layers detached from each other during sample handling, and images could only be captured from individual layers, obscuring the visibility of the interface. Nevertheless, the intact slice observed on day 4 indicates that no substantial changes occurred at the interface. Throughout the hydrogel, bacterial growth appeared uniformly distributed, without localized hotspots or clustering, which further supports the observed difference at higher initial cell concentrations. *C. kluyveri* are homogeneously distributed in the lower layer and *C. carboxidivorans* in the upper layer. The leftmost image shows the situation directly after seeding at day 0. In comparison to Figure 3.1a, it can be seen that *C. carboxidivorans* forms aggregates at these high seeding densities. In liquid cultures, it was already observed that *C. carboxidivorans* formed aggregates at high cell densities. Figure 3.2c,d show fluorescence images capturing the distribution and viability of the bacteria within the hydrogel over time, using Cy3 and Cy5, respectively, for the same image section. As for day 1 to 3, no intact interface could be measured. Figure 3.2b shows only the lower part of the image sections presented in Figure 3.2c,d. To quantify cell growth within each layer, 400×400 -pixel regions of interest were selected from each layer to analyze and measure the cells within these areas. The results of these measurements are presented in Figure 3.2a. Additionally, no growth of one of the microorganisms into the other layer of the synthetic biofilm could be observed. Figure 3.2c,d for day 0 and day 4 show that no fluorescence signal could be observed for *C. kluyveri* in the upper part of the hydrogel, and vice versa, no signal for *C. carboxidivorans* could be observed in the lower part of the hydrogel. These fluorescence images reveal the dynamic distribution of the two bacterial species: *C. kluyveri* exhibits a gradual reduction in signal intensity after 2 days, indicating a decrease in cell viability or population density, while *C. carboxidivorans* maintains consistent fluorescence intensity throughout the first 2 days of the cultivation period, suggesting a stable population and viability over this time. After 2 days, *C. carboxidivorans* also decreases in cell viability. The presumed viability of *C. carboxidivorans* and the assumed decline in cell viability of *C. kluyveri* towards the end of the batch process can be explained by the pH measured throughout the process. It remained within a range where *C. carboxidivorans* is viable [144]. However, for *C. kluyveri*, the pH at the end of the process was too low [145].

3.3 Discussion and Conclusion

FISH and fluorescence microscopy offered valuable tools for monitoring bacterial distribution and viability within the anaerobic biofilms, enhancing our understanding of growth dynamics in biofilms.

The clustering observed in *C. kluyveri* at low initial cell concentrations, as well as the stable distribution at higher concentrations, highlighted the impact of cell density on bacterial behavior. Overall, the results underscore the potential of synthetic biofilms for studying microbial interactions in anaerobic processes and optimizing biofilm-based bioprocesses. The method we developed has broader applications in biofilm research, particularly in examining interactions within synthetic co-culture systems and enhancing microbial process efficiency. Future experiments could focus on optimizing conditions to promote the metabolic activity of *C. carboxidivorans* within the biofilm, e.g., adding CO to the gas phase for autotrophic growth and product formation (acetate and ethanol). These adjustments could reveal new insights into the spatial behavior and interactions with *C. kluyveri*, deepening our understanding of the metabolic interplay between these species. Bioprinting of hydrogels with metabolically complementary cells will offer new possibilities for the design of synthetic biofilms with high local resolution [136]. A motivation for the future design of synthetic biofilms is the use of cooperative relationships between selected (and metabolically designed) microorganisms such as mutualism or commensalism, for instance, by cross-feeding, without the necessity of ensuring growth conditions for the cells in the synthetic biofilm [146]. Synthetic biofilms are promising to improve microbial process efficiency, particularly in biochemical production. Future work will aim to refine the biofilm system, explore spatial dynamics of bacterial migration and cooperative relationships, and extend this approach to other microbial consortia.

Beyond microbial systems, FISH can also be applied for labeling and analyzing eukaryotic cells, making it a valuable tool in the development of complex microphysiological systems. By enabling the visualization of spatial cell arrangements and gene expression patterns in engineered tissues, FISH could support the design and functional assessment of multicellular constructs in synthetic tissue environments.

3.4 Materials and Methods

3.4.1 Preparation of Synthetic Biofilms

The two bacteria strains *Clostridium kluyveri* DSM 555 and *Clostridium carboxidivorans* DSM 15243 were cultivated at 37 °C using Hurst medium in anaerobic flasks in the lab of Prof. Weuster-Botz by Josha Herzog. The cells were harvested during exponential phase and resuspended in anaerobic phosphate-buffered saline (PBS) (pH 7.4) for inoculation in the hydrogel. For production of the synthetic biofilm 18 g of agar-agar were dissolved in 1 L of modified Hurst medium (details can be found in the methods section of the publication [130]) and autoclaved at 121 °C for 20 min. After cooling the hydrogel under nitrogen atmosphere to 45 °C the hydrogel was mixed with the bacteria diluted in PBS. The double layered biofilm was produced by applying first 10 mL of the hydrogel

with one type of the bacteria in a 500 mL anaerobic flask and let it solidify. Then another 10 mL of hydrogel with the other type of bacteria were applied on top. The biofilm was then cultivated with 100 mL culture medium on top and under anaerobic conditions for up to 7 d.

3.4.2 Monitoring of Immobilized Bacteria Using FISH

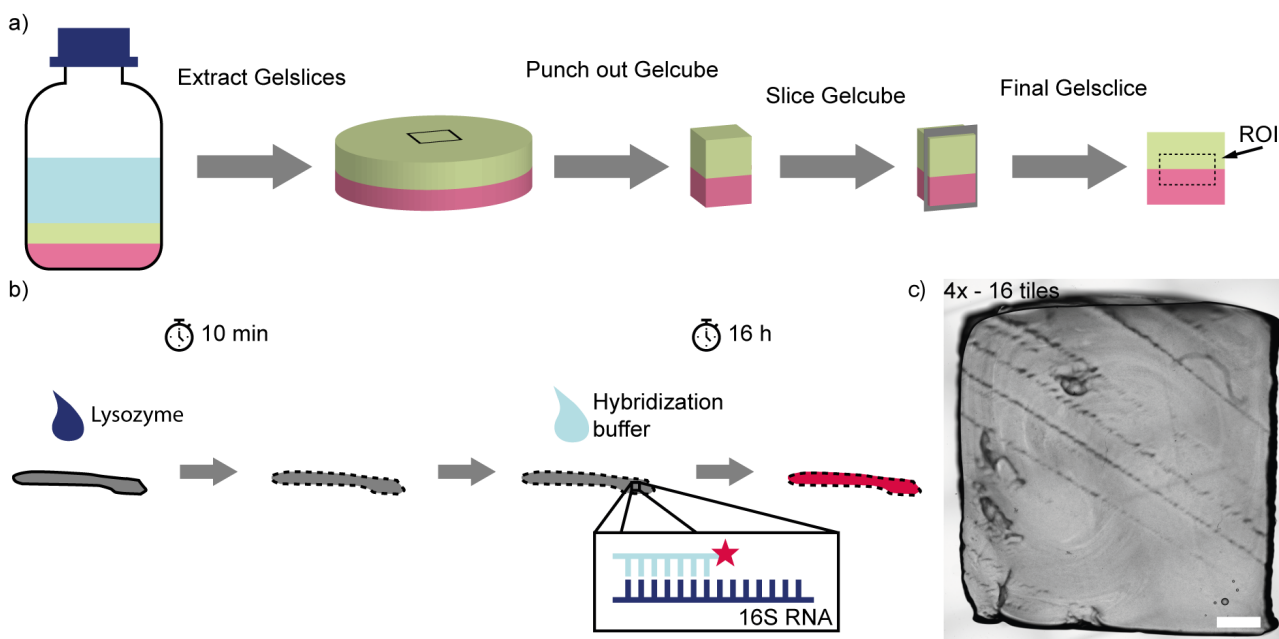


Figure 3.3: Preparation of gel slices to monitor the growth of bacteria. (a) Gel cubes are extracted from the bottle cultures with an edge length of 10 mm with a custom-made polylactide (PLA) punch. These cubes were cut into 1 mm-thick slices with a microtome so that the interface between the two different bacteria cultures could be observed. The figure shows the ROI which is later observed with a microscope. (b) To visualize the different types of bacteria, they are stained with FISH probes. The membrane of the bacteria was penetrated with lysozyme to make it permeable for the FISH probes, which were designed to bind to specific 16S or 23S rRNA of the two types of bacteria. The probes were functionalized with a fluorescent dye (red star) at the 5 prime end to measure the intensities with a confocal microscope. (c) Gel slices with a 10 mm-long edge length were cut; scale bar: 1000 μm .

The procedure for monitoring the growth of immobilized bacteria within the synthetic biofilm, using FISH and confocal microscopy, is outlined in Figure 3.3. Gel slices were carefully prepared from the bottle cultures to enable observation of bacterial growth at the interface between the two species. Initially, gel cubes, each with an edge length of 10 mm, were punched out from the anaerobic bottles (Supplementary Information of reference [130] - Figure S2) using a 3D-printed custom-made PLA punch (Supplementary Information of reference [130] - Figure S3) which could be connected with a motoric pipette filler (Pipetus, Hirschmann, Eberstadt, Germany). These cubes were then precisely

sliced into 1 mm-thick sections (Supplementary Information of reference [130] - Figure S5) using a microtome with a razor blade (Classic, Wilkinson Sword, High Wycombe, UK) (Supplementary Information of reference [130] - Figure S4a), ensuring the bacterial interface was preserved. To differentiate between the two bacterial types, FISH was employed, utilizing probes designed to bind specifically to the 23S rRNA of each species. Prior to probe application, the bacterial membranes were treated with lysozyme to increase permeability, allowing the probes to penetrate the cells. The FISH probes, functionalized with fluorescent dyes at the 5' end, enabled visualization of the bacterial species under a confocal microscope by measuring fluorescence intensity. A universal FISH probe, EUB338 [147], was modified with the fluorescent dye Alexa488 to stain both bacteria species as a positive control. To distinguish the two bacterial species, FISH probes that specifically bind either *C. carboxidivorans* or *C. kluyveri* were used. The probes "ClosCarb_1516" and "ClosKluy_1516" were modified with the fluorescent dyes Cy 3 and Cy5, respectively. As shown in Figure 3.3c, the prepared gel slices retained their 10 mm edge length, with the scale bar indicating 1000 μm for reference. This method, adapted from Schneider et al. (2021) [143], was validated through preliminary tests, which confirmed its efficacy (see Supporting Information Figure S1). Subsequently, this approach was employed to analyze bacterial growth patterns and spatial distribution within the synthetic biofilm, allowing for precise monitoring of the co-cultivated species.

3.4.3 FISH Probes and Buffer

To stain the bacteria, a general FISH probe "EUB338" that targets the 16S rRNA of most bacteria strains was used [143], [147]. To differentiate the two cultures, a specific 23S rRNA oligonucleotide probe for *C. carboxidivorans* and *C. kluyveri* were used. The sequences were developed by Schneider et al. (2021) [143]. The probes were ordered from Integrated DNA Technologies, B.V. (IDT, Leuven, Belgium). They were synthesized with a fluorescent dye at the 5 prime end and purified with high performance liquid chromatography (HPLC). To be able to image all three probes at the same time, the orthogonal fluorescent dyes Alexa488, Cy3, and Cy5 were used to label the probes (Table 3.1).

Table 3.1: FISH probes used in this study.

Name	Target Organism	Target Molecule	Fluorophores	Sequence
EUB338	Most bacteria	16S rRNA	Alexa488	TTGC TGCC TCCC GTAG GAGT
ClosCarb_1516	<i>C. carboxidivorans</i>	23S rRNA	Cy3	AGCC ACTC CCCA TCAC AC
ClosKluy_1516	<i>C. kluyveri</i>	23S rRNA	Cy5	GCGG ACTC CCCT TCAA AG

The hybridization buffer (cf. Table 3.4) was prepared by combining the following components: 180 mL of NaCl (5 M), 20 mL of Tris-HCl (1 M), 500 mL of VE-H₂O, 300 mL of formamide, and 1 mL of SDS (10% w/v). The wash buffer (cf. Table 3.3) was prepared by mixing 20.4 mL of NaCl

(5 M), 20 mL of Tris-HCl (1 M), 948.6 mL of VE-H₂O, 10 mL of EDTA (0.5 M), and 1 mL of SDS (10% w/v).

Table 3.2: Buffers used for FISH analysis, prepared according to reference [143].

Table 3.3: Wash buffer.

Component	Concentration
NaCl	0.1 M
Tris-HCl	20 mM
EDTA	10 mM
SDS	0.01 %

Table 3.4: Hybridization buffer.

Component	Concentration
NaCl (pH 8)	0.9 M
Tris-HCl	20 mM
Formamid	30 %
SDS	0.01 %

3.4.4 Staining Procedure of Bacteria in Hydrogel

The gel slices with bacteria were placed in a 24-well plate and dehydrated sequentially with 80% ethanol followed by 99% ethanol, each for 3 min, removing the solution after each dehydration step. Cell lysis was performed by adding 800 μ L of PBS and 200 μ L of lysozyme solution (10 mg mL⁻¹) to the samples and allowing them to react for 10 min on ice. In previous experiments, the timescale for lysozyme incubation needed for gel samples was screened (Supplementary Information of reference [130] - Figure S6): No visible difference could be observed for 10 min to 5 h of incubation. The liquid was then removed with a pipette, and the gel samples were washed with 1000 μ L of double-distilled water for 3 min by adding the volume with a pipette. After removing the wash solution with a pipette, 500 μ L of hybridization buffer (Supplementary Information of reference [130] - Table S2) containing 50 ngmL⁻¹ of the respective probes was added with a pipette, and the samples were incubated overnight at 46 °C, protected from light. The following day, the samples were washed with 1 mL of prewarmed washing buffer (Supplementary Information of reference [130] - Table S2) by replacing the liquid with a pipette, incubated for 3 min, and then washed again with 1 mL of ice-cooled PBS. Samples were sealed in airtight containers to prevent drying (Section 3.4.5) and imaged directly.

3.4.5 Sample Preparation for Imaging

For sample preparation, liquid cultures were flushed into microscopy slides (15 μ -slide VL 0.4, uncoated, Ibidi, Munich, Germany), while gel cultures were carefully placed on coverslips. To prevent drying, the samples were stored in an airtight environment (Supplementary Information of reference [130] - Figure S4b). A homemade PLA frame, printed using a 3D printer (X1E, Bambulab, Shenzhen, China), was glued around the slices using liquid glue (UHU Sekundenkleber, UHU GmbH

& Co. KG, Bühl, Germany). Finally, the chamber was sealed with a cover slip and made airtight using dental glue (Addition-curing duplicating silicone, Picodent twinsil, Wipperfürth, Germany). This meticulous preparation ensured that the samples remained in optimal condition and were ready for immediate imaging.

3.4.6 Image Acquisition and Analysis

Inverted Fluorescence Microscopy

Images of liquid cultures were taken with an inverted fluorescence microscope (Ti2-2, Nikon, Tokyo, Japan) equipped with a 40 $\times$ objective. To measure the FISH probes, the following filter cubes were used: Excitation 472/30, Emission 520/35, and Dichroic Mirror 495 for Alexa488; Excitation 540/20, Emission 575/29, and Dichroic Mirror H559 LP for Cy3; and Excitation 640/30, Emission-690/50, and Dichroic Mirror T660 LPXR for Cy5.

Confocal Microscopy

Image acquisition for the gel slices was conducted using a lightning confocal microscope (SP8, Leica, Wetzlar, Germany) with the software LAS X version 3.5.7.23225 with a 10 $\times$ /0.45 air objective and HC PI APO 40 $\times$ /1.10 water immersion objective. Laser excitation was performed with diode lasers at wavelengths of 405 nm, 488 nm, 552 nm, and 638 nm. The system operated in photon integration mode, using a PMT detector for optimized signal detection. Emission detection was configured to collect wavelengths from 493 to 548 nm, 557 to 643 nm, and 646 to 700 nm for specific fluorophores (e.g., Cy3 and Cy5). The confocal system used a pinhole setting equivalent to 1.00 Airy units for enhanced resolution. Frame accumulation and averaging were set to 1, with unidirectional scanning at a speed of 100 Hz.

Data Analysis

Images were analyzed using the image processing package Fiji (version 1.54f, ImageJ, Open Source). Initially, the images were duplicated, and a background image was generated by applying a Gaussian blur with a width of 15 pixels. This background image was then subtracted from the raw image to enhance the signal-to-noise ratio. Subsequently, the look-up tables (LUTs) were adjusted to optimize contrast and visualization. A ROI measuring 400 by 400 pixels was selected within the area of interest for detailed analysis. One ROI was selected for each gel slice, and three gel slices per sample have been analyzed. A threshold was applied to the image to distinguish particles from the background. The particle analyzer tool of Fiji was then used on the thresholded image, with the analysis being back directed onto the raw data. This process enabled accurate counting of particles

within the defined ROI. A detailed description of the specific steps can be found in the Supporting Information (Supplementary Information of reference [130] - Figures S12 and S13).

4. Genetic Modification of Cells in Hydrogel-Based Microenvironments

This chapter is based on the content of the publication [38]:

Genetic Manipulation of Mammalian Cells in Microphysiological Hydrogels

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4.1 Introduction

For the realization of synthetic tissues and organ-like systems from living cells, two general approaches can be applied, as discussed in Section 1.2.2. Either external scaffold structures are generated via biofabrication techniques, such as soft lithography and 3D bioprinting, or intricate structures are formed by natural self-organization of cells through processes such as differentiation, growth, and intercellular communication. Ideally, as we state, both approaches would be combined – using geometric and mechanical boundary conditions provided by the scaffold, while simultaneously allowing cellular self-organization processes to unfold. These self-organization processes are often governed by pattern-forming genetic programs, which need to be appropriately regulated to guide tissue formation. A major challenge in the creation of thick, tissue-like constructs is the limited effectiveness of passive diffusion over larger length scales, as we discussed in detail in Section 1.2.3. This limitation impairs nutrient supply, slows the removal of waste products, and hampers genetic control over cellular self-organization, including the exchange of intercellular signaling molecules. In natural tissues, this challenge is overcome by the integration of vascular systems, which span from microvasculature (with channel diameters ranging from approximately 5 to 100 μm) to macrovasculature (with diameters up to several cm) [148].

In the present work, we aim to demonstrate that cells can be cultured within Gelatin methacryloyl (GelMA) hydrogels for extended periods, while enabling in situ genetic manipulation. Both nutrient supply and the delivery of genetic constructs can be facilitated via synthetic channel structures, which ultimately could allow to combine externally guided tissue formation with genetically programmed self-organization, enabled by synthetic biology tools (cf. Figure 1.9c). GelMA is a versatile hydrogel scaffold widely used in tissue engineering due to its biocompatibility, bioactivity, and tunable mechanical properties. Its arginine-glycine-aspartate (RGD) motifs promote cell adhesion and spreading, while its rapid photo-crosslinking allows precise structural control at physiological temperatures [73]. By adjusting concentration and crosslinking density, GelMA can be tailored for applications ranging from 3D tissue constructs to bioreactor systems. Concentrations between 10–15 % have been shown to balance structural integrity with cellular ingrowth and viability [149], [150]. GelMA has a pore size of order $\approx 100 \mu\text{m}$ [151]. The diffusion coefficient D for small molecules with typical sizes of 1 nm in 10 % GelMA has been found to be on the order of $10^{-6} \text{ cm}^2/\text{s}$ [152], which is reduced roughly tenfold with respect to free diffusion. This results in diffusion times $t \sim L^2 D^{-1}$ exceeding $t = 100 \text{ h}$ over distances of $L = 1 \text{ cm}$, rendering passive diffusion impractical for nutrient delivery in thick constructs. Rather than using typical tissue engineering cell lines, we focused here mainly on human embryonic kidney 293T (HEK293T) cells due to their well-established genetic manipulation methods. HEK293T cells are widely used for synthetic gene circuit testing, protein expression, and genome editing [153], [154], making them an ideal starting point before transitioning to primary or stem cell-based systems. Moreover, they can be co-cultured with fibroblasts, endothelial cells, or mesenchymal stem cells (MSCs) to study heterotypic cell-cell interactions, and they function as bioactive factories, secreting growth factors, cytokines, or signaling molecules that influence neighboring cells. We use our model system to demonstrate in situ gene induction, gene delivery as well as genome editing within 3D hydrogel matrices. Gene delivery within 3D cell cultures is an emerging tool in tissue engineering and gene therapy [155]. While transfection in 2D is well established, adapting these strategies to 3D hydrogels poses additional challenges, including reagent penetration, diffusion kinetics, and vector stability. Previous work has tested various transfection reagents for hydrogel cultures [156], [157], showing that mRNA transfection can be more efficient than plasmid DNA delivery in certain 3D models [158]. Beyond transient gene expression, permanent genomic modifications allow long-term functional control of engineered tissues. As a step towards this goal, we demonstrate CRISPR/Cas-based prime editing [60] within GelMA hydrogels, with its necessary components delivered either as plasmid DNA or, alternatively, RNA.

4.2 Results

4.2.1 Growth Dynamics of Cells Seeded in Hydrogel

Cell growth and viability within 3D gel matrices are strongly influenced by structural properties of the matrix such as pore size, porosity, and interconnectivity, which impact nutrient transport, waste removal, and overall scaffold stability. Smaller pores enhance cell attachment and intracellular signaling, while larger pores facilitate gas diffusion and vascularization, necessitating an optimal balance between porosity and mechanical integrity [159]. As we envisioned already the inclusion of vascular structures in our hydrogel constructs we chose a GelMA concentration of 15 %, as this has to be proven to show well shape fidelity [160].

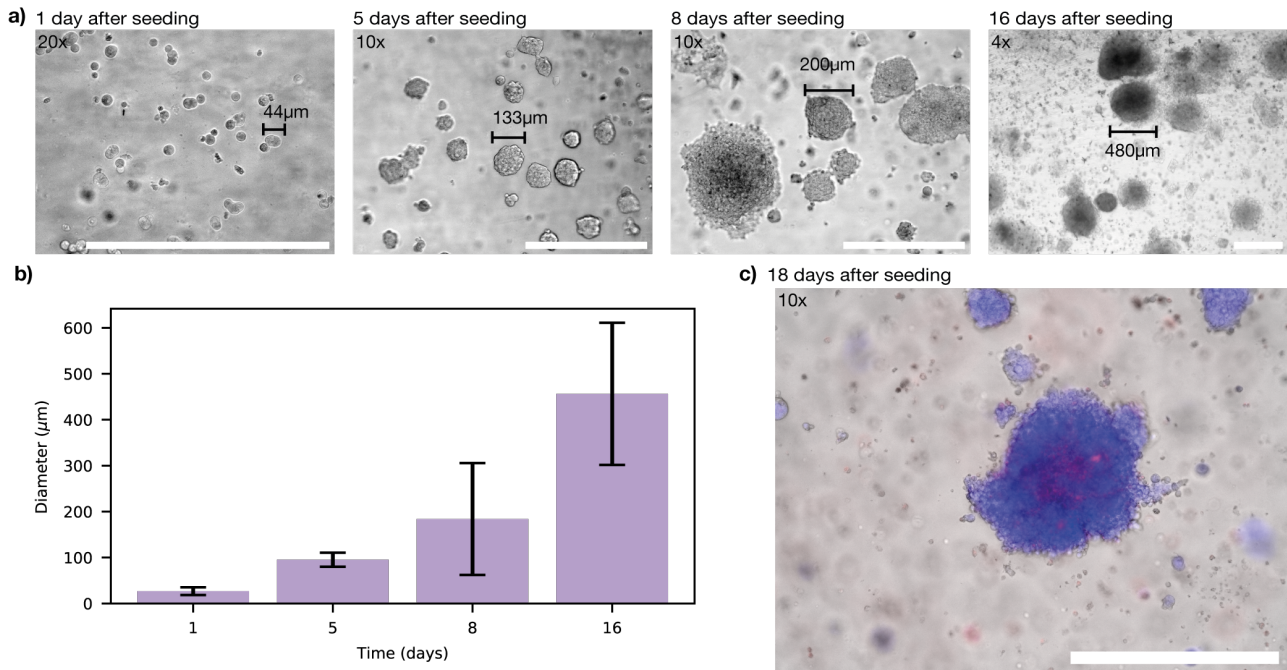


Figure 4.1: Cell growth in 15 % homemade GelMA crosslinked with 0.25 % LAP using 405 nm UV for 30 s. a) Proliferation of HEK293T cells embedded in GelMA over a 16-day culture period. b) Formation of multicellular clusters within the hydrogel, with an estimated doubling time of approximately two days. Error bars represent standard deviation, for each timepoint 10 clusters were measured. c) Live/dead staining using Hoechst (blue, live) and Propidium Iodide (red, dead) to assess cell viability. The majority of cells remain viable, with minimal cell death observed, primarily localized at the center of cell clusters. Scale bar: 500 μm for all images.

To evaluate cellular behavior over an extended period of time, HEK293T cells were embedded in 15 % GelMA containing 0.25 % Lithium-Phenyl-2,4,6-Trimethylbenzoylphosphinate (LAP) for crosslinking and cultured for 16 days, with medium exchange every 3–4 days. The rapid crosslinking of GelMA provided a stable 3D structure, enabling cell proliferation in a physiologically relevant

environment. One day after seeding, cells were observed as singlets, doublets, and triplets, with cluster sizes reaching up to 44 μm in diameter (Figure 4.1a). Over 18 days, cells proliferated into spherical clusters, reaching diameters of approximately 500 μm .

A quantitative analysis of the microscope images (Figure 4.1b) confirms consistent cell growth within GelMA indicating that the matrix provided adequate structural and biochemical support for proliferation. Assuming roughly spherical clusters, we can calculate the cluster volume $V_{cl} = \pi d_{cl}^3/6$ from the measured diameter d_{cl} . V_{cl} is proportional to the number of cells in the cluster, i.e., $V_{cl} = N_c \cdot V_c$, where V_c is the cell volume. For constant growth the number of cells increases over time as $N_c(t) = N_0 \cdot 2^{t/t_D}$. We can thus extract an estimate for the cell doubling time in the gel from our data, which results in $t_D \approx 2$ days (Supplementary Information of reference [38] Figure S3). The final clusters contain $N_c \approx 5000$ cells.

Live/dead staining performed on day 18 (Figure 4.1c) confirmed high cell viability throughout the culture period. Minimal cell death was observed, primarily localized at the cluster centers. This observation is consistent with previous studies that have reported a maximum cell cluster size of 500 μm before central necrosis occurred [161], likely due to diffusion limitation of nutrients.

To compare if the proliferation behavior into clusters in our homemade GelMA is equal for different cell lines, we assessed the compatibility of our hydrogel with two other cell lines: NIH cells and hMSCs. The results, presented in the Supplementary Information of reference [38] (Figure S9), demonstrate that both HEK293T and NIH cells proliferated within the hydrogel and exhibited cluster formation, whereas hMSCs distributed homogeneously throughout the matrix without forming clusters.

For subsequent studies, HEK293T cells were selected as the primary model due to their well-established suitability for genetic modification.

4.2.2 Induction of Gene Expression in Matrix-Embedded Cells

Inducible gene expression in cells embedded in hydrogel was assessed using the Tet-On 3G system [162], [163]. HEK293T cells were genetically modified to stably express the fluorescent protein mGreenLantern as a baseline reporter, while mScarlet-I expression could be induced with doxycycline via the Tet-On 3G system (Experimental section). In these cells, both components of the Tet-On 3G system are integrated into a single vector (Figure 4.2a). mGreenLantern is expressed under a CAG promoter in the forward orientation to enhance contrast for improved visualization of the cells in hydrogel. The Tet-On 3G transactivator is driven by the constitutive human phosphoglycerate kinase 1 promoter (hsPGK1) in reverse orientation, alongside the gene of interest — mScarlet-I -, which is put under the control of the inducible TRE3GS (TRE 3G) promoter. Cells in adherent culture displayed homogeneous constitutive expression of mGreenLantern as well as induced expression of

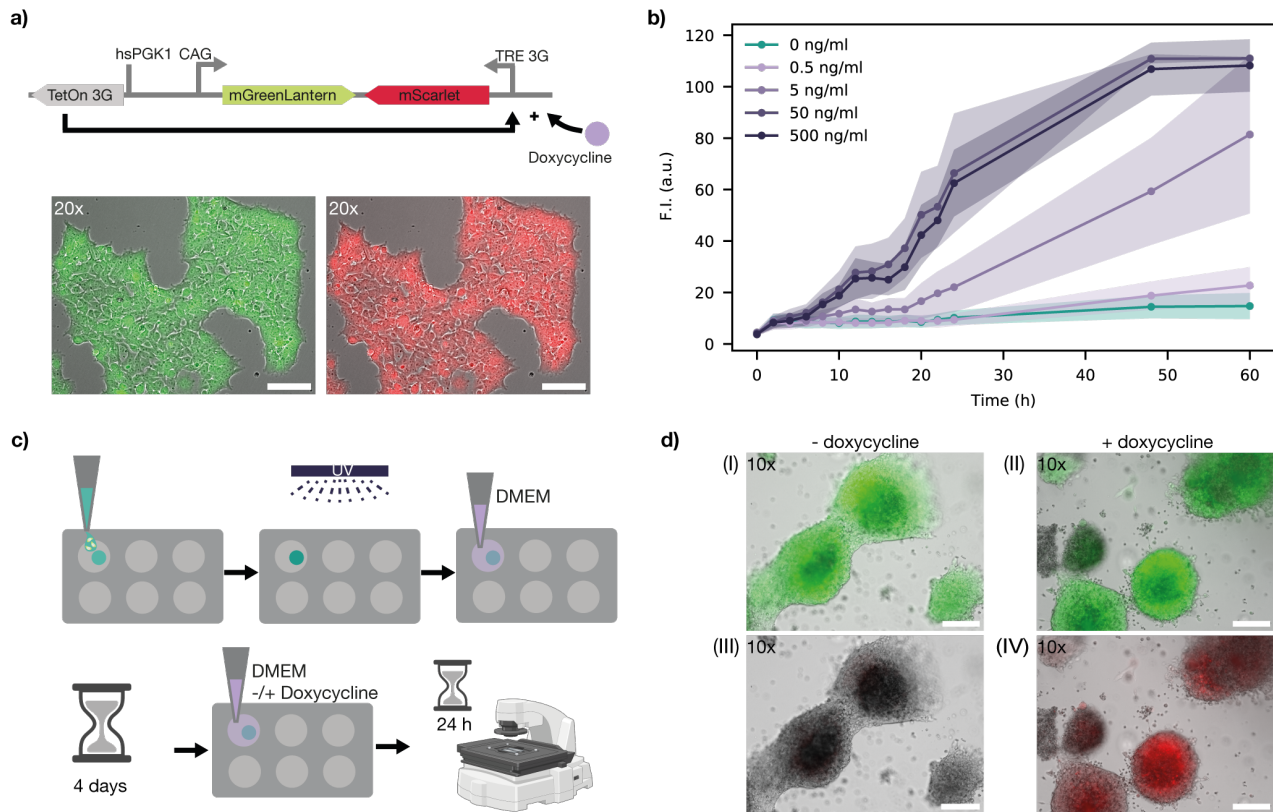


Figure 4.2: In situ gene induction. a) For gene induction experiments a genetically modified HEK293T cell line was used that constitutively expressed mGreenLantern from a CAG promoter, while mScarlet-I expression could be induced by the addition of doxycycline via the Tet-On system. The microscope panels show composites of brightfield and fluorescence channels for mGreenLantern expression (left) and mScarlet-I expression (right), 24 h after treatment with 500 ng/ml doxycycline. b) Dose-dependent induction of mScarlet-I in adherent HEK293T cells. A doxycycline concentration of 50 ng/ml is sufficient for full activation. c) HEK293T cells embedded in 15 % GelMa at a density of 1 million cells per ml were cultured in well plates containing DMEM and induced with 500 ng/ml doxycycline after four days of incubation. Fluorescence intensities were recorded 24 h post-induction. d) Microscopy images of Gel-embedded cell clusters (overlays of brightfield and fluorescence channels). (I–II) Control and induced samples show mGreenLantern fluorescence. (III–IV) mScarlet-I fluorescence is only observed in the doxycycline induced sample. Scale bars: 200 μ m.

mScarlet-I 24 h after induction with 500 ng/ml doxycycline, with fluorescence levels being roughly similar across the cell population (Figure 4.2a).

Although already 50 ng/ml doxycycline were sufficient for full induction of adherent cells (Figure 4.2b), we chose 500 ng/ml for all experiments in hydrogel-embedded cells to ensure sufficient doxycycline penetration through the gel matrix for full induction.

Cells were mixed into 15% GelMA at 10^6 cells/ml, seeded into a 48-well plate, and crosslinked using UV light, as illustrated in Figure 4.2c. 300 μ l of the cell gel mixture was applied to the well. As this has a surface of 1.1 cm² the height of the hydrogel was $\sim$ 270 μ m. UV exposure was limited to only a few seconds to keep its effect on the cells as low as possible. Culture medium was applied on top, and cells were grown until clusters reached a size of approximately 100 μ m, typically after 4 days. Doxycycline was then added, and fluorescence was measured 24 h later (Figure 4.2d). Only doxycycline-exposed cells exhibited robust mScarlet-I fluorescence, demonstrating that in situ gene induction proceeds effectively within the gel. Induced cell clusters could be observed in the upper part (roughly 200 μ m) of the gel which was exposed to 500 μ l of DMEM.

4.2.3 In Situ Plasmid and mRNA Delivery

We next evaluated the influence of the GelMA matrix on transfection efficiency. While transfection protocols are well established in 2D cultures, adapting these strategies to 3D hydrogels presents additional challenges, including restricted diffusion and reagent penetration, reduced vector stability, and altered cell-matrix interactions that can affect vector uptake. Previous studies have explored transfection methods in 3D environments [156] and tested various reagents for hydrogel-based cultures [157]. Notably, some studies have reported higher mRNA transfection efficiencies in specific 3D models [158].

To evaluate transfection performance in our system, we tested both pDNA and mRNA vectors, using jetOPTIMUS for plasmid DNA transfection and Lipofectamine 2000 for mRNA delivery. Both vectors must undergo several steps before yielding a measurable expression signal (Figure 4.3a). First, the vectors must diffuse through the hydrogel matrix to reach the cells. The transfection complexes are expected to exhibit sizes in the range of 100 to 200 nm, with pDNA complexes likely being slightly larger than mRNA lipoplexes. As a result, minor differences in diffusivity can be expected.

Further, plasmid DNA is more stable compared to mRNA, but has to be processed by the cell before the gene of interest is expressed. After cellular uptake, pDNA must enter the cell's nucleus, where it undergoes transcription, followed by mRNA nuclear export before the protein can be translated. In contrast, mRNA transfection bypasses these steps and can be directly translated after entering the cytoplasm.

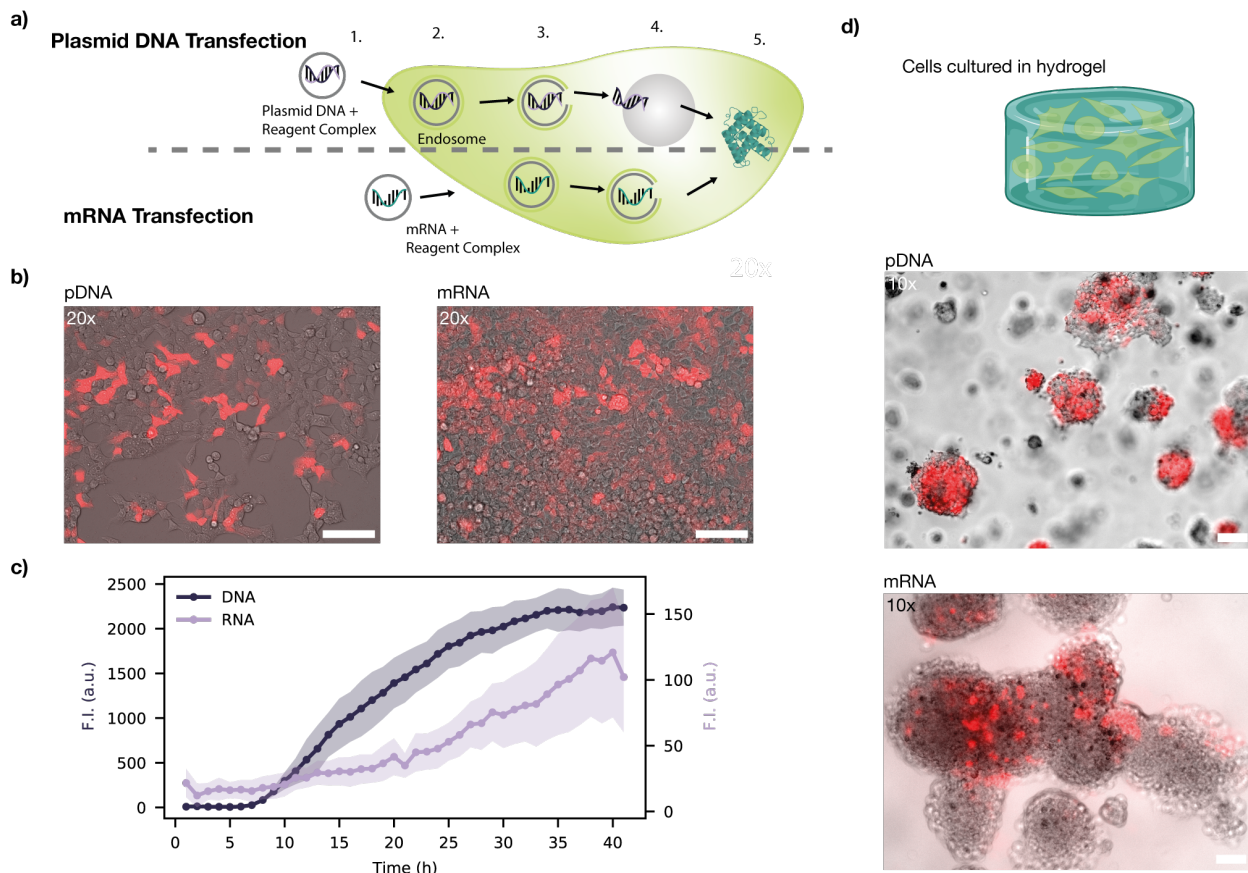


Figure 4.3: Transfection of HEK293T cells cultured in hydrogel with plasmid DNA (pDNA) versus mRNA. a) Schematic representation of the steps involved in pDNA vs. mRNA transfection: (1) Complexation of pDNA or mRNA with transfection agent, (2) cellular uptake via endocytosis, (3) endosomal escape, (4) for pDNA only: nuclear entry, transcription, and mRNA export (5) translation of mRNA into protein in the cytoplasm. b) Composite images of brightfield and mScarlet-I fluorescence in cells transfected with pDNA (left) and mRNA (right). Scale bar: 100 μ m. c) Fluorescence intensity of mCherry expression following transfection with pDNA versus mRNA. For each curve fluorescence was determined in an ROI measured containing several cells (see SI Figure S1 for details.) The shaded area indicates the standard deviation, for each timepoint 3 ROIs were measured. d) Cells were mixed with 15 % GelMA, pipetted into a well, and GelMA containing 0.25 % LAP was crosslinked using 405 nm UV light for 30 s before the medium was applied on top. Composite images of brightfield and mScarlet-I fluorescence show HEK293T cells cultivated in GelMA and transfected with either pDNA or mRNA. Scale bar: 100 μ m.

Using mCherry as a fluorescent reporter, we first tested transfection in adherent HEK293T cells (Figure 4.3b). Transfection with pDNA resulted in a signal increase after 6 h and leveled off after ≈ 30 h, resulting in an overall sigmoidal time-course of the fluorescence intensity. In contrast, mRNA transfection produced a weak signal with no apparent lag time and the fluorescence intensity followed a linear trend. mRNA transfection resulted in overall lower tenfold fluorescence intensities than when using pDNA as a vector (Figure 4.3c).

Next, we investigated the feasibility of in situ transfection of hydrogel-embedded cells. Transfection efficiency, defined as the percentage of cells that successfully take up and express the introduced genetic material, was used to evaluate the success of gene delivery. We found that pDNA vectors led to significantly higher transfection efficiencies compared to mRNA vectors (Figure 4.3d).

In pDNA transfection, we consistently observed uniform expression within a cell cluster. In contrast, mRNA transfection primarily led to fluorescent protein expression in individual cells, but not in whole clusters.

4.2.4 Precision Genome Editing in Hydrogel-Embedded Cells

To further advance the genetic manipulation of gel-embedded cells, we explored the possibility of in situ genome engineering via CRISPR-based prime editing (Figure 4.4a) [60]. In prime-editing, a prime-editing guide RNA (pegRNA) containing a primer binding site (PBS) and a reverse transcriptase template binds with a Cas9-derived nickase (SpCas9n from *Streptococcus pyogenes*) fused to a reverse transcriptase (RT) domain. This complex binds to the target DNA at a sequence complementary to the pegRNA spacer, where SpCas9n cuts the unbound target strand.

The PBS of the pegRNA binds the nicked strand and the sequence of the RT template is reverse transcribed onto the nicked DNA strand. A DNA repair mechanism then leads to the integration of the newly synthesized DNA at the target site.

For readout, we used a genetically modified cell line that constitutively expresses a mutant mGreenLantern, in which two amino acids were altered at positions 65 and 66 (G65S and Y66H), resulting in a weakly blue fluorescing mutant [62]. Prime-editing can be used to revert this mutation and thus recover fluorescence emission in the green (Figure 4.4b).

To enable direct monitoring of its expression, mScarlet-I was encoded on the same transcript as the prime editor. As in our transfection experiments, we investigated the delivery of the prime editor using both plasmid DNA and RNA vectors. For pDNA delivery, we transfected cells with one plasmid encoding the prime editor and mScarlet-I, and a second plasmid encoding the pegRNA. For RNA lipoplexes, we transfected cells with mRNA encoding the prime editor and mScarlet-I, mixed with pegRNA in a ninefold excess and packaged using Lipofectamine 2000.

The presence of green fluorescent cells confirmed successful prime editing with both pDNA and

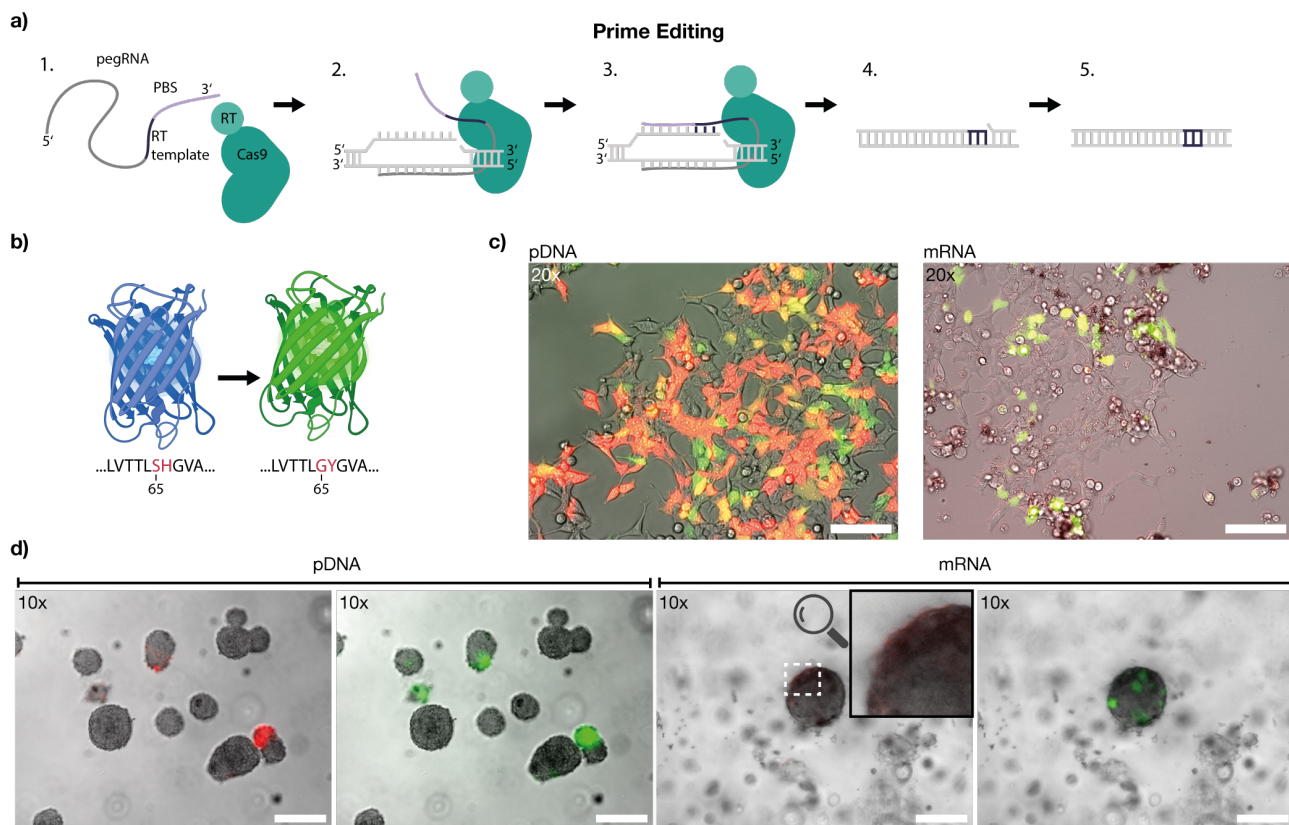


Figure 4.4: In situ prime editing. a) Prime editing mechanism: a prime editing guide RNA (pegRNA) contains the sequence for the desired edit along with a primer binding site (PBS), while the Cas9 protein is fused to a reverse transcriptase. Upon binding to the target DNA, one of the target DNA strands is nicked, the RT template is reverse-transcribed and the new DNA sequence is inserted at the edit position utilizing endogenous host DNA repair machinery. b) Two mutations in the mGreenLantern amino acid sequence (G65S and Y66H) shift its fluorescence from green to blue. Prime editing restores the original sequence, reverting the fluorescence to green. c) Left: HEK293T cells transfected with plasmid DNA encoding pegRNA and prime editor on two separate plasmids. mScarlet-I is co-expressed from the same plasmid as the prime editor as a control. Scale bar: 100 μm . Right: HEK293T cells transfected with pegRNA and prime editor mRNA (capped, polyadenylated, and transcribed with pseudouridine), co-expressing mScarlet-I. Scale bar: 100 μm . d) HEK293T cells seeded in 15 % GelMA were transfected three days post-incubation, and images were acquired 24 h later to assess fluorescent protein expression. Left (pDNA): Composite images of brightfield and RFP channel, and composite image of brightfield and GFP channel of cell clusters in GelMA, for which cells were transfected with plasmid DNA, showing expression of mScarlet-I and mGreenLantern in different clusters. Right (mRNA): Corresponding composite images of cell cluster after transfection with mRNA. Expression of mScarlet-I is barely detectable (see faint mScarlet-I signal highlighted at the upper left side of the cell cluster), whereas mGreenLantern expression is clearly visible. Scale bar: 100 μm for all images.

mRNA (Figure 4.4c). However, pDNA transfection appeared to yield a higher proportion of successful prime-editing events. Notably, in pDNA-transfected cells most cells expressed mScarlet-I ≈ 6 h after transfection, but initially lacked a green mGreenLantern signal, which appeared with a time delay of ≈ 4 h. This can be attributed to the sequential nature of protein expression: the prime editor must first be transcribed, simultaneously with mScarlet-I, and translated before the actual genomic editing occurs, leading to a delayed mGreenLantern signal. In contrast, mRNA-transfected cells exhibited mScarlet-I expression almost immediately, while an mGreenLantern signal became detectable after ≈ 6 h. In this case, the prime editor must first be translated from the mRNA before it can bind to the co-transfected pegRNA. As a result, prime editing efficiency is influenced by pegRNA degradation within the cell, contributing to the overall lower prime editing efficiency observed.

As shown in Figure 4.4d, we observed successful editing events also in gel-embedded cell clusters. However, the fraction of cells expressing a fluorescent protein after prime editing appeared to be lower than after simple transfection with a mCherry encoding plasmid (cf. Figure 4.3). Crucially, prime editing leads to a permanent change in the genome of the cells and thus has a permanent effect on gene expression than the transient transfection with pDNA or mRNA. This is particularly evident in the lower mScarlet-I signal following mRNA delivery, whereas the recovered mGreenLantern signal in successfully edited cells remains comparable to the intensity level of pDNA-modified cells. This stands in stark contrast to the vastly different fluorescence levels of both reporters observed in the transfection experiments.

4.2.5 Stimulation of Hydrogel-Embedded Cells via Channel Perfusion

Diffusion limitations prevent tissues above a certain size from being functional without vascularization. Distances larger than a few hundred micrometers cannot be sufficiently supplied with nutrients, signaling molecules, and other reagents by passive diffusion. To investigate how cells would grow in a hydrogel that was vascularized with a channel structure, we seeded cells in hydrogel around a channel with a diameter of 600 μm and flushed it with culture medium at a flow rate of 200 $\mu\text{l/h}$ (Figure 4.5a). Even though the cells were initially homogeneously distributed throughout the gel matrix, the cells only formed clusters in proximity to the channel. A region of ~ 500 μm around the channel was densely populated by cell clusters. This shows that the supply with nutrients over channel structures increases the area in which cell proliferation can be observed. The region increases from 200 μm in well plate experiments to a final tissue thickness of 1600 μm including the channel with diameter of 600 μm . When subtracting the channel the tissue height increased still 5 fold.

To assess the diffusion profile in the gel we introduced fluorescein by flushing the central supply channel with the dye at a constant flow rate of 200 $\mu\text{l/h}$. Fluorescein has a size comparable to that of small metabolites in the culture medium. Figure 4.5c shows fluorescence images of the

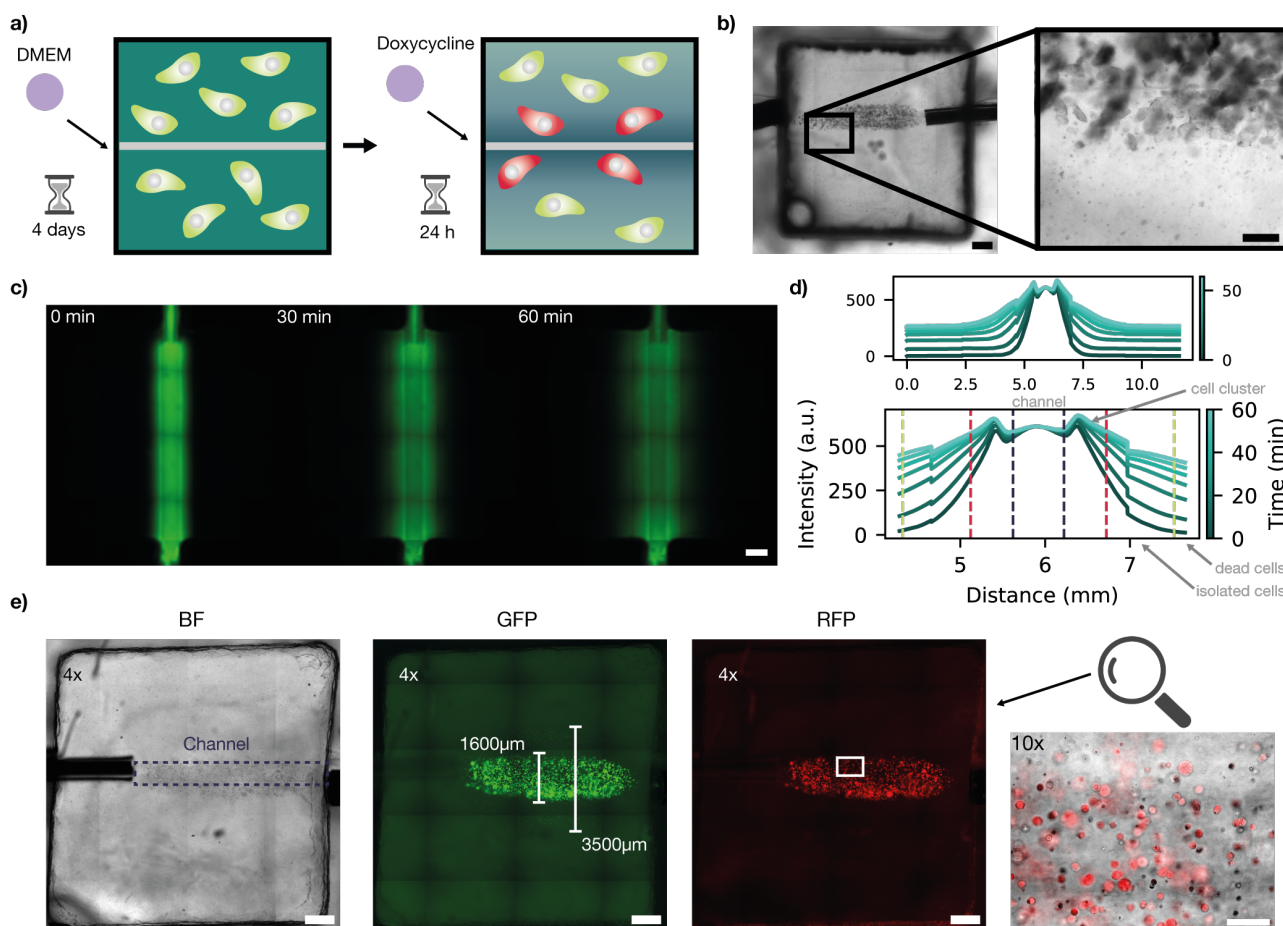


Figure 4.5: Construction of vascular-like structures. a) Schematic representation of the experiment. Cells were mixed with GelMA (Cellink) containing 0.25 % LAP and seeded in a homebuilt bioreactor. DMEM was perfused through the channel at a flow rate of $200 \mu\text{l/h}$ for four days. Doxycycline at a concentration of 500 ng/ml was introduced into the medium and perfused for 24 h. b) Brightfield image of the printed channel. Cells proliferate around the channel. Scale bar: 1 mm. c) To investigate the diffusion of small molecules in the hydrogel matrix, fluorescein was perfused through the channel. Representative images at 0 min, 30 min, and 60 min are shown. Scale bar: 1 mm. d) Time evolution of the fluorescein diffusion profile around the channel within 1 h. Images were acquired every 5 min. The dashed lines indicate regions with different growth conditions - purple: central channel, red: region with high concentration of nutrients supporting cell growth, green: nutrients support growth of isolated cells. Outside of the region marked with green dashed lines, cells are not viable. e) From left to right: Brightfield, green and red fluorescence images of the bioreactor. The central channel is barely visible, and therefore indicated with a dashed line in the brightfield image. Cells extend up to a distance of $1750 \mu\text{m}$ from the channel center, regions with high cell density and larger cell clusters are confined within $800 \mu\text{m}$ from the channel. The rightmost image provides a magnified view of this region in the RFP channel, showing that cells within its proximity express mScarlet-I upon addition of doxycycline. Scale bar: 1 mm.

vascularized gel taken at different time points, the corresponding mean fluorescence values in the direction perpendicular to the channel are plotted in Figure 4.5d.

We used the channel structure to regulate gene expression in gel-embedded HEK293T cells via a diffusible inducer. Cells were seeded in the bioreactor, cultured under continuous DMEM flow for four days, and then exposed to 500 ng/ml doxycycline to induce gene expression.

The diffusion zone (red-dashed lines in Fig. 4.5d) closely matched the region where cells proliferated and formed clusters, extending up to approximately 800 μm from the channel center. Beyond this, isolated live cells were detected up to 1750 μm , expressing fluorescent proteins but not forming clusters.

Further from the channel, gene expression and proliferation ceased. Isolated live cells were observed up to 3500 μm (green-dashed line, Fig. 4.5d), while only dead cells were found beyond this range.

For future applications, more advanced channel structures will be needed to optimize nutrient supply, waste removal, and spatially controlled gene expression via diffusible inducers. As a proof of concept, we implemented a 3D bioprinting strategy using a sacrificial ink approach [164] to fabricate vascular-like channels. This allowed us to investigate complex vascular architectures and assess their influence on small molecule diffusion within the gel matrix (cf. Supplementary Information S11–S13).

4.3 Discussion and Conclusion

Our study demonstrates that GelMA hydrogel scaffolds support cell proliferation, localized stimulation, and genetic manipulation in a 3D environment. HEK293T cells embedded in GelMA remained viable and formed dense clusters over 18 days with minimal cell death. Additionally, vascular-like supply channels facilitated controlled molecular delivery and enabled in situ genetic manipulation of matrix-embedded cells. We systematically progressed from chemical gene induction to more advanced genetic modifications, evaluating how embedded cells respond to external stimuli. By delivering genetic constructs via both plasmid DNA and mRNA, we demonstrated conventional fluorescent protein expression as well as in situ prime editing, leading to permanent genetic modifications. The emergence of the final gene product follows distinct kinetic processes: mRNA enables rapid expression but has a short half-life, whereas plasmid DNA requires multiple processing steps, introducing a time delay. When combined with mRNA delivery, prime editing provides a transient initial stimulus while inducing a permanent genomic modification in targeted cells. We also explored how embedded channels within GelMA hydrogels influence spatial control over gene expression and molecular transport. While vascular-like structures have the potential to improve nutrient delivery and gene induction, further optimization will be necessary to enhance their physiological relevance.

Future research has to focus on refining hydrogel properties, optimizing gene delivery strategies, and improving molecular transport dynamics through synthetic vasculature to better mimic native tissue environments. Advancing these aspects could further drive applications in tissue engineering, regenerative medicine, and drug testing.

4.4 Materials and Methods

Cell Lines and Plasmids

Three HEK293T cell lines were used for growth, transfection, doxycycline induction, and prime editing experiments:

i) A standard HEK293T cell line (ATCC, CRL-3216™) was employed for growth studies and general transfection experiments due to its high transfection efficiency and suitability for protein expression analyses. A plasmid encoding mCherry under the J23119 promoter was used [165] for transfection studies. The plasmid map is available in the appendix Figure A.4. Two additional genetically modified HEK293T cell lines were engineered using the piggyBac transposon system, which enables stable genomic integration via two plasmids: one encoding the piggyBac transposase and another carrying the gene of interest.

ii) Doxycycline-Inducible Cell Line: This cell line constitutively expresses mGreenLantern and switches to mScarlet-I upon doxycycline induction via the Tet-On 3G system [162], [166], [167]. This system allows for precise control and visualization of inducible gene expression. The plasmids used for stable transfection are provided in the appendix Figure A.5.

iii) Prime Editing Cell Line: This cell line was designed for prime editing studies by modifying the green fluorescent protein (GFP) in G65S and Y66H, resulting in a hypsochromic spectral shift from green to blue fluorescence. Successful prime editing restores the original SH sequence, reverting fluorescence back to green. This fluorescence switch provides a visual and quantitative readout of editing [168]. Plasmid maps used for prime editing of the prime editor and pegRNA can be found in the appendix Figures A.6-A.8.

Plasmid Preparation and RNA Synthesis

Plasmids were prepared using the ZymoPURE Plasmid Miniprep Kit (Zymo Research) following the manufacturer's protocol without modifications. The purified plasmid DNA was used directly for experiments or as a template for in vitro transcription (IVT) to generate mRNA.

For mRNA synthesis, the RiboMAX™ Large Scale RNA Production System (Promega) was used. Plasmid DNA templates were linearized with AsiSI and BasI-HF-v2 (NEB) to ensure proper tran-

scription termination. Following digestion, DNA was purified using the DNA Clean & Concentrator kit (Zymo Research) and used as transcription template.

IVT was performed according to the manufacturer's instructions, incorporating pseudouridine (Φ) (Jena Bioscience) for enhanced RNA stability for the prime editor only. Afterwards, plasmid DNA was removed through DpnI digestion and RNA was isolated using the RNA Clean & Concentrator kit (Zymo Research). Transcribed pegRNA was stored directly at -80°C and mRNA for the prime editor was capped with the FCE Capping system (NEB) and polyadenylated using Poly A Polymerase (NEB) to improve translational efficiency. The processed RNA was purified using the RNA Clean & Concentrator kit (Zymo Research), quantified with a Nanophotometer (Implen), and stored at -80°C until use.

Cell Culture Conditions

Cells were initially seeded in 75 cm^2 cell culture flasks in Dulbecco's Modified Eagle Medium (DMEM, high glucose, Gibco™) supplemented with 10 % v/v Fetal Bovine Serum (FBS, Sigma-Aldrich) and 1 % Penicillin-Streptomycin (P/S, Gibco™) to support growth and prevent bacterial contamination. Cultures were maintained at 37°C with 80 % humidity and a 5 % CO_2 atmosphere. The medium was fully exchanged every 3 to 4 days to replenish nutrients and remove waste products. Upon reaching 80 % confluence, cells were passaged using 0.25 % Trypsin-EDTA (Gibco™) and maintained up to passage 20.

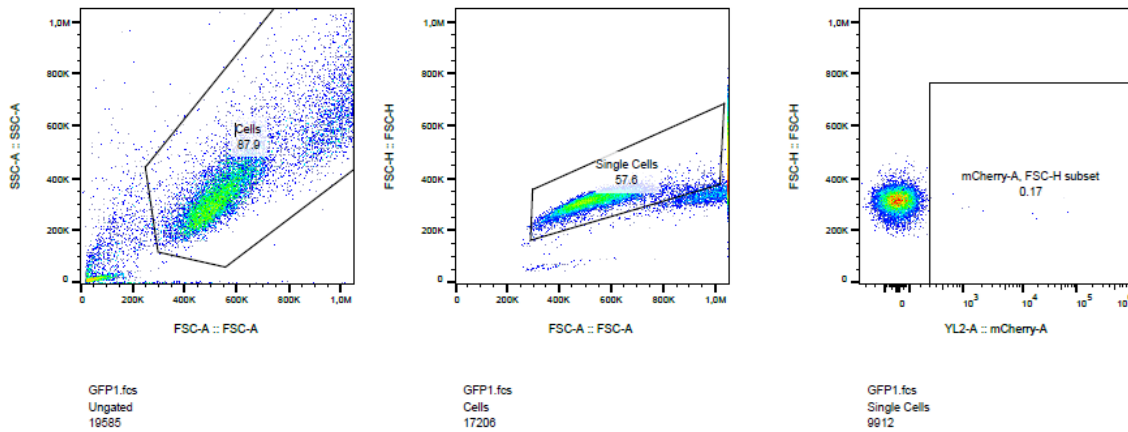
For three-dimensional culture, cells were encapsulated in GelMA (gelatin methacrylate) pre-warmed to 37°C to facilitate mixing. To achieve a final concentration of 1×10^6 cells/ml, 1 ml of GelMA was combined with 100 μl of a cell suspension containing approximately 1.1×10^6 cells. The mixture was homogenized by gentle pipetting, then transferred to a well plate or bioreactor depending on the experiment. Constructs of varying thickness (1 mm to 3 mm) were crosslinked under 405 nm UV light for 10 s to 30 s, depending on the thickness.

Two types of GelMA were used: a homemade formulation adjusted to 15 % with 0.25 % Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, Sigma-Aldrich) for well plate experiments, and commercially sourced GelMa (GelMa A, Cellink) containing 0.25 % LAP. Both formulations exhibited similar behavior above 35°C , as confirmed by rheometer analysis (Figure 4.7).

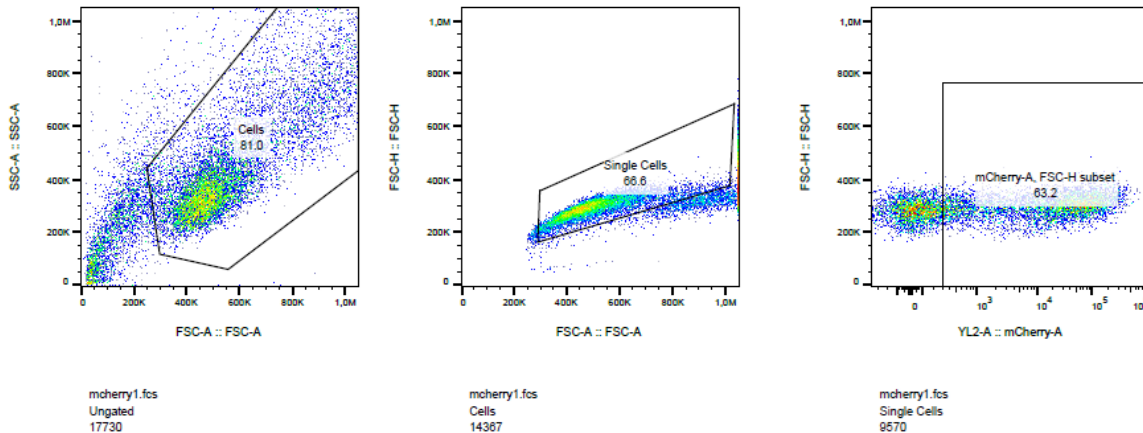
Transfection of HEK293T Cells

Transfection of HEK293T cells was performed to introduce genetic material and assess the efficiency of different transfection reagents in 2D and 3D environments. In 2D cultures, DNA transfection was carried out using jetOPTIMUS (Polyplus), while RNA transfection was performed with Lipofectamine 2000 (Invitrogen™). In 3D GelMa hydrogels, DNA and RNA transfection efficiencies were

directly compared using Lipofectamine 2000 (Invitrogen™). The amounts for buffer, DNA, and the transfection reagent were used according to the manufacturers protocol for DNA transfection. A 24-well plate was used and 0.7 μ L of reagent were used. For RNA transfection with Lopfectamin amounts for transfection reagent and Optimem were also used according to the manufacturers protocol for a 24-well plate. Slightly higher amounts of RNA were used compared to the manufacturers protocol: 800 ng instead of 500 ng. When transfection RNA for prime editing 720 ng of the RNA for the prime editor and 80 ng of pegRNA were used. Transfection efficiency was determined with a flow cytometer (Attune NxT, Invitrogen) to yield $\approx 63\%$ (see Figure 4.6).



(a) Flow cytometer measurement of HEK293T cells as control for the transfection efficiency.



(b) Flow Cytometer measurement of cells transfected with plasmid DNA encoding the protein mCherry.

Figure 4.6: Flow cytometer measurement to detect efficiency of transfection with plasmid DNA.

Homemade GelMa

Homemade GelMa foam was synthesized using 10 g of Type A gelatin (300 bloom, Sigma-Aldrich) which was dissolved in 100 ml of 1x phosphate-buffered saline (PBS tablets, Sigma-Aldrich) at room temperature with moderate stirring, then heated to 50 °C in a water bath until fully dissolved. While stirring continuously, 0.6 g of methacrylic anhydride (MAA, Sigma-Aldrich) per gram of gelatin was gradually added using a glass pipette to prevent plastic interaction. The reaction proceeded for 3 h under continuous stirring to achieve a high degree of methacryloyl functionalization ($(75 \pm 9) \%$) according to the protocol of Loessner et al. [169].

Following the reaction, the solution was centrifuged at 3500 g for 3 min to remove unreacted MAA. The supernatant was diluted with two volumes of preheated (40 °C) 1x PBS and transferred into a 12 kDa molecular weight cut-off dialysis membrane. Dialysis was performed against 5 L of 1x PBS at 40 °C for 5 to 7 days, with daily PBS changes.

After dialysis, the pH was adjusted to 7.4 using 1 M NaHCO_3 . The solution was filter-sterilized using 0.2 μm syringe filters, aliquoted into 50 ml tubes, and snap-frozen in liquid nitrogen. Without thawing, aliquots were lyophilized for 4 to 7 days until fully dehydrated. The lyophilized GelMa foam was stored at -20°C in sealed screw-top tubes, protected from light and moisture.

Preparation of Bioinks

For preparing the homemade GelMA solution, lyophilized GelMA foam was sterilized with UV light for 30 minutes before use. A 1.25 % LAP stock solution was prepared by dissolving 50 mg of LAP in 4 mL of DPBS (Gibco™) under constant stirring at 70 °C for 30 minutes, with the solution protected from light using aluminum foil. The appropriate volume of LAP stock solution was then added to achieve a final concentration of 0.25 % (e.g., 2 mL LAP stock for 10 mL total GelMA solution). Separately, GelMA precursor solution was prepared by dissolving the desired amount of lyophilized GelMA foam in PBS at 70 °C for 30 minutes under magnetic stirring (e.g., 1.5 g GelMA in 8 mL PBS for a 15 % GelMA solution). Finally, the LAP and GelMA solutions were combined and stirred at 60 °C for 10 minutes to produce the final hydrogel solution. The characteristics of our homemade GelMA were analyzed using a rheometer (Anton Paar, at the 'Lehrstuhl für Systemverfahrenstechnik' - TUM School of Life Science) and compared with a GelMA purchased from Cellink (SKU: IK3521020303). Methylcellulose was used as a control material for rheological measurements due to its well-characterized viscoelastic properties and stability under shear stress. As a semi-synthetic polysaccharide, methylcellulose forms a thermoreversible gel and exhibits shear-thinning behavior, making it a suitable reference for benchmarking soft hydrogel-like materials. Three tests were conducted: An amplitude sweep was used to determine the linear viscoelastic region of the material by measuring how the storage modulus (G') and loss modulus (G'') respond to increasing

strain. A temperature sweep was conducted to assess how the material's viscoelastic properties change with temperature, providing insights into gelation, melting, or thermal stability. Lastly, a thixotropy test was done to evaluate the material's ability to recover its structure after shear stress by applying a high-shear phase followed by a low-shear recovery phase. The results are presented in Figure 4.7 and show that above 27-30 °C the hydrogels behave the same. Because of this we kept the working temperature always above this value for mixing, printing, and casting.

Bioreactor Setup

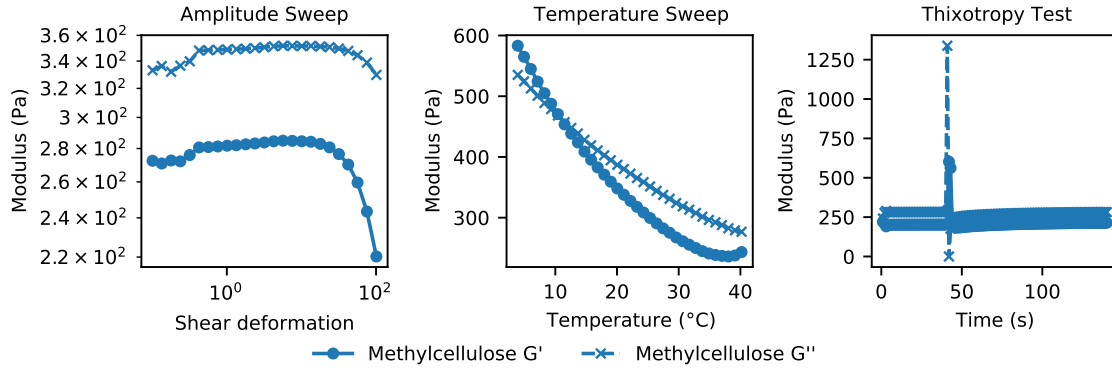
The bioreactor system was constructed using custom chambers made of polydimethylsiloxane (PDMS, SYLGARD 184, Biesterfeld) to support printed GelMA structures. Each PDMS chamber was bonded to a glass microscope slide (VWR) via oxygen plasma treatment, providing a stable and sealed base. A needle tip was punched through the bioreactor chamber, and GelMA mixed with cells was cast within the PDMS chamber. A second glass slide (coverslip, 18x18 mm, DWK Life Science) was then secured on top using silicone glue (SI 595, Loctite, Henkel), creating a fully enclosed environment for cell culture. The glue was selected for its ability to bond glass and PDMS while maintaining biocompatibility. Biocompatibility was confirmed experimentally (see Figure 4.8).

The bioreactor, shown in Figure 4.9, was cross-linked under 405 nm UV light for 30 s and incubated for 5 min to allow glue solidification. The needle was then carefully removed, and catheters (indwelling venous cannula) were inserted through the PDMS to create inlets and outlets for media flow. A 20 ml syringe (Braun) filled with DMEM (or Fluorescein for the diffusion experiments in Figure 1.9) was connected via tubing (extension line, Braun) to the catheters, and channels were flushed with DMEM. Media flow was maintained using a programmable multichannel syringe pump (Darwin) at a flow rate of 200 $\mu\text{l/h}$. The direct catheter-to-PDMS connection established a continuous, airtight flow system, facilitating nutrient exchange and supporting cellular growth within the GelMA matrix.

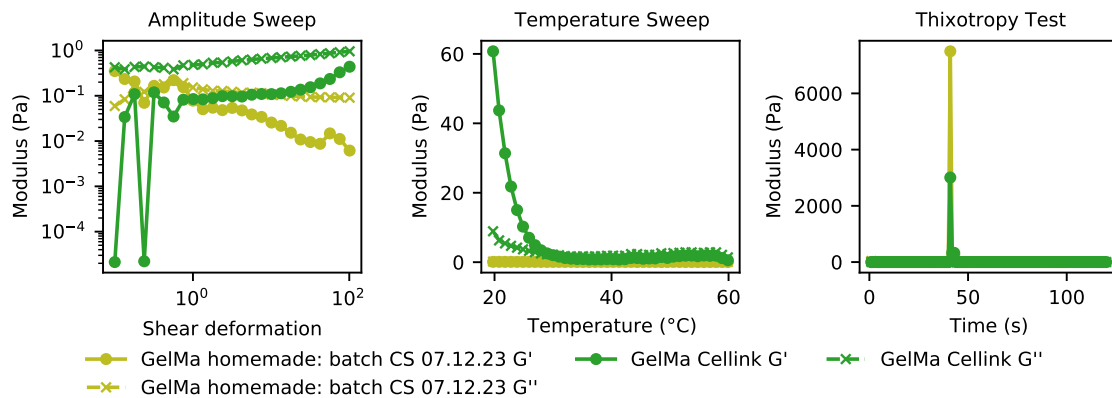
Microscopy

Fluorescence imaging was performed using an M7000 microscope (Evos) equipped for live-cell imaging. To maintain optimal cell viability during imaging, samples were kept at 37 °C with 80 % humidity and 5 % CO₂ using a Stage Top Incubation System, simulating physiological conditions. The microscope was equipped with GFP, RFP, and DAPI filter sets.

For live/dead analysis, cells were stained with Hoechst 34580 (Invitrogen) to label nuclei in live cells and Propidium Iodide (Life Sciences) to identify dead cells with compromised membrane integrity. This dual-staining approach enabled the assessment of cell viability within 3D GelMA



(a) Methylcellulose was measured as benchmark. The amplitude sweep provides insight into the viscoelastic properties of methylcellulose by evaluating its storage modulus (G') and loss modulus (G'') across increasing strain amplitudes. In the linear viscoelastic region (LVR), G' remains higher than G'' , indicating that the material behaves as a predominantly elastic gel. As strain increases beyond the critical strain, G' decreases, marking the onset of structural breakdown and a transition to more liquid-like behavior. This suggests that methylcellulose maintains its gel-like properties under low deformation but undergoes yielding at higher strains. The temperature-dependent rheological behavior of methylcellulose is characterized by an increase in G' at higher temperatures, indicative of thermogelling properties. Initially, at lower temperatures, the material behaves as a viscous solution with $G'' > G'$. As temperature rises, polymer interactions strengthen, leading to gel formation, reflected in a crossover point where G' surpasses G'' . This transition temperature is a critical parameter for applications requiring thermal responsiveness. The thixotropy test assesses the recovery of methylcellulose after shear-induced breakdown. Upon applying high shear, both G' and viscosity drop significantly, reflecting structural disruption. When shear is reduced, a gradual recovery of G' is observed, indicating partial structural reformation. However, if full recovery is not achieved within the measured timeframe, this suggests that methylcellulose exhibits a degree of irreversible structural breakdown or slow rebuilding dynamics. This behavior is relevant for applications where shear-induced fluidization and recovery kinetics are important.



(b) Rheological analysis of GelMA purchased from Cellink and our homemade GelMA. Amplitude-, temperature sweep, and thixotropy test. The temperature sweep shows similar behavior of the hydrogels above 30°C . The thixotropy test shows that both hydrogels recover its structure and viscosity after deformation.

Figure 4.7: Rheometer analysis.

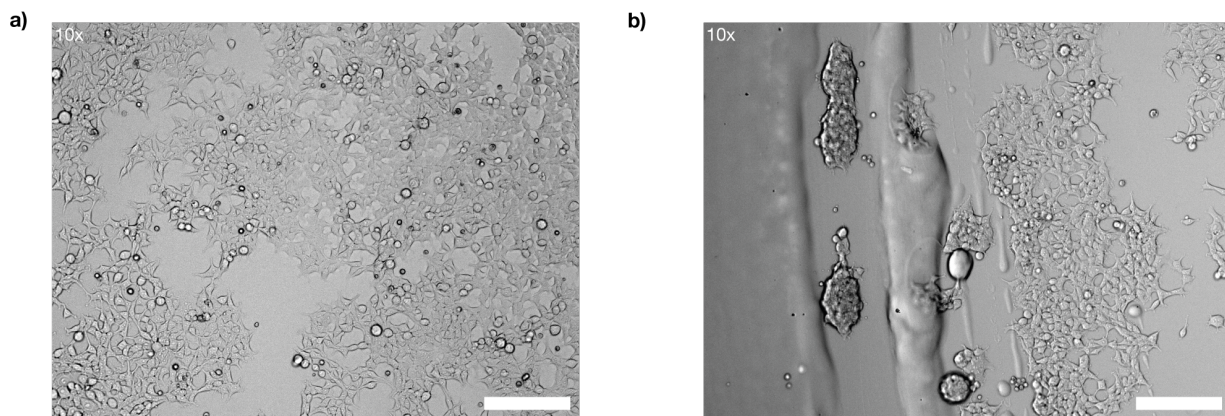


Figure 4.8: a) Cell growth in a well plate next to a glue drop. b) Cell growth inside the gel drop. Cells do grow in clusters similar to cells growing in GelMA. Scale bar: 200 μm .

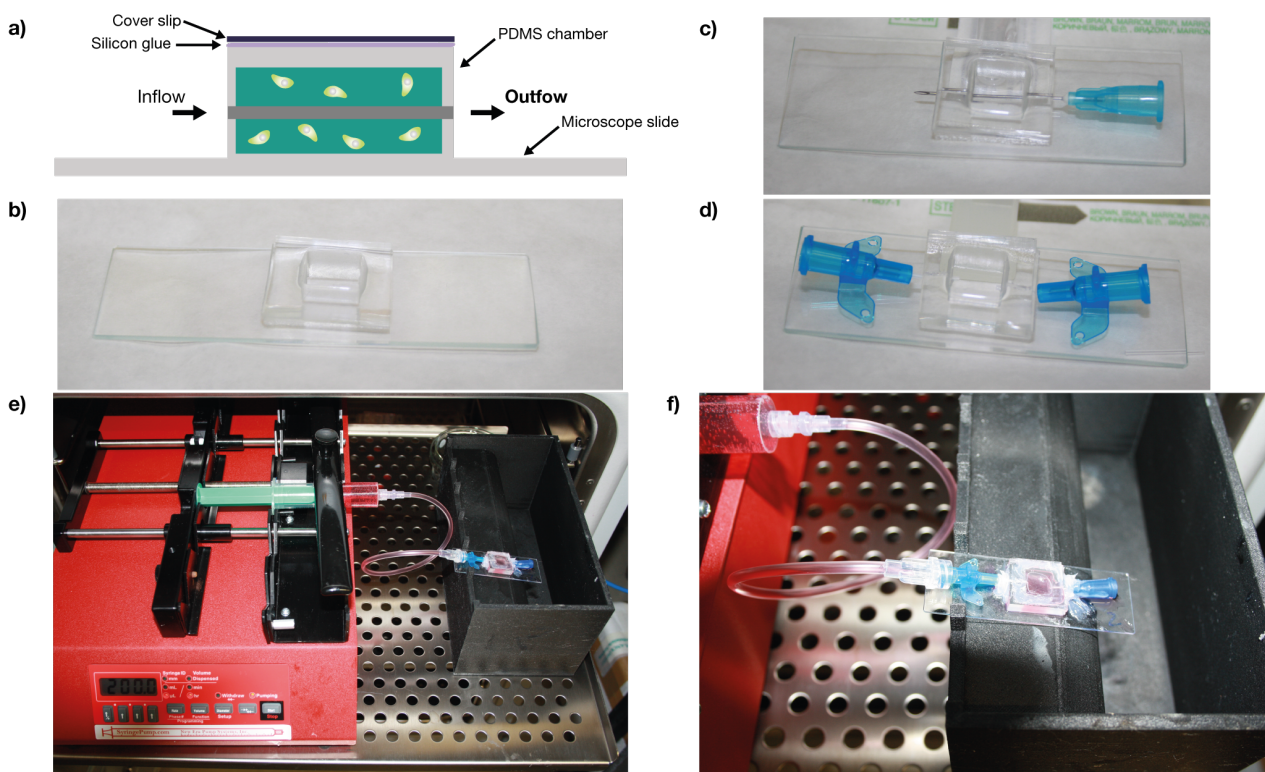


Figure 4.9: a) Schematic of bioreactor. b) PDMS chamber bonded to microscope slide with O₂ plasma. c) Needle (ID 0.6 mm) punched through PDMS for casting gel to create a channel. d) In- and outlet connection with catheter 22G. e) Photograph of the whole setup. A syringe pump is used to be able to run 6 bioreactors in parallel. A 3D printed PAHT chamber is used as collecting tank for material flushed through the bioreactors. f) Zoom in on the bioreactor itself.

constructs under real-time conditions.

Data Analysis

Data analysis was performed using Fiji (ImageJ, version 1.54f) to process and quantify fluorescence images. Cell viability, spatial distribution, and expression levels were assessed through fluorescence intensity measurements. For doxycycline titration experiments, regions of interest (ROIs) were selected, see Figure 4.10, to ensure 100% confluence over time. Cell clusters for growth analysis were manually selected (Figure 4.11), and their sizes were approximated by fitting ellipsoids. Ten clusters per sample were analyzed for statistical evaluation.

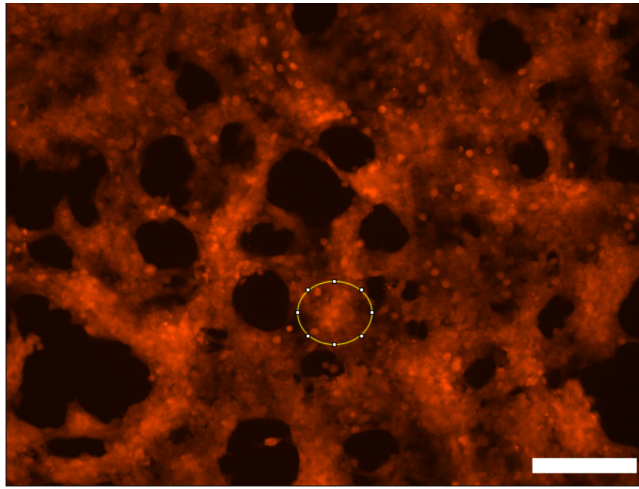
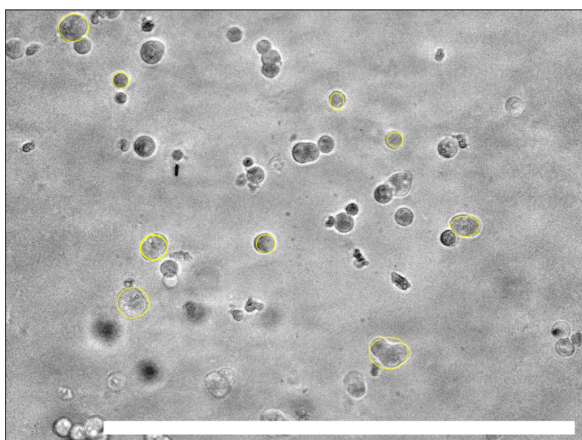
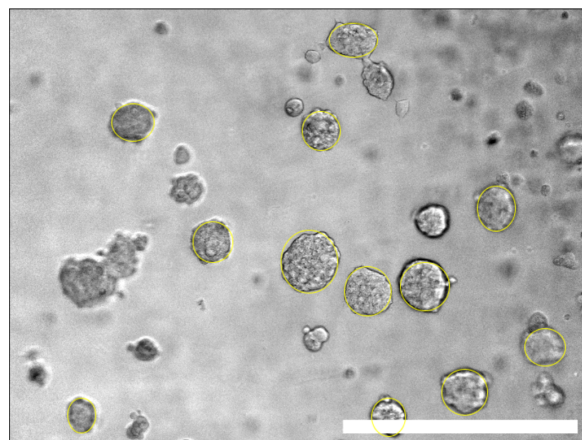


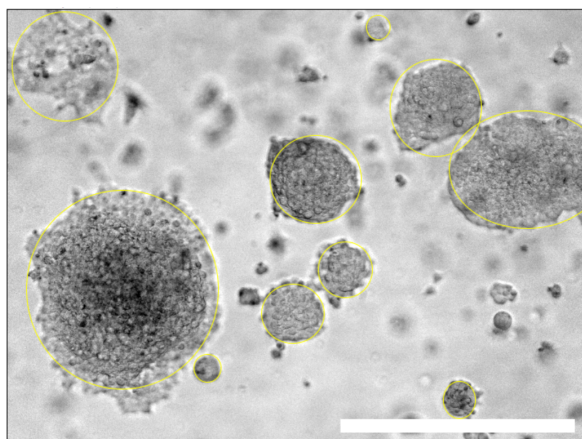
Figure 4.10: ROI selected for time measurements. Scale bar: 500 μm



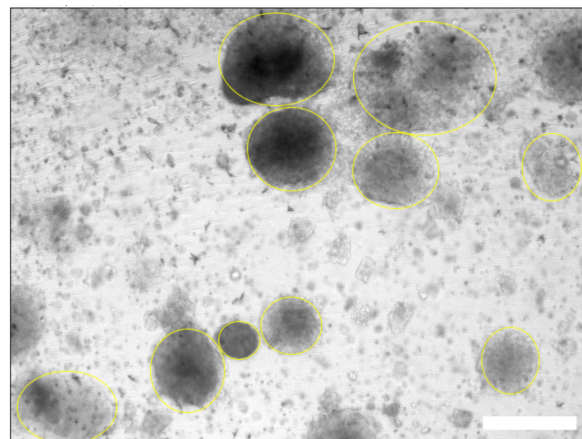
(a) Day 1 after seeding cells in GelMA.



(b) Day 5 after seeding cells in GelMA.



(c) Day 8 after seeding cells in GelMA.



(d) Day 16 after seeding cells in GelMA.

Figure 4.11: For each sample 10 clusters were selected to measure its size. The feret radius was determined with Fiji and the mean was calculated for each sample. Scale bar: 500 μm

5. Biofabrication of Vascular Structures for Microphysiological Systems

5.1 Introduction

The development of functional tissue constructs critically depends on maintaining cellular viability, establishing proper spatial organization, and ensuring efficient nutrient supply and waste removal. Both, natural and synthetic hydrogels, have been shown to effectively mimic the extracellular matrix (ECM), and various manufacturing techniques can be employed to engineer such constructs. However, a key limitation remains: the insufficient supply of nutrients and the removal of metabolic waste, particularly in larger constructs. To address this, the integration of vascular-like structures is essential for sustaining tissue viability and enabling the formation of larger, more physiologically relevant models.

As a foundation for such engineered tissues, we previously demonstrated that genetic circuits can be designed to induce spatial pattern formation. This approach enables the pre-programming of cellular behavior and offers control over differentiation and tissue development. The fact that these patterns emerge under non-equilibrium conditions highlights the importance of a dynamic exchange of nutrients and waste products to maintain functional activity within the system.

In parallel, we investigated how living organisms can be incorporated into hydrogel-based environments. We showed that bacterial populations can be spatially resolved and identified without genetic modification by using fluorescence in situ hybridization (FISH). Extending this work to mammalian systems, we successfully achieved genetic modification of cells while they remained embedded in the hydrogel matrix, demonstrating the feasibility of manipulating cellular function in situ.

To further support these complex cellular systems on a larger scale, we introduced simple channel structures into the hydrogel constructs. These channels enabled localized stimulation and efficient removal of metabolic byproducts, thereby enhancing viability in thicker constructs and laying the groundwork for more advanced vascular integration.

Building on these initial steps, we now focus on the integration of more complex, vascular-

like networks within hydrogel scaffolds. Such structures are critical to support long-term tissue functionality, especially in larger constructs where passive diffusion alone is insufficient. To this end, we evaluate three bioprinting strategies for generating perfusable networks: the use of sacrificial templates to form hollow channels, direct dual-material extrusion using a pneumatic printer, and embedded printing into a support bath following the FRESH technique.

Each method is assessed with respect to its biological compatibility, material constraints, and suitability for creating functional vascular-like structures. We place particular emphasis on the technical challenges involved in printing fine features, the choice and optimization of support materials, and the structural and biological performance of the resulting scaffolds. By comparing these approaches, we aim to identify practical design considerations and highlight promising directions for the advancement of engineered tissue constructs with integrated vascular networks.

5.2 Construction of Vascular Structures Through Casting

In our bioreactor, presented in Chapter 4 we employed a casting technique to construct simple channels in our hydrogel environments. To extend this to more complex vascular-like structures we employed a sacrificial casting technique utilizing the thermoreversible properties of Pluronic F-127. This approach allowed us to print vascular-like structures within GelMA matrices, as schematically illustrated in Figure 5.1a.

The process involved printing Pluronic F-127 using a commercial 3D bioprinter to define the desired channel geometry. A 3D-printed PLA frame was then affixed around the printed template. Subsequently, GelMA, with or without embedded mammalian cells, was cast over the Pluronic template and crosslinked under 405 nm UV light. Cooling the constructs to 4 °C facilitated the liquefaction and removal of the Pluronic template, leaving behind hollow channels suitable for perfusion. The PLA frame included inlet and outlet fittings compatible with catheters, enabling connection to a syringe for fluid introduction.

Perfusion tests demonstrated that the channels remained intact after the removal of the Pluronic ink (Figure 5.1c) and introducing fluorescent solutions through the channel network allowed visualization of flow and diffusion processes within the hydrogel matrix (Figure 5.1d). These experiments provided insights into the stability of the constructs and the diffusion characteristics of small molecules.

An uneven network structure was utilized to assess the efficacy of channel designs in delivering nutrients to specific regions. As shown in Figure 5.1d, fluorescein distributed homogeneously in the lower half of the construct after approximately 15 min, while the upper half exhibited areas lacking fluorescence signal. It took around 30 min for the entire construct to achieve approximately homogeneous fluorescence distribution. When examining the diffusion more closely (see Figure 5.1e

and f), it becomes evident that the single channel in the channel-sparse region of the construct was perfused less than the two channels located in the channel-dense region. Consequently, the overall intensity in the channel-sparse area is lower compared to that in the channel-dense region. Additionally, intensity artifacts appear at the borders of the channels, likely caused by light scattering at the interface between the liquid and the hydrogel.

This method was successfully applied in our earlier work [164], where we embedded vascular-like channels into hydrogels to investigate cell-free systems in a 3D matrix.

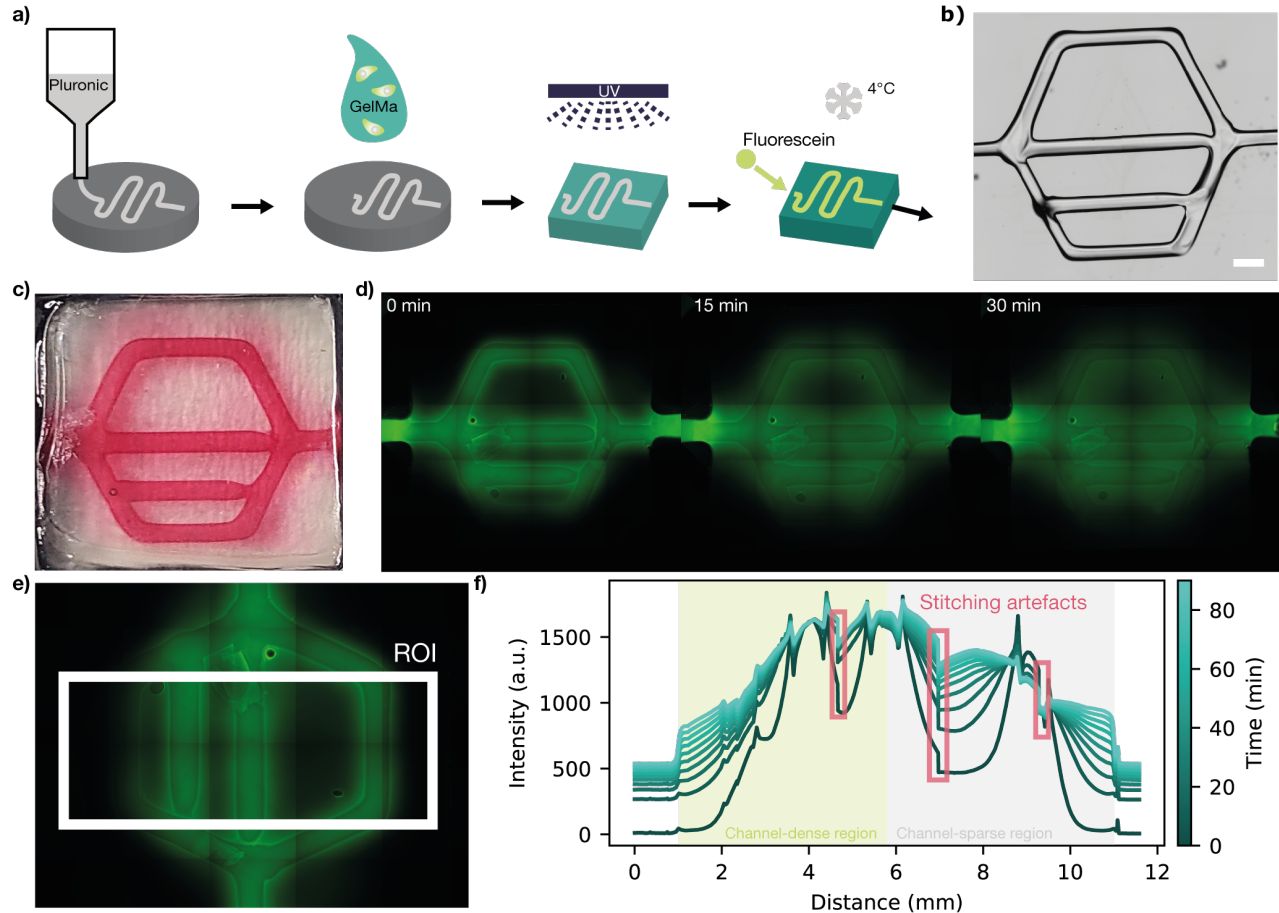


Figure 5.1: Construction of vascular-like structures. a) Schematic representation of the method for preparing vascular structures using a commercial 3D bioprinter. Pluronic F-127 is printed, followed by casting GelMA with or without cells on top. The GelMA is crosslinked using 405 nm UV light for 30 seconds. Subsequently, the construct is cooled to 4 °C for 5 minutes, liquefying the Pluronic to enable channel perfusion with a material of interest. b) Brightfield image of the printed channel before hydrogel application. Scale bar: 1000 μm. c) Photograph of a constructed channel filled with red food dye to visualize perfusion within the GelMA matrix. d) Channels perfused with fluorescein to observe diffusion into the surrounding hydrogel over time. e) Selected ROI for a diffusion analysis over two regions: Channel-dense and channel-sparse. f) Quantitative analysis of fluorescein diffusion.

However, the use of Pluronic F-127 at high concentrations necessary for structural stability poses

challenges for cell compatibility. While Pluronics are widely reported to support biocompatibility at low concentrations, they are also known to inhibit cell adhesion on surfaces, particularly in tissue engineering applications [170]. At elevated concentrations, Pluronics can disrupt the normal architecture of the lipid bilayer and potentially lead to an increased membrane permeability and even cell lysis [171]. In one study, a reduction in the fresh-to-dry cell weight ratio was observed with high concentrations of Pluronic F-68, suggesting osmotic stress [172]. Residual Pluronic in the culture environment, combined with such osmotic imbalances, can impair cell viability, especially when cells are embedded in close proximity to printed structures where diffusion is limited.

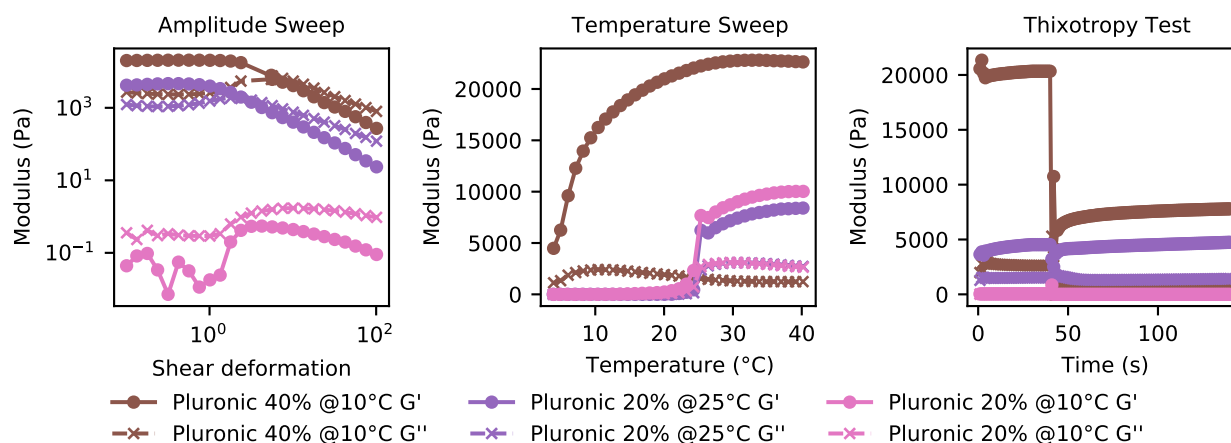


Figure 5.2: Pluronic is printable at 20 % and 40 % at room temperature for ≈ 15 min when stored before at -4°C . An amplitude sweep of Pluronic hydrogels at different concentrations and temperatures are shown. Compared to methylcellulose, the Pluronic curves exhibit a less steep decline in the storage modulus (G') and loss modulus (G'') with increasing strain, indicating a more gradual structural breakdown. Additionally, Pluronic 20 % at 10°C shows significantly lower G' values compared to Pluronic 20 % at room temperature or Pluronic 40 % at 10°C . This suggests a temperature-dependent shift in mechanical strength and reflects the thermoresponsive nature of Pluronic, where gel stiffness varies with both concentration and temperature. Interestingly Pluronic with a concentration of 40 % at 10°C behaves similar as Pluronic with a concentration of 20 % at 25°C . The temperature sweep of Pluronic hydrogels at different concentrations shows that in contrast to methylcellulose, which shows a more linear increase in modulus with temperature, Pluronic exhibits a non-monotonic "hill-shaped" curve. The storage modulus (G') initially increases with rising temperature, reaches a peak, and then declines at higher temperatures. This trend suggests a gelation process followed by structural weakening. Additionally, the difference between storage (G') and loss modulus (G'') is more pronounced compared to methylcellulose, highlighting distinct viscoelastic behavior and phase transitions in Pluronic hydrogels. The thixotropy test shows that Pluronic at 40 % does not recover its structure and viscosity as the 20 % Pluronic does.

To address this, we explored diluting Pluronic concentrations and observed the rheological parameters using a rheometer (Anton Paar), comparing different concentrations (Figure 5.2). Our prints thus far have used Pluronic at a concentration of 40 % at 10°C , allowing printing within 15 min

until it transitions into a gel state that is no longer printable. This formulation behaves similarly to Pluronic at a concentration of 20 % at 25 °C, as evidenced by their comparable stress and shear moduli. Dilution offers a broader timeframe but less stability; printing at 20 % concentration directly at room temperature provides similar moduli without the 15 min limitation imposed by the 40 % formulation heating up to room temperature. The temperature sweep indicates that the curve for 40 % is smooth, whereas the curve for the 20 % formulation shows an abrupt change at approximately 25 °C. It remains uncertain whether we are above or below this critical point when printing at room temperature. Excessive dilution compromises structural stability, rendering the formulation too liquid to be printable.

This limitation restricts the direct inclusion of cells during the casting process due to the toxicity of high concentrations and underscores the need for alternative approaches or protective strategies to enable cell-compatible vascular integration. Washing the fabricated channels and applying coatings could improve cell compatibility, as demonstrated by Zhou et al. [76].

Given the adverse effects of this approach on cell viability and our goal to transition from a quasi-3D to a true 3D method not limited by the casting technique, alternative strategies are warranted.

5.3 Construction of Vascular Structures With Dual Extrusion

As the casting approach limits us to designing and constructing structures in a more 2D than 3D manner, we investigated dual extrusion. In this method, two extruders are used simultaneously: One filled with bioink to construct the channel structures, and the other filled with the material for the surrounding matrix (i.e., the support structure).

3D filament printers with a stepping motor typically exhibit higher precision compared to pneumatic-controlled bioprinters. To evaluate our pneumatic driven bioprinter, we compared the structures with PLA prints fabricated with another commercial printer (Bambulab X1E), which uses a stepping motor to drive filament extrusion (cf. Figure 5.3a).

To investigate the resolution, we chose PVA as a support material because it can be removed in water, allowing the fine structures to become visible once the support is gone. The larger features had diameters of approximately 4 mm, while the finer structures reached down to 0.5 mm. However, after removal of the PVA, it became evident that only structures with diameters of 1 mm or more remained stable without additional support.

Relatively coarse structures ($d \approx 2 - 4$ mm) are well printable with the bioprinter. Figure 5.3b and c show images of a heart vascular structure [173] printed with a test bioink. The sidearms are well printed, but the printer's resolution is insufficient to print hollow channels. When attempting to

print finer structures ($< 2\text{ mm}$), such as kidney vasculature [174], shown in Figure 5.3d and e, under- or over-extrusion become a challenge. In the case of under-extrusion, many defects are visible, with parts of the channel structures missing. In the case of over-extrusion, too much material is extruded, causing the channels to become larger than intended.

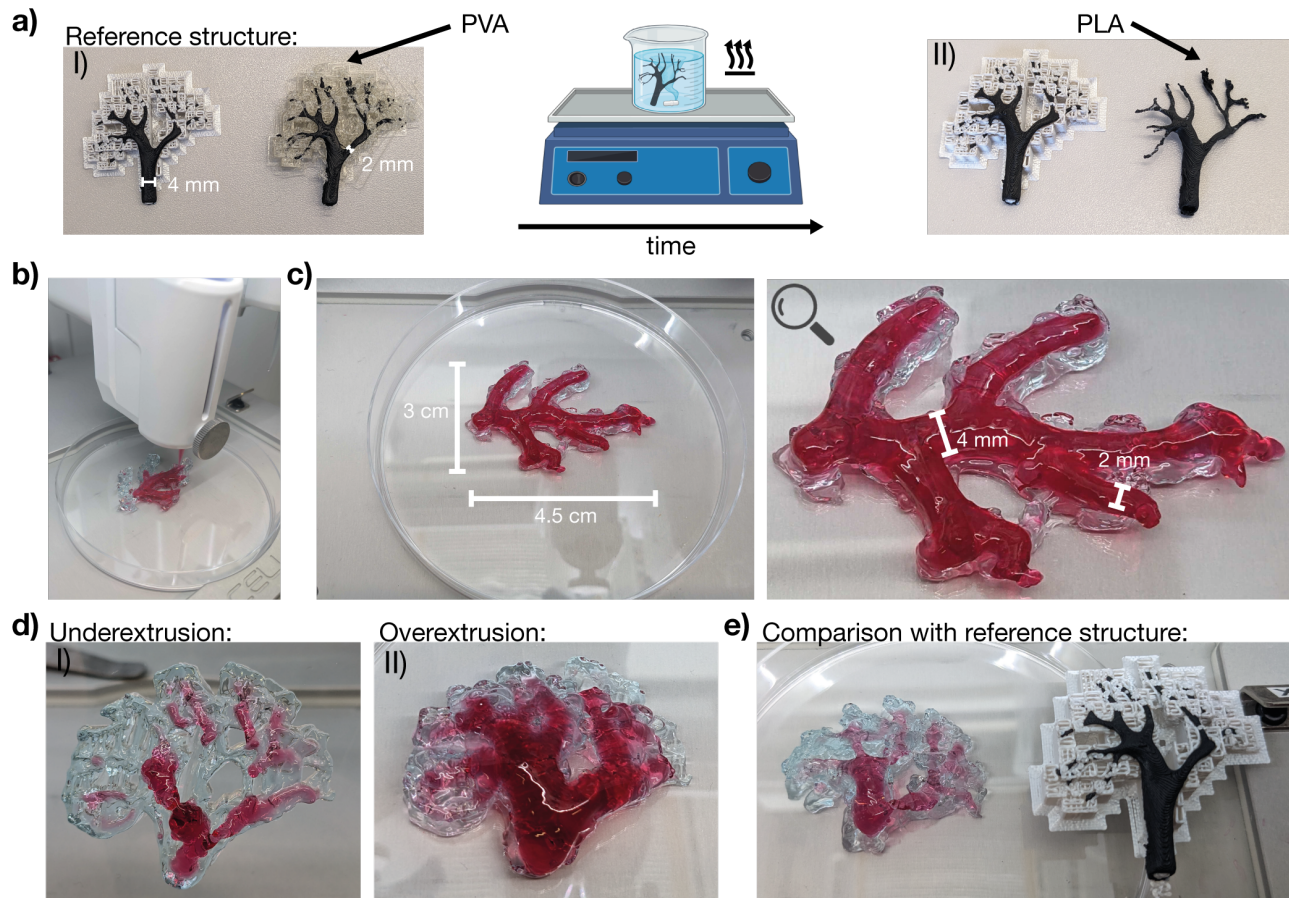


Figure 5.3: Dual extrusion in 3D printing used for the construction of vascular structures. a) Kidney vascular structures [174] were printed with a commercial 3D filament printer (Bambulab, X1E) with PLA in black. On the left the support structure was printed with PLA in white and on the right the support was printed with PVA. Image I) shows the structures before incubation in water and II) after. The PVA dissolves in water over time. b) A coarser structure of heart vasculature [173] was used as a test print for the bioprinter (Cellink, BIOX2). A bioink (Cellink, Cellink Start) was stained with red food color for better contrast. The support was printed with unstained bioink. c) Zoom of the final construct. d) For finer structures it is more difficult to find the best pressure. Image I) shows under extrusion and II) over extrusion of the stained bioink. e) Final result with optimized printing parameters and comparison of the same vascular structure with the print produced by the filament printer. Figures were partly created in BioRender. Jäkel, A. (2025) <https://BioRender.com/mk677sq>.

The pressure needs to be precisely adjusted to match the print speed so that the optimal amount of material is extruded. When comparing this structure with the print from the thermoplastic printer

(Figure 5.3e), we can see that the resolution is not as high as that of the thermoplastic printer. This demonstrates that there is still significant room for improvement in bioprinter technology.

Bioprinting is especially complicated due to the use of hydrogels, as they exhibit shear thinning behavior, meaning their viscosity decreases under shear stress. This characteristic makes the extrusion process more challenging, as the material's flow becomes more difficult to control. Unlike traditional 3D printing materials like PLA, which maintain relatively consistent viscosity during extrusion, hydrogels require precise regulation of both extrusion pressure and temperature. The dynamic nature of hydrogels, combined with the need for real-time adjustments to maintain structural integrity, adds an extra layer of complexity, making bioprinting less predictable compared to classical fused filament fabrication 3D printers.

5.4 Construction of Vascular Structures Using FRESH Printing

As the casting approach with Pluronic lacks in biocompatibility and the dual extrusion lacks in resolution we investigated another approach called FRESH printing which was invented by the *Regenerative Biomaterials & Therapeutics Group* led by Adam Feinberg.

FRESH is an advanced 3D bioprinting technique designed to fabricate intricate biological structures using soft, cell-laden hydrogels. As traditional bioprinting methods often struggle with the structural collapse of delicate materials during printing, in FRESH bioprinting bioinks are extruded into a supportive, thermoreversible gelatin-based support bath. This bath holds the soft constructs in place, maintaining their shape until they are stabilized through crosslinking. Once printing is complete, gentle warming liquefies the support bath, allowing for its removal without damaging the printed structure. This approach enables the creation of complex, high-resolution tissue constructs that closely mimic native biological architectures.

The results presented in this section were gathered during a research stay at this lab under the supervision of Jacqueline Bliley.

5.4.1 Vascular Structures in Collagen

We investigated printing vascular structures in collagen. Figure 5.4a shows a schematic of a hydrogel block containing a vascular structure. It has one inlet and one outlet, with the sidearms extending to the sides of the hydrogel block, allowing for potential tissue supply.

The construct was printed in a support bath and had a length of approximately 2 cm (cf. Figure 5.4c). After removing the print from the slurry, the fine resolution of the printer becomes evident. A commercial printer, originally developed for thermoplastic extrusion, was modified with a different

extruder to regulate hydrogel extrusion using a stepping motor. The prints (cf. Figure 5.4d) show much higher resolution compared to the bioprinter with pneumatic control that we used in for the prints in the previous section. Features as small as 0.6 mm in diameter can be printed, and the grid structure of the ECM mimicking hydrogel is clearly visible, indicating that a linewidth of 150 μm can be resolved.

This combination of using a precision-driven stepping motor for hydrogel extrusion, along with the support bath, is crucial for maintaining the fine resolution required for bioprinting applications. The stepping motor ensures that the material flow rate is accurately regulated, while the support bath helps maintain the structural integrity of delicate hydrogel features. Achieving the desired resolution without material deformation depends on the careful balance between extrusion speed and the bath's viscosity.

To demonstrate the perfusability of the vascular structure, the released print was flushed with 1xPBS stained with red food coloring. Over time, diffusion into the side channels was observed, and on longer timescales, diffusion into the collagen material itself became visible.

This observed perfusability demonstrates the potential for creating functional tissue models. The channels can simulate nutrient and oxygen delivery within a tissue engineering setup. This perfusability is also critical for supporting the growth of endothelial cells *in vitro*, which is a key feature for developing vascularized tissue constructs in regenerative medicine applications.

5.4.2 FRESH Printing of GelMA

Since collagen is not the best choice for some cell types, we wanted to test if these structures could also be printed using GelMA. We chose a vascular structure to be printed in a slurry support bath, followed by crosslinking with UV light after printing. Figure 5.5a shows the print with collagen, providing a comparison, while Figure 5.5b displays the released GelMA print after crosslinking with UV light and removal from the support bath. Figures 5.5c and 5.5d show a zoomed-in view and an image from a different angle. The next step will be to print the structure, as shown in the sketch in Figure 5.4a, using GelMA.

5.5 Discussion and Conclusion

The biofabrication of vascular-like structures remains a key challenge in the development of functional microphysiological systems, particularly when aiming to sustain larger constructs that require efficient nutrient and waste exchange. In this chapter, different strategies for generating perfusable channels within hydrogel matrices were explored, with a focus on material compatibility, structural integrity, and fabrication precision.

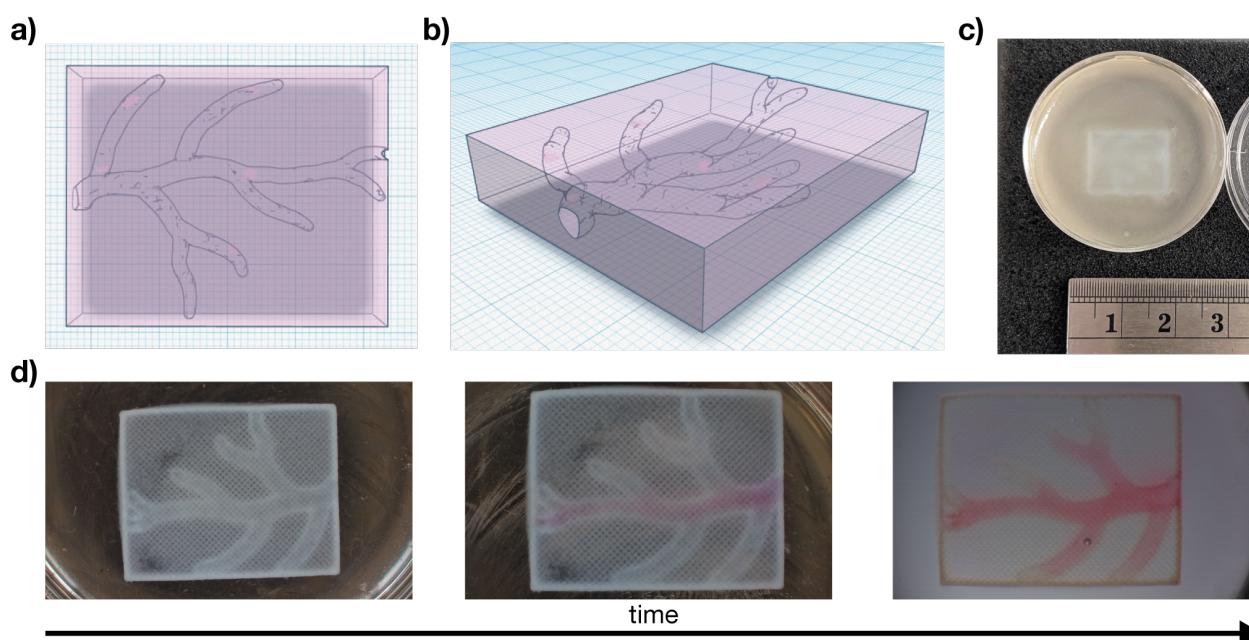


Figure 5.4: Fabrication of vascular structures using FRESH printing. a) Schematic of a vascular structure in a hydrogel block shown from the top. The negative of the vascular heart structure [173] was used. b) Same schematic shown from a different angle. c) Image of the collagen print still embedded in slurry, Ruler units are in centimeters. d) Microscope image of the construct after being released from the slurry. e) A stained 1xPBS solution (red food color) was applied to the right side of the vascular network. f) The stained solution diffusion over time in the side channels, and from there, in the surrounding tissue.

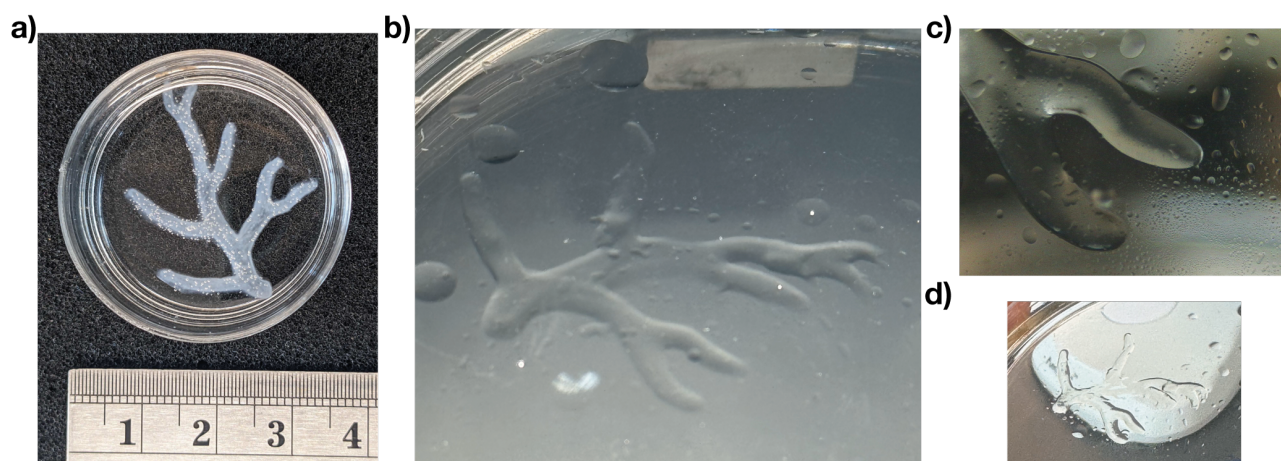


Figure 5.5: a) Collagen print of the vascular network [173]. Collagen shows higher shape fidelity compared to GelMA. b) - d) Show the same print consisting of GelMA. The print was conducted together with Caner Distol and images were captured by Caner Distol.

Sacrificial casting using Pluronic F-127 enabled the formation of well-defined channels but revealed a critical limitation: the high Pluronic concentrations required for structural stability are not compatible with cell viability. This restricts the use of this method to acellular casting or necessitates additional steps to remove residual Pluronic before cell seeding, which adds additional complexity to the workflow.

In contrast, materials such as GelMA and collagen demonstrated favorable biocompatibility and sufficient mechanical stability for supporting embedded cells and maintaining printed structures. However, the choice between these materials should be guided by the specific requirements of the target cell type, as different cells exhibit varying sensitivity to stiffness, degradation rates, and biochemical cues.

To address the challenge of printing complex vascular geometries, the FRESH printing approach was evaluated. The support bath offered by FRESH significantly improved the printing resolution and fidelity of fine structures, making it a promising technique for generating intricate vascular networks in soft, cell-laden bioinks.

In summary, while no single strategy fulfills all criteria for vascular integration in biofabricated constructs, this work highlights key trade-offs between structural precision and biological compatibility. These insights contribute to the development of customizable approaches for integrating vascular structures into 3D-printed microphysiological systems and underscore the importance of material selection and process optimization tailored to the desired application.

5.6 Materials and Methods

5.6.1 GelMA Casting with Sacrificial Pluronic Used to Fabricate Channel Structures

To fabricate vasculature-like structures, a 40% (w/v) Pluronic F-127 solution was prepared by dissolving Pluronic powder (Sigma-Aldrich) in double-distilled water at 4°C overnight. Periodic vortexing was applied to ensure complete dissolution. This temperature-responsive bioink served as a sacrificial support material, facilitating the creation of vascular channels within the GelMa matrix.

A BIOX 3D bioprinter (Cellink) equipped with a 200 µm nozzle was used to print the vascular structures. The Pluronic solution, stored at 4°C, was printed at room temperature, to maintain its optimal viscosity. To prevent premature gelation, printing was performed within 15 minutes after removal of the solution from cold storage. If the solution warmed beyond a critical threshold, re-cooling was necessary before continuing the process. Printing parameters were optimized, including a pressure of 100 kPa, a printing speed of 5 mm/s, and a preflow adjustment of −50 ms to ensure controlled extrusion.

The bioprinting workflow for generating vascular-like channels is illustrated in Figure 5.1a. First, Pluronic F-127 (40 % w/v) was printed as a sacrificial ink to define the channel structure within GelMA. The construct was overlayed with GelMA, and the hydrogel was crosslinked using 405 nm UV light for 30 seconds. Following crosslinking, the construct was cooled to 4 °C for 5 minutes, liquefying the Pluronic and enabling its removal, thereby creating perfusable channels.

5.6.2 Dual Bioprinting

Dual extrusion experiments were performed using a BIO X2 bioprinter (Cellink) equipped with two pneumatic extrusion heads. To test system performance and printability, both printheads were loaded with the standard bioink Cellink Start provided by the manufacturer. To distinguish between the two materials during printing, one cartridge was supplemented with red food dye.

G-codes were generated using the manufacturer's slicing software and adapted to allow sequential deposition from both printheads. Printing was performed with 22 G nozzles at room temperature. Pneumatic pressure (20-40 kPa) and print speed (5-10 mm/s) were manually adjusted to match extrusion rates between the two materials and avoid under- or over-extrusion. All constructs were printed onto a cooled printbed set to 10 °C to support structure formation.

For resolution benchmarking, identical designs were printed using PLA filament on an fused filament fabrications (FFF) printer (X1E, Bambu Lab) and with PVA support structures where needed. These served as reference models for structural fidelity and feature resolution.

5.6.3 FFF Printing With PLA and PVA

3D-printed structures were fabricated using a FFF printer (X1E, Bambu Lab). PLA (Prusament PLA) and PVA (Bambu Lab PVA) filaments were used to create the main structures and sacrificial supports, respectively. Printing was performed on a textured PEI build plate with a 0.4 mm nozzle and a standard layer height of 0.2 mm. All prints were sliced using Bambu Studio software with default profiles for the respective filaments, unless otherwise specified.

PVA was chosen for sacrificial support structures, as the fine geometry of the PLA prints made mechanical removal of PLA supports difficult. To dissolve the PVA, the entire construct was submerged in tap water heated to 40 °C and stirred for approximately 4 h.

5.6.4 FRESH Bioprinting

The protocol for FRESH printing was followed with small modifications according to reference [175]. A MakerGear 3D printer modified to enable bioprinting according to this reference [69] was used (Figure 5.6c).

3D models were sliced using PrusaSlicer with modified parameters suitable for hydrogel printing. Printing was performed using a 30 G (150 μm) needle, with the extrusion width set to the needle diameter. The extrusion multiplier was adjusted depending on the bioink; for collagen, a value of 0.9 was used. Retraction distance was set to 0.15 mm, and layer height to 0.06 mm (40 % of the needle diameter). The filament diameter in the slicer was set to the internal diameter of the syringe (7.28 mm, Hamilton syringe). Printing speed was set to 10 mm/s, and travel speed to 20 mm/s. The printer was homed prior to each run.

The printed bioink consisted of collagen Type I (Cellink, LifeInk 200) mixed with 0.24 mM acetic acid. To enable gelation of the bioink during the printing process, the support slurry was prepared with 70 mM HEPES buffer to gradually neutralize the collagen during extrusion. In case of dual printing the bioink was stained with blue food color to visualize different printing regions (Figure 5.6f).

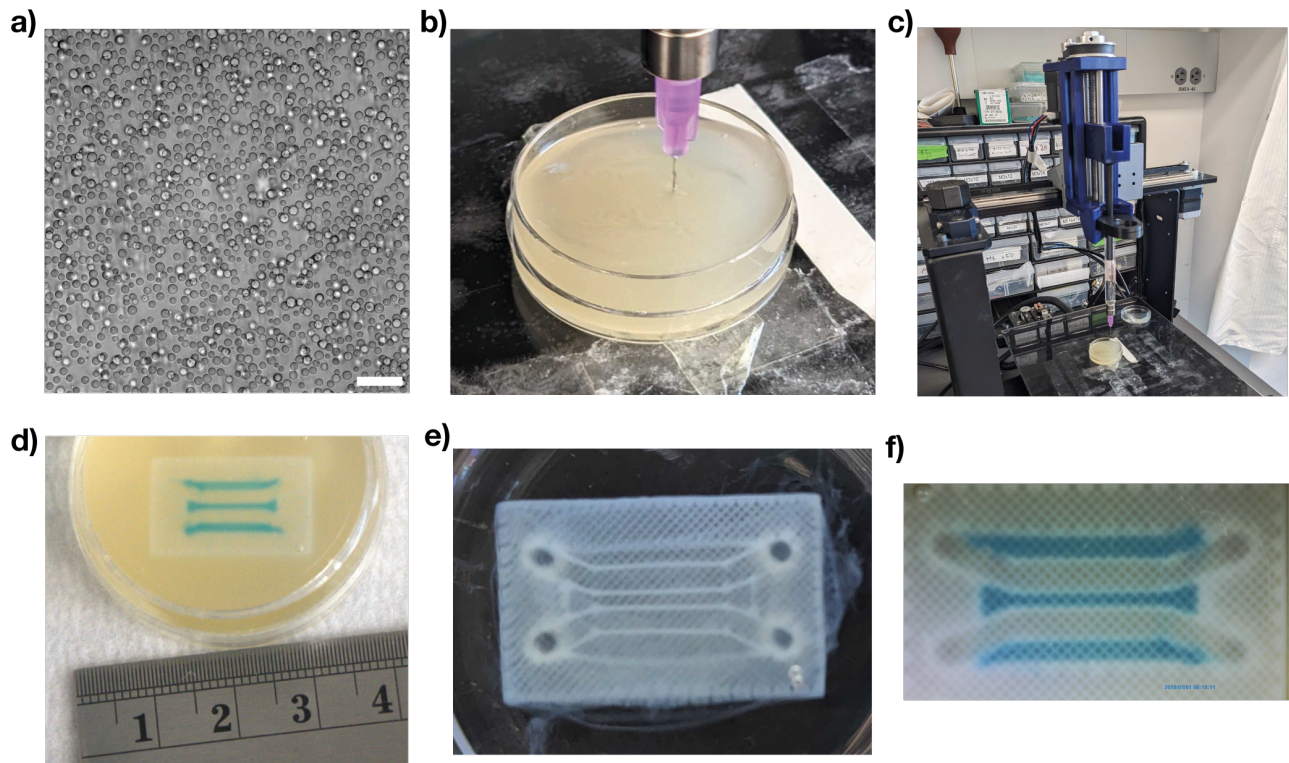


Figure 5.6: FRESH printing. a) Microscopy image of the slurry used as support bath for FRESH printing. Scale bar: 100 μm . b) A petridish is filled with slurry, in which is printed with a needle tip. c) For printing, a commercial filament printer is used which is equipped with custom made extruders according to reference [69]. d) Collagen print that was produced with dual extrusion. To highlight the parts where cells would be deposited a collagen ink stained with blue food color was used. Design was reproduced from reference [19] c) Microscopy image of a similar print where all parts were printed with unstained collagen. d) Microscopy image of the dual extrusion print shows the mesh structure.

A support bath for FRESH bioprinting was prepared by dissolving gelatin Type B (3%), gum arabic (0.3%), and Pluronic F-127 (0.125%) in a 1:1 mixture of ethanol and water pre-heated to 60 °C. All powders were sterilized by UV exposure for 15 minutes prior to mixing. The solution was stirred for 1 minute, adjusted to pH 5.6 using 2 M HCl, and stirred overnight at 540 rpm.

The next day, the slurry was washed with 70 mM HEPES buffer using the following protocol: vortex, centrifuge at 750 rpm for 3 minutes, remove the supernatant, and add double the slurry volume of fresh buffer. This wash step was repeated three times, with foam removed after the second step. The final slurry was transferred to 3 mL syringes, placed in a desiccator for 15 minutes to remove air bubbles and centrifuged at 1000 g for 3 minutes. A plunger was then inserted while gently degassing with a wire to maintain compact packing. The prepared slurry (Figure 5.6a) was transferred into a petri dish and used as a support bath for printing (Figure 5.6b).

After printing, constructs were left at room temperature for 30 minutes to allow for gelation of the bioink (Figure 5.6d). They were then incubated at 37 °C for 1 hour to accelerate fibrillogenesis and solidify the bioink, and liquefy the support slurry. The support bath was subsequently removed (Figure 5.6e), and the printed structures were washed six times with 70 mM HEPES buffer. The final washing step included 1% penicillin/streptomycin to prevent bacterial contamination.

6. Conclusion

The precise spatial organization of biological signals and components is essential for building functional tissue-like systems in vitro. Yet replicating features found in natural tissues, such as complex capillary networks, hierarchical organization, mechanical heterogeneity, or dynamic cell–matrix interactions, remain a major challenge. Challenges like the establishment of complex gradients, the control and design of feedback loops in genetic networks, and the development of vascularization strategies still need to be overcome.

In particular, the controlled formation of patterns within 3D environments and the integration of dynamic biological elements, as genetic circuits or living cells, demand new strategies that bridge molecular programming and material design.

Current approaches in synthetic biology and tissue engineering have made progress in generating spatial patterns using morphogen gradients and synthetic gene networks, mostly in 2D or well-controlled microenvironments [5], [31], [95], [125], [176]. Similarly, 2D cell cultures are still the most prominent way of doing research [177]. But 3D cell culture has, in recent years, shown the potential to completely change how new drugs and treatments could be tested [178], how diseases could be modeled, stem cells utilized, and how organs might eventually be transplanted [179].

Bioprinting has advanced the field of tissue engineering: Functional materials [180] and approaches to position individual cells in complex 2D and 3D patterns [181] have been developed. Tissues have also been fabricated for medical applications, such as bone constructs [182] and models of aortic valves [183].

These constructs show spatial resolution but often lack dynamic biological function or long-term viability due to limited nutrient exchange and insufficient integration of active cell behavior. For the creation of larger tissues, vascularization strategies are crucial. Various approaches have been investigated in recent years [18], [39], [65], [76], but they are typically optimized for specific challenges and not yet generalized. Significant progress has been made recently in the fabrication of artificial tissues with vascularized structures using FRESH printing [184]. New types of bioreactors supporting cell migration, remodeling, and the formation of capillary-like networks have been developed [19].

In this thesis, new strategies for integrating genetic networks and living organisms into 3D-printed environments, that aim to replicate and study functional aspects of natural tissues, are

presented. Through a combination of synthetic biology and biofabrication techniques, we explored how programmable genetic components and structural design can be harnessed to generate complex, spatially organized biological systems.

First, we demonstrated that synthetic gene circuits can be used to create spatial patterns in response to environmental gradients. By operating under non-equilibrium steady-state conditions in a cell-free context, these circuits enabled robust and tunable pattern formation. To achieve operation under these conditions, a microfluidic chip was employed that allowed for well-controlled mixing and precise inflow and outflow volumes. This work contributes to our understanding of how spatial information can be encoded and processed in synthetic systems, laying the groundwork for controlled morphogenesis in engineered tissues. Next, we established a platform for the long-term immobilization and monitoring of bacteria within synthetic biofilms. Using FISH, we were able to observe bacterial growth and spatial distribution over time, as well as distinguish between different bacterial species without the need for genetic modification. This enables new possibilities for studying microbial or eukaryotic behavior and interspecies interactions in defined environments. The ability to study bacterial spatial arrangements without genetic engineering provides a valuable tool for microbiological research and biotechnological applications. Building on these findings, we investigated methods to embed mammalian cells within hydrogel-based constructs, focusing on both cell viability and functional manipulation. We evaluated DNA and RNA transfection strategies in 3D, demonstrating that genetic modification of embedded cells is feasible. In particular, prime editing revealed that transfection with mRNA encoding the prime editor and pegRNA induced permanent genomic modifications in targeted cells. This approach facilitates localized control of gene expression in complex tissues, which is critical for regenerative medicine and tissue engineering applications. Finally, we addressed a key limitation in tissue engineering: the lack of vascularization in larger constructs. We developed methods for integrating perfusable vascular structures into the hydrogel matrix, supporting nutrient exchange and enhancing long-term cell survival. The successful incorporation of vasculature not only improves functional outcomes but also enables the fabrication of larger and more physiologically relevant tissue models. Together, these contributions advance the integration of synthetic biology and tissue engineering, demonstrating how programmable biological functions can be embedded into 3D-printed systems to create living materials with increasing complexity and utility.

These tissue models offer a platform to study fundamental biological principles in a more realistic setting. With more complex and layered environments, it becomes possible to investigate specific regulatory mechanisms, test different network topologies, and analyze how cells interact with each other and their surrounding ECM. Processes like diffusion, communication, and proliferation can be studied in a spatially resolved and dynamic manner.

As a next step, integrating engineered logic circuits into cells could pave the way for true 4D

bioprinting. While time is typically considered the fourth dimension, most current approaches focus only on material properties that change post-printing[185]. However, as we've shown that genetic manipulation of embedded cells is possible, it would be exciting to push this further: Toward systems where cellular behavior, such as differentiation, unfolds over time in a controlled way. This could bring us closer to printing entire functional organs. Growth factors have already been included in bioprinted constructs, but their precise spatial and temporal delivery through vascular networks remains a promising area for development.

In the long term, creating smart hybrid materials that respond to their environment and self-organize based on genetic programming could lead to major advances in therapeutics and biomaterials. Especially personalized medicine could improve through the use of patient-derived iPSCs [186] and in particular, 3D bioprinting of organs could help address the shortage of donor organs and reduce the risk of immune rejection.

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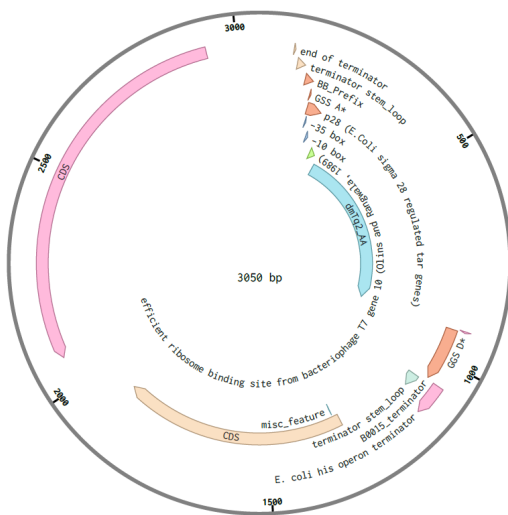
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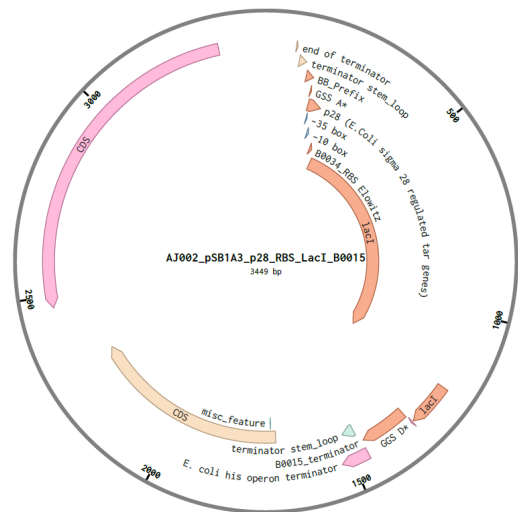
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Appendix

A1 Plasmid Maps Used for the Genetic Circuit

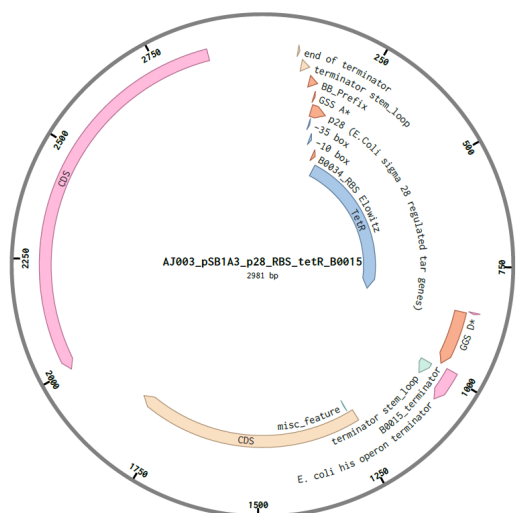


(a) Plasmid map of the sequence for the fluorescent protein dmTurquoise2 expressed under a p28 promoter.

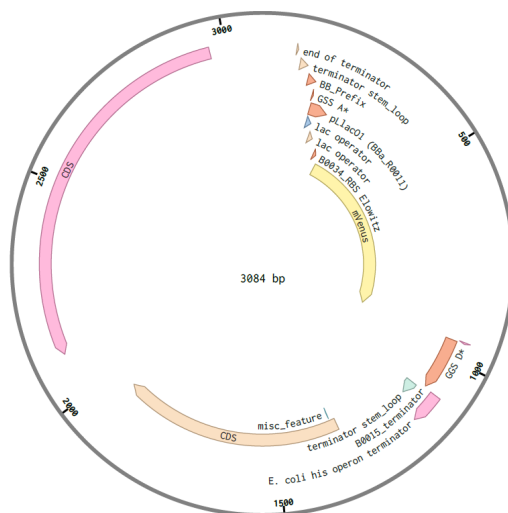


(b) Plasmid map of the sequence for the transcription factor LacI expressed under a p28 promoter.

Figure A.1: Plasmids used to engineer Node 1 of the I-FFL used in chapter 2.

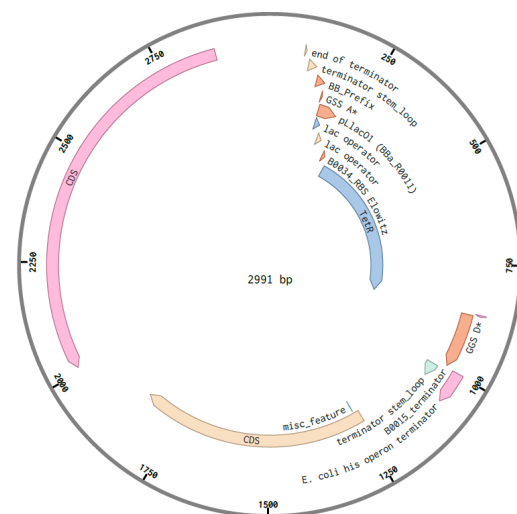


(a) Plasmid map of the sequence for the transcription factor TetR expressed under a p28 promoter.

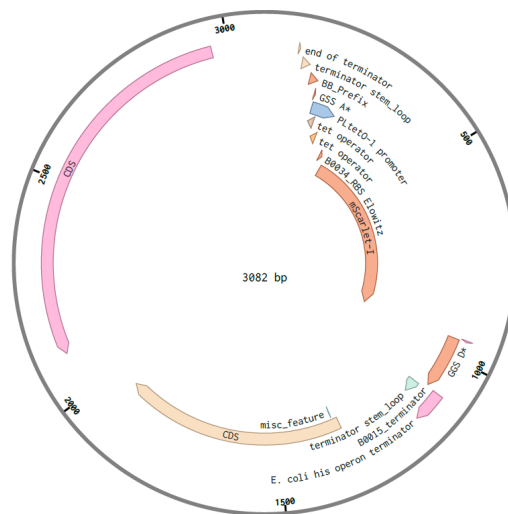


(b) Plasmid map of the sequence for the fluorescent protein mVenus expressed under a pLac promoter.

Figure A.2: Plasmids used to engineer Node 1 and 2 of the I-FFL used in chapter 2.



(a) Plasmid map of the sequence for the transcription factor LacI expressed under a pLac promoter.



(b) Plasmid map of the sequence for the fluorescent protein mScarlet expressed under a pTet promoter.

Figure A.3: Plasmids used to engineer Node 2 and 3 of the I-FFL used in chapter 2.

A2 Plasmids Maps Used for the Genetic Manipulation of Mammalian Cells

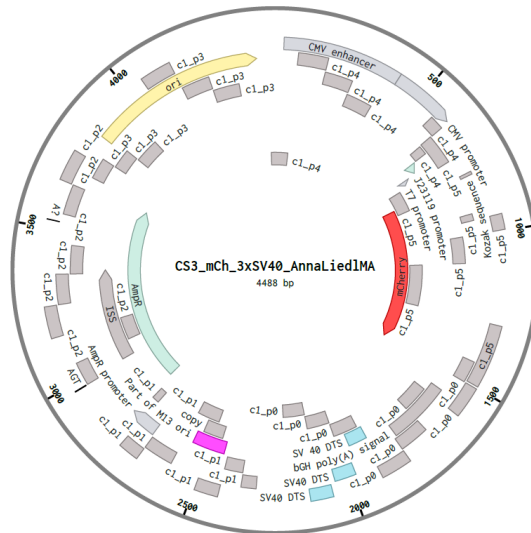
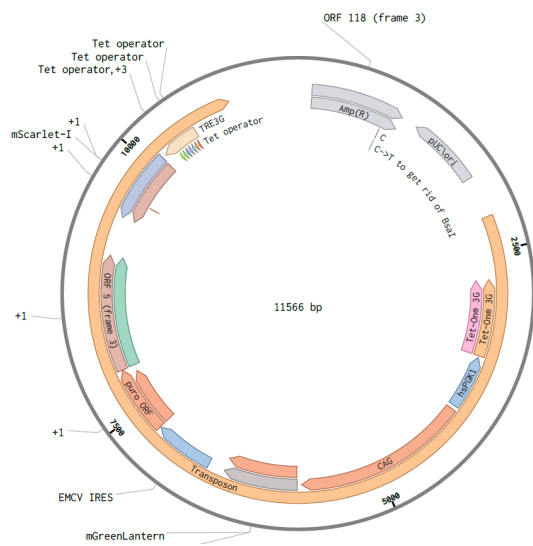
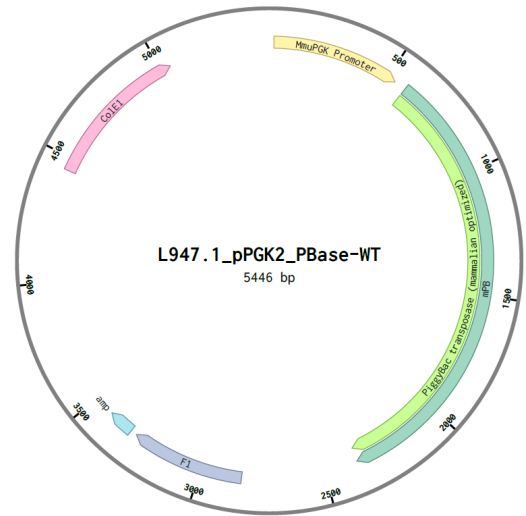


Figure A.4: Plasmid map of the sequence for the fluorescent protein mCherry. The plasmid was a kind gift from the Dietz lab engineered by Anna Liedl, already published in this reference [165].

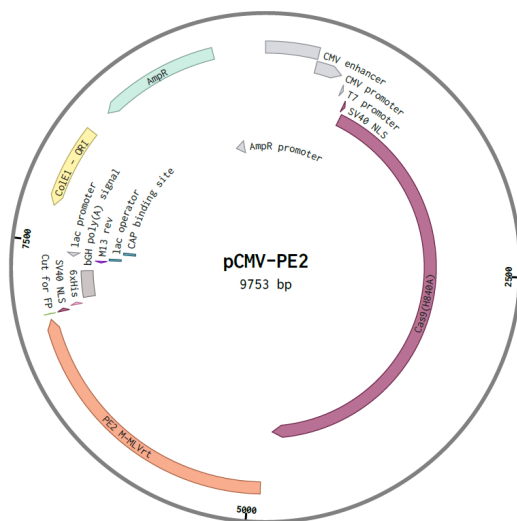


(a) Plasmid map of the sequence used to engineer a stably cell line that expresses the fluorescent protein mGreen Lantern by default and mScarlet upon doxycycline induction.

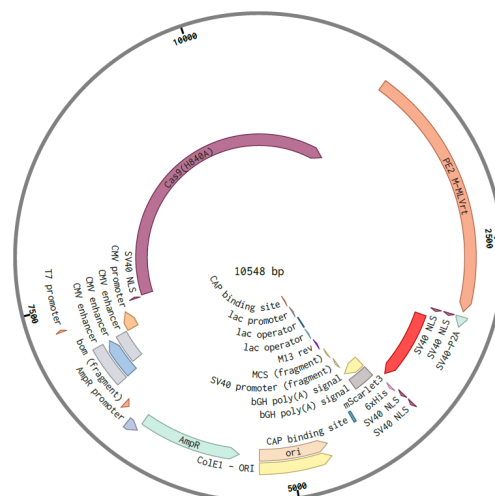


(b) Plasmid map of the sequence for the transposase to engineer a stably cell line with piggy bac that expresses the fluorescent protein mGreen Lantern by default and mScarlet upon doxycycline induction.

Figure A.5: Plasmid maps to engineer a cell line that is inducible with doxycycline. The plasmids were a kind gift from the Westmeyer lab engineered by Dong-Jiunn Jeffery Truong and Julian Geilenkeuser, already published in this reference [38].

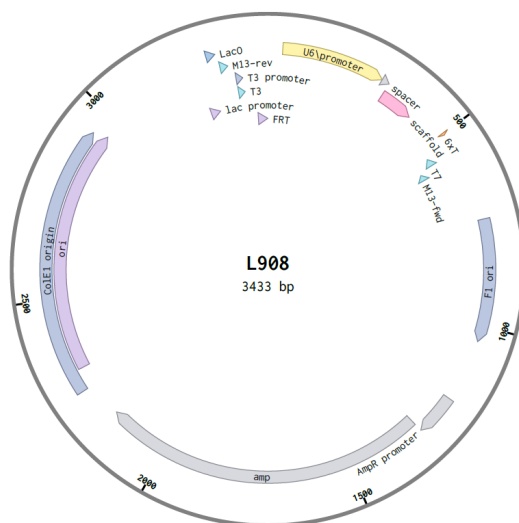


(a) Plasmid map of the prime editor sequence used to modify cells of an engineered cell line, that expresses by default a fluorescent protein BFP and can be edited to express GFP instead.

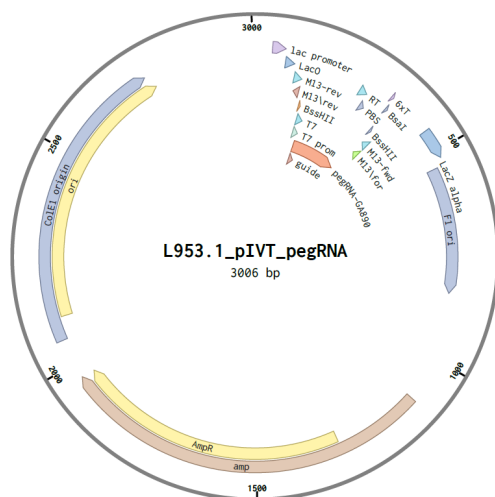


(b) Plasmid map of the prime editor sequence used to modify cells of an engineered cell line, that expresses by default a fluorescent protein BFP and can be edited to express GFP instead. The expression of the prime editor can be tracked as mScarlet is additionally expressed with using this sequence.

Figure A.6: Plasmid maps of the prime editors. The plasmids were a kind gift from the Rädler lab already published in these references [38], [168].



(a) Sequence of the pegRNA used for prime editing with the sequences of A.6.



(b) Plasmid map of the sequence for the pegRNA used to produce mRNA of it with an IVT reaction.

Figure A.7: Plasmid map of the pegRNA used for prime editing. The plasmids were a kind gift from the Westmeyer lab engineered by Dong-Jiunn Jeffery Truong and Julian Geilenkeuser, already published in this reference [38].

(a) Plasmid map of the prime editor sequence A.6 a) used to produce mRNA of the prime editor with an IVT reaction.

(b) Plasmid map of the prime editor sequence A.6 b) used to produce mRNA of the prime editor with an IVT reaction.

Figure A.8: Plasmid maps used to produce mRNA of the prime editors with an IVT reaction. The plasmids were a kind gift from the Westmeyer lab engineered by Dong-Jiunn Jeffery Truong and Julian Geilenkeuser, already published in this reference [38].

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