Building a Tissue Segment by Segment

Pushing the frontier of synthetic developmental biology

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Abstract

The novel field of synthetic development arose from synthetic biology and developmental biology. The field aims to gain control over the developmental process and gain a general understanding of development while simultaneously creating tools with potential therapeutic uses. In the past decade, most research in the field has focused on simple isolated gene regulatory networks, which were rarely linked to morphogenesis. While there is ample room for researchers to explore these simple regulatory networks further, in this review, I want to push the frontier of synthetic development by proposing the pursuit of building a segmented elongated tissue. This would be the largest and most complex structure built using the principles of synthetic development. By exploring this pursuit, we can create a better perspective on what tools we need to develop and which ones we already have to create more complex tissues, in general. In particular, I will discuss module construction, linking modules, and computational tools. Overall, the ability to engineer complex tissues would open up the true potential of synthetic development.

Layman's Summary

Being able to control development has been an age-old desire of humankind. We are still far from controlling development on an organism scale, nor should we want to create a whole organism for ethical reasons. However, if we could control development to create designer tissues and organs, there would be a host of exciting applications for this newfound ability. Examples of initial use cases for these synthetic tissues include *in vitro* meat for consumption, improving life support machines, or synthetic organs for biotechnology. As of now, we are starting to gain control of development on the sub-tissue scale. Synthetic development is one of the key contributing fields to gaining control in designing tissues. This field combines advances in synthetic biology, a field that aims to make biology easier to engineer, and developmental biology, which tries to understand how bodies are formed.

Most scientists in synthetic development focus on small-scale pattern formation and very basic morphogenesis (the shaping of tissues). However, if we want to create larger tissues, we need to start making them. This is easier said than done. This review highlights some of the tools we will need to develop so designer tissues can become a reality. I divide the overall goal of creating designer tissues into three subgoals. The first subgoal is to create more modules with specific developmental behavior, which we can control. The second subgoal is that we need to start combining these modules of development to create more complex behavior. The third subgoal is that we need to improve the computational tools for designing complex tissues.

Furthermore, in this review, I propose that we construct an elongated segmented tissue as one of the first designer tissues as this would allow us to address all three of these subgoals. In investigating how we might construct such a tissue, I find that many modules required for tissue elongation are lacking in our toolbox. Especially, the lack of control over extracellular matrix deposition will need to be addressed soon. Additionally, I note that many insights have come from computational studies. However, these studies generally focussed on explaining a natural phenomenon and did not aim to produce a tool for designing tissues. Such a computational tool would be particularly interesting for *de novo* tissue designs.

Introduction

Synthetic development is at the interface between developmental and synthetic biology (Davies, 2017). Developmental biology is the study of how multicellular bodies are formed, which, historically, has been mainly studied in a top-down fashion. Here, the necessary components are mapped by removing parts of developmental systems. For example, experiments in Drosophila, where the function of a specific gene was disturbed, have led to the identification of many important developmental genes (Roberts, 2006). Importantly, however, these kinds of studies cannot answer whether the found components are sufficient drivers of the observed phenomenon. In contrast, when assembling a system from a set of parts (**Box 1**) or principles, one can usually identify if something is missing. This approach to test sufficiency by building something from the ground up is called bottom-up, and this is where synthetic biologists come into the picture (Bashor & Collins, 2018).

Generally, synthetic developmental biologists use living mammalian or bacterial cells as a "chassis" to carry out instructions written in the form of synthetic gene regulatory networks (GRNs, **Box 1**). Most synthetic development studies' end goal is to capture a natural pattern formation or morphogenesis behavior in a simple module (**Box 1**). Some studies try to prove the sufficiency of these simple modules to drive a developmental process (Davies, 2017). Testing for sufficiency in this way is often seen as complementary to computational approaches, which are generally faster and cheaper. However, the synthetic biology approach requires more close consideration of biological and physical practicalities, such as what kind of molecules can enable a certain diffusion speed. This testing for sufficiency is one of the two primary ways synthetic development can impact science and society.

Box 1: Levels of Complexity in Synthetic Developmental Biology.
Synthetic biology aims to make biology easier to engineer by using a modular design approach to build-up complexity gradually. Described below are the three conceptual levels of complexity used in synthetic development.

Part | A small section of DNA with a well-defined function is considered to be a Part. Examples include an open reading frame that codes for a protein or the binding site of a transcription factor (Ellis et al., 2011).

Gene Regulatory Network (GRN) | By combining the well-defined modular parts, one can create artificial gene regulatory networks. These networks can transform one or more inputs into a specific output or function autonomously (Kærn et al., 2003). An example of an autonomous GRN is the repressilator (**Fig. 2B**).

Synthetic Development Module | A well-defined GRN that can drive a specific pattern formation or morphogenic behavior is called a synthetic developmental module, or module, in this review. These modules could be used to create more complex behavior by sequentially linking them. Ideally, each module is under the control of a single master regulator. Not entirely unlike the master regulator *eyeless*, which can drive the formation of an eye wherever expressed in Drosophila (Halder et al., 1995).

The second, and arguably the more impactful, way synthetic development could contribute is by creating novel tools with medical or biotechnological applications. More specifically, the GRNs for pattern formation and morphogenesis could mimic natural tissues or create *de novo* tissue designs with potential applications in *in vitro* meat production or life-support machines. This does not have to stop there. Imagine biotechnological processes that run more efficiently in a de novo designed organ instead of a typical bioreactor. As it is very early days in the field, the gap between our current ability to dictate simple pattern formation and actually designing structured tissues or even organs is so large that those applications seem more like fiction than a possible future reality. This gap needs to be bridged to truly envision the potential of synthetic developmental approaches for tissue engineering.

To push the frontier of synthetic development towards tissue engineering, we need to explore what it will take to create a more complex tissue using the tools from synthetic development. Three subgoals will help push the frontier. First, the field will need to expand the tool kits of patterning and morphogenesis modules. Second, proof of concept studies that link multiple morphogenesis and patterning steps will need to be done. Third, we need to grow the limited suite of computational tools that specifically aid GRN design for tissue engineering. In this review, I map a road towards creating an elongated sequentially segmented tissue. While doing so, I will touch upon all three of the subgoals.

The choice for a sequential segmented elongated tissue as the foundation for this thought experiment has three main reasons. 1) Some of the synthetic modules needed for the construction are not yet available from the toolbox of synthetic development. 2) Creating the final tissue will require linking multiple patterning and morphogenesis modules under strict spatio-temporal control. 3) Many computational models can instruct initial design. In this paper, I propose a target for synthetic developmental biology to create a sequentially segmented tissue. This would highlight required and desired innovations in the previous paragraph's points and drive the field forward in its mission to engineer complex tissues *de novo*.

Steps Towards a Sequentially Segmented Tissue

The “Chassis”

As is the case for any project that builds something from the ground up, you have to decide where that ground level is. For example, when building a car, one might decide that the most fundamental part is a bolt or sheet of metal, not the iron ore used to construct it. The same goes for the sequentially segmented tissue we aim to construct. For the engineering of multicellular structures, it is reasonable to select the cell as the fundamental central component.

Choosing to construct entire cells from scratch is not viable since fully functioning synthetic cells have yet to be built (Ivanov et al., 2021). Alternatively, one might decide to start from the most minimal cell available. Such minimal cell systems have been constructed from bacterial genomes by removing non-essential genes (Hutchison et al., 2016). However, these minimal cells, and bacterial cells in general, lack important morphogenic components needed to construct our multicellular tissue. Replicating these essential morphogenic components in these cells, if at all possible or practical, is outside the scope of this review. Animal eukaryotic cells do generally have the capacity to orchestrate large morphogenesis. From an application-oriented perspective, it makes sense to choose mammalian cells. The objectives of this review are to push the frontier of tissue engineering. Most applications in this field will likely be using mammalian cells either in food or medical applications.

When opting to work with mammalian cells, one has to specify which cell type would be best suited for the application. The two primary branches are differentiable cells (e.g., embryonic stem cells) and differentiated cells (e.g., fibroblasts). The advantage of starting with differentiable cells is that you could differentiate them while creating a tissue and thereby create a more complex tissue containing multiple cell types. Furthermore, such a tissue would resemble *in vivo* tissues better as they are generally composed of a host of different cell types. However, the disadvantage of these differentiable cells, like embryonic stem cells, is that they are more challenging to handle and will differentiate unless certain factors in the medium are provided. In contrast, differentiated cells are generally easier to work with and more predictable in their behavior. Since we aim to create an elongated tissue with a segmentation pattern and are not interested in creating multiple tissues, like somites or vasculature, we do not need to use multiple cell types. It would thus be more practical to opt for a differentiated cell type such as fibroblasts.

While choosing a differentiated cell type constrains the parameter space of cell behavior, such as cell-to-cell adhesion and locomotion, a reasonable amount of freedom within the parameter space can be achieved. A study by E. Cachat and colleagues showed that by activating effector genes (master regulators of cell behavior), one could regulate cell behavior to a large extend (Cachat et al., 2014). For example, they show they can significantly up-regulate locomotion in their T-REx-293 cells by activating Crk-II. The paper also includes effector genes for proliferation, cell death, cell-cell adhesion, and cell fusion. While this paper provides a strong foundation to expand from, true control over other cell behavior such as extracellular matrix (ECM) production remains elusive (Toda et al., 2019). The design of larger 3D multi-cellular structures would likely require tight control over ECM formation and its features. A particularly relevant example of the necessity for control over ECM production for our construction of a sequentially segmented tissue comes from an in vitro study on somitogenesis. To generate a segmented gastruloid *in vitro,* it is required that they are embedded in Matrigel, a medium for in vitro culturing composed of ECM components (van den Brink & van Oudenaarden, 2021). However, it should be noted that it remains unclear whether Matrigel triggers the morphogenic changes because of a chemical or physical cue.

In summary, to create a sequentially segmented tissue, we need to start the design from the level of individual cells. The most practical choice would be to use a differentiated mammalian cell type. The behavior of these cells can then be modulated by activating effector genes. However, to create larger tissues, tools for strict control over ECM production and architecture are likely needed and will have to be developed.

Elongation

Starting from an aggregate of the chosen “chassis” cells, we can begin to think about elongation. There are three basic approaches to elongating a tissue (**Figure 1A**). The first option is to elongate the cells in the tissue. For example, during elongation of the vertebrate embryo, this mechanism takes place in the notochord, where the inner cells of the notochord begin to expand their vacuoles along the AP axis due to ECM constriction (Ellis et al., 2013; Stemple, 2005). Deploying a similar mechanism in our elongated segmented tissue would require control over vacuoles and ECM. Synthetic biology tools for both are currently lacking. Furthermore, this tissue elongation method is conceptually not as appealing as the primary approach since it reduces the number of cells per length unit in the elongated tissue. This limits further morphogenesis steps and increases the stochastic effects of cellular decision-making.

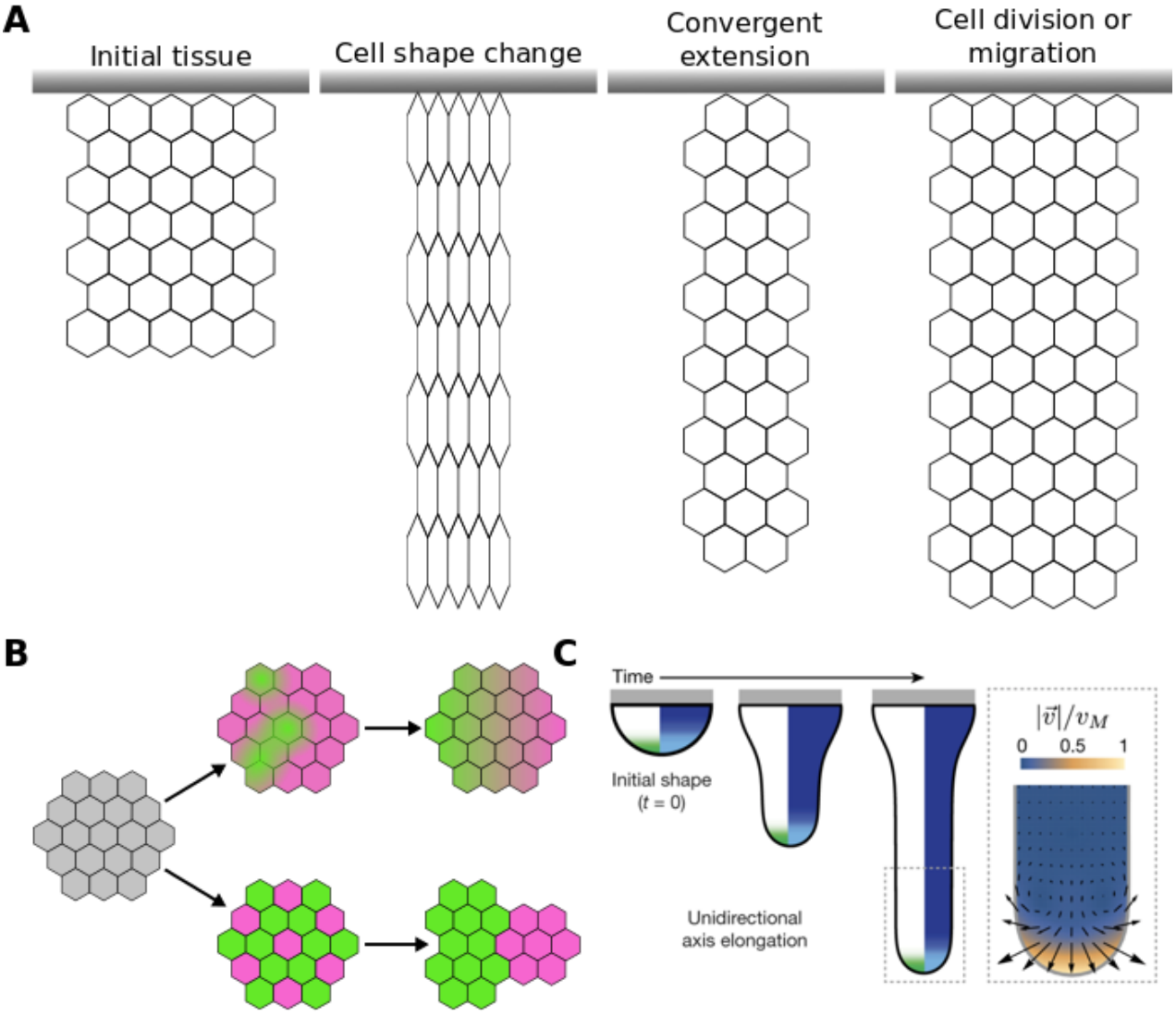

Figure 1 | Symmetry breaking and elongation. Illustrated in **A** are the three ways a tissue can extent itself. Without an external symmetry breaking event there are two main ways to break symmetry. Either an autonomous patterning mechanism creates a morphogen gradient after stochastic induction (top), or cells are stochastically patterned and then sort themselves out into two groups (bottom). In **B**, these two ways are depicted. **C** shows a simulation of tissue jamming, which can create the mechanical properties needed to grow an elongated tissue (adopted from (Mongera et al., 2018)). The green gradient indicates the rate of increase in cell number, and the blue gradient shows the difference in viscosity. The vectors indicate the velocity field of the expanding tissue.

The second basic approach for body elongation is to rearrange cells within a tissue by moving cells from the sides of the tissue towards the middle (**Figure 1A**). This process is called convergent extension, and it does not raise the conceptual problems that the previous approach had. A proposed unified model suggests that polarised cell-crawling and polarised junction shrinkage are the foundation of this tissue remodeling process (Huebner et al., 2018). This again would require significant development of tools for the synthetic developmental toolbox since both synthetic control over cell polarization and cell crawling between cells are lacking. However, there is a noteworthy study on planar cell polarity using synthetic biology, but this system is not entirely orthogonal, limiting control (Loza et al., 2017). Alternatively, a computational study shows that convergent extension can also be driven by segment-specific adhesion (Vroomans et al., 2015). However, since our segmented elongated tissue will lay down the segmentation pattern during elongation, this method cannot be the driving force of elongation.

The third basic approach of elongation is to have cells divide on the posterior side of the tissue or migrate from other more anterior tissues to the posterior (**Figure 1A**). The latter method is thought to be the primary mode of body elongation in vertebrate development (McMillen & Holley, 2015). Here, cells from an anterior pool migrate on the dorsal side of the embryo to the posterior tailbud. After reaching the tailbud, the cells move towards the ventral side of the embryo and into the presomitic mesoderm (Dray et al., 2013). To deploy this approach for constructing our synthetic segmented elongated tissue, we would need to define two separate tracks of cellular motion, which would likely require some form of physical separation, such as an ECM boundary. Furthermore, we would need to establish an AP gradient to enable chemotaxis or durotaxis. While a synthetic orthogonal tool has been developed for chemotaxis, it uses a biologically inert drug not made in cells (Park et al., 2014). Durotaxis is the mechanism where cells move along a stiffness gradient generally towards the stiffer tissue, frequently made more rigid by cells increasing ECM depositions (Sunyer & Trepat, 2020). Again, we run into the issue that synthetic tools for ECM production and regulation are not available.

The other method of posterior growth, local cell division, requires a significantly less complex setup and would therefore be the preferred approach for constructing a segmented elongated tissue. The main difficulty would be to create a mechanism that robustly defines the region where cell division happens and where not. This would require setting up the AP axis. Conceptually there are two ways to establish this axis without outside sources of symmetry breaking (**Figure 1B**). Option one is to generate a stochastic morphogen field that stabilizes into two regions. Turing patterns can generate such behavior by creating one full standing wave (Kondo & Miura, 2010). A study using Nodal and Lefty provides the tools for a synthetic Turing-like pattern (Sekine et al., 2018). The alternative option is that cells can stochastically obtain a cell identity and then sort themselves out. This approach has been shown to work *in silico* (Lam et al., 2019) and the synthetic tools modeled in that study are available (Toda et al., 2018). With an established AP axis, we can begin to think about moving this division front. A computational study on the design of multicellular synthetic circuits shows that synNotch can define a moving interface where cell division occurs (Lam et al., 2019). However, this limits the number of cells that divide to a 2D plane, thus resulting in slow growth. Ideally, we would have two opposing morphogen gradients similar to the situation in vertebrate somitogenesis (Maroto et al., 2012). Cell division could then decrease along one gradient, resulting in faster growth. The construction of these gradients should be achievable since synthetic tools for gradient formation and interpretation are readily available (Santos-Moreno & Schaerli, 2018).

When opting for this third basic approach to create an elongated structure, it is critical to prevent the formation of a sphere. In vertebrate body elongation, one of the key processes that drive elongation is the decrease in cell motility and the increase of tissue stiffness along a gradient (**Figure 1C**, (Mongera et al., 2018)). FGF is thought to provide this gradient (Bénazéraf et al., 2010). Furthermore, an *in vitro* study has shown that Integrin-Fibronectin interactions can modulate a viscoelastic liquid to viscoelastic solid transition in tissues (Caicedo-Carvajal et al., 2010). This would require tight control over ECM construction, which we currently do not have any tools for in synthetic biology. Alternatively, a computational study shows that the morphogenesis of elongation can also be achieved by differential adhesion (Lam et al., 2019). Reasonable control over differential adhesion to direct morphogenesis has been achieved in the field of synthetic development (Toda et al., 2018). Thereby making it likely the easiest path to create an elongated tissue.

In conclusion, the essential synthetic biology tools for creating an elongated tissue are available if we opt for the elongation approach based on cell division and using differential adhesion as a driver for morphogenesis. This would be a clear proof-of-concept study linking patterning and morphogenesis models in constructing an *in vitro* tissue. However, this review serves to highlight places where synthetic developmental tools are lacking, and this section shows that tools for tight control over many important morphogenesis modules need to be created. Especially, the development of synthetic biology tools for the control of ECM depositions will be vital in the construction of larger and more complex tissues.

Sequential Segmented Patterning

When we can create an elongating tissue, we can begin thinking about patterning it sequentially as it grows. The transformation of continuous growth into a discrete pattern is one of the fundamental patterning problems in developmental biology (**Fig. 2A**). More than four decades ago, Cooke and Zeeman proposed their clock-and-wavefront model to solve this problem (Cooke & Zeeman, 1976). In this model, the tissue grows, and a morphogen gradient moves along with it. A genetic oscillator creates locally synchronized waves that move from the posterior to the anterior and arrest after passing a certain morphogen threshold (**Fig. 2D**). The oscillator thus solves the problem of transforming a continuous state into a set of discrete states or segments. This kind of temporal patterning has received little attention from synthetic biologists (Santos-Moreno & Schaerli, 2018). A notable exception comes from a paper by Potvin-Trottier and colleagues. They used a repressilator and the arrest of gene expression of *E. Coli* in its stationary phase to generate a segmented pattern (**Fig. 2B & C**, (Potvin-Trottier et al., 2016)). Since our tissue would be constructed from mammalian cells, we will not be able to use their approach. Furthermore, an arrest of gene expression would severely limit our ability to expand the complexity of this elongated segmented tissue in the future. In this section, I will discuss the suitability of the clock-and-wavefront model and two alternative models as the basis for the development of a novel synthetic sequential segmentation module.

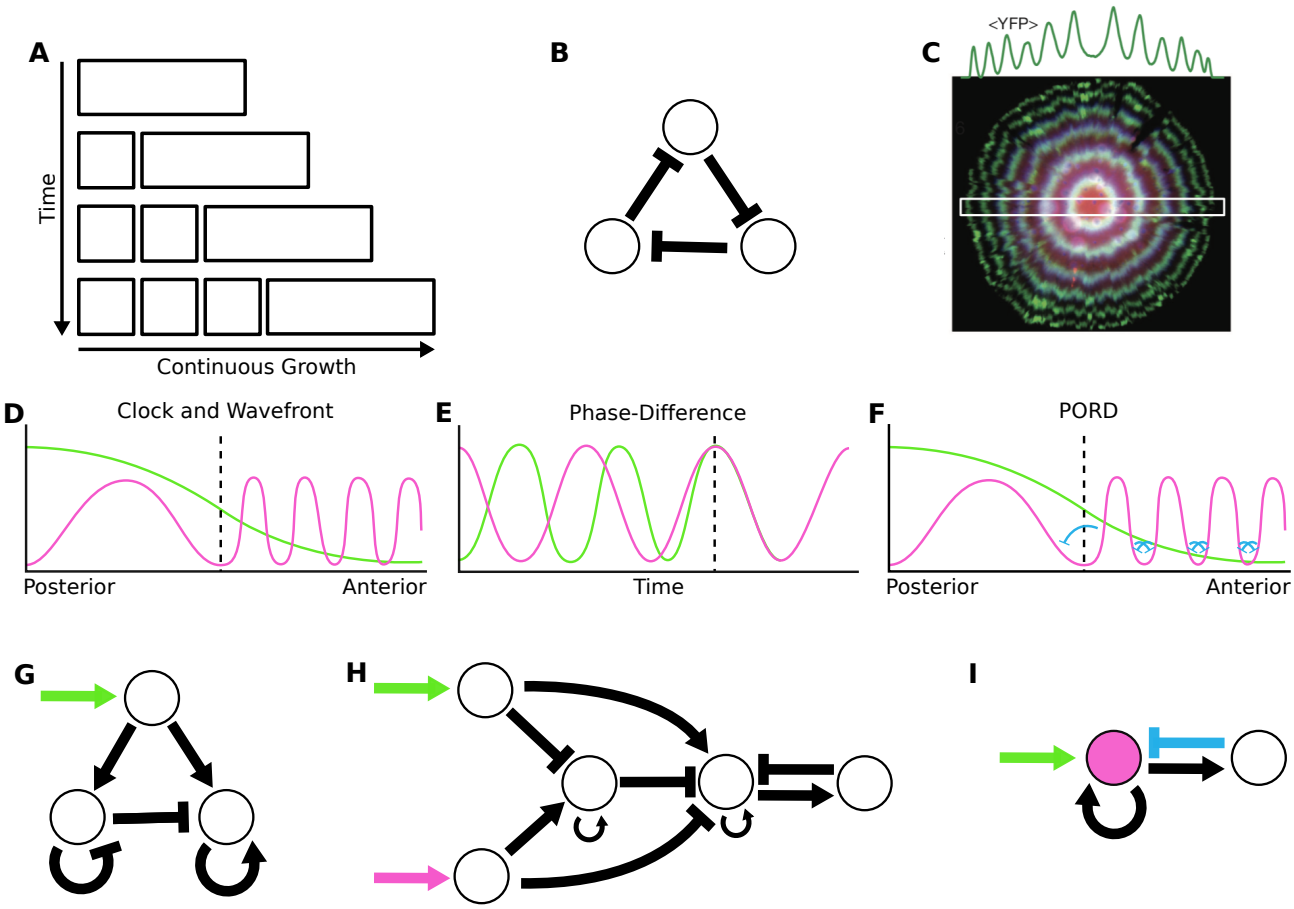

Figure 2 | Sequential segmentation patterning. Depicted in **A** is the problem of turning a continuously growing tissue in segments sequentially. **B** shows the GRN design of the repressilator used to generate the pattern in **C** (adopted from (Potvin-Trottier et al., 2016)). In **D-F** three different models of sequential segmentation are illustrated. The dotted lines indicate the moving arrest front. The GRNs depicted in **G-I** correspond to the models directly above.

Since synthetic developmental biology has no sequential segmentation modules available, we need to build a synthetic module from scratch. Computational models that are well studied come with more information and insights, which could provide the fertile ground for developing a new synthetic module. This makes the 40-year old clock-and-wavefront model an attractive choice (**Fig. 2D**, (Cooke & Zeeman, 1976)). For example, in a computational study, Hester and colleagues show that the clock-and-wavefront model indeed can generate a segmented pattern in a more complex multi-scale model (Hester et al., 2011). This paper partially verifies that we would be able to create a synthetic elongated segmented tissue if we put together a similar network. Furthermore, a study by Francois et al. found through *in silico* evolution a more simple GRN topology that can transform the information from oscillations and a gradient into a segmented pattern (**Fig. 2G**, (François et al., 2007)). Moreover, this same topology was also found in a systematic search for segmentation networks (Cotterell et al., 2015). However, recent work has shown that the clock-and-wavefront model has problems with scaling the size of somites when growth halts (Lauschke et al., 2012). This has prompted Lauschke and colleagues to propose a more robust phase-gradient model as an alternative model for sequential segmentation (Lauschke et al., 2012).

Box 2: Network Motifs (Shoval & Alon, 2010).
To get a more intuitive grip on the behavior of complex GRNs, it is common to divide them into smaller reoccurring GRNs. When these simpler networks are categorized by pattern or structure, they are called a network motif. The primary network motifs are described below.

Autoregulation | One of the simplest motifs is positive or negative autoregulation. Here, a node in the network enhances or inhibits its production. Typically, positive autoregulation decreases network responsiveness, and negative autoregulation increases responsiveness.

Cascades | When nodes activate each other sequentially, it is called a cascade. Typically, this causes a time delay between activation of upstream and downstream nodes.

Single-Input Module | When a node directly activates multiple other nodes, this is called a single-input module. This motif allows for the coordinated activation of genes with a similar function.

Positive-Feedback Loops | When two nodes feedback on each other, they create a loop. If the interaction between the nodes is double-negative or double-positive, it is a positive feedback loop. This motif can be used as a kind of memory.

Negative-Feedback Loop | This motif is similar to the positive-feedback loop, but instead of having edges of similar type between the nodes, they are of the opposite type. Additionally, the inhibition or activation edge is often faster than the other. This difference results in a motif that can generate oscillations.

Feedforward Loop (FFL) | This is a prevalent motif in natural genetic networks. It is essentially a cascade where the first node also directly inhibits or activates the last node. There are two types of FFLs: coherent and incoherent. This motif can be responsible for a wide variety of behaviors. For example, it can function as a signal persistence detector or a pulse generator. In a french flag-like fashion, incoherent FFLs are also known to turn a gradient into positional information (Santos-Moreno & Schaerli, 2018).

In the proposed phase-difference model, cells use the difference between the phases of two oscillators to calculate positional and temporal information (**Fig. 2E**). One of the oscillators entrains the other, thereby reducing the phase difference between the two over time and space (Beaupeux et al., 2016). The wavefront is thus implicit in the behavior of the oscillators and not an explicit gradient as in the clock-and-wavefront model. Like the clock-and-wavefront model, this phase-shift model was postulated about half a century ago as a means for cells to obtain positional awareness (Goodwin & Cohen, 1969). But until recently, the phase-difference model has been much less popular in the study of segmentation than the clock-and-wavefront model. The gain in popularity comes from *ex vivo* studies which imply that the phase-difference model will be more representative of the *in vivo* system (Lauschke et al., 2012; Sonnen et al., 2018). Reconstructing an evolutionarily preferred model makes sense to manage risk. Furthermore, an *in silico* study has found GRN topologies capable of transforming information from the phase difference of two oscillators into a segmented pattern (**Fig. 2H**, (Beaupeux et al., 2016)). While this phase-difference model seems to be closer to the in vivo system and more robust, it will likely be more complex to construct as it requires two oscillators instead of one, and the identified GRN that is capable of transforming the information has significantly more parts compared to the other models (**Box 2**). Recently, an alternative model was proposed that can generate a segmented pattern but with a very simple GRN.

This novel model is called the Progressive Oscillatory Reaction-Diffusion (PORD) system (**Fig. 2F**), and it was identified in a systematic analysis of GRN typologies that can generate a segmented pattern in an elongating tissue (Cotterell et al., 2015). Interestingly, this Turing-type reaction-diffusion model finds its origins in the same seminal paper from 1976 by Cooke and Zeeman as the clock-and-wavefront model (Cooke & Zeeman, 1976). A slow diffusing activator and a rapidly diffusing repressor form zones of repression and activation in the model. However, the early model was quickly rejected based on its inability to account for the scaling of somites upon manipulation. A major difference between the previous reaction-diffusion and the PORD models is that a global gradient directly activates the fast diffusing activator (**Fig. 2I**). While this gradient is not needed to create the somites, it does resolve the scaling issue seen in previous models. Furthermore, in the PORD model, the differentiation front is determined locally instead of globally, as is the case for the clock-and-wavefront model. A key advantage of this model is that it can also generate segments simultaneously, which might be helpful in the formation of the first few segments. However, the limited complexity at which the PORD model has been simulated does make the reader question if it will hold in more complex simulations or *in vitro*. Furthermore, a Turing reaction-diffusion system requires such a large difference in diffusion constants that only a single reaction-diffusion system has been constructed using synthetic biology (Sekine et al., 2018). While workarounds can significantly influence the diffusion parameter, for example, by increasing adhesion to cells, it is likely that it will still be difficult to optimize a Turing reaction-diffusion system for this application.

In summary, all three models should generate a sequentially segmented pattern *in vitro*. The clock-and-wavefront model is the most well studied of the three and thus comes with the most information on flaws, design, and parameters. In contrast, the phase-difference model has only recently gained popularity as it is likely more representative of the *in vivo* system and has more robust segment scaling characteristics. While the phase-difference model is possibly the most robust, it is also the most complex. The PORD model, with its limited number of parts, could be the easiest of the three models to construct. However, due to the strict parameter requirements on its parts, it might actually be more challenging to build than the other three. As each of the three models has its advantages and disadvantages, there is no clear recommendation. If a lab

considers constructing one of these synthetic modules, the lab should also look into recent developments of the neighbor level-difference model (Boareto et al., 2021). As of the time of writing, the information on this model of sequential pattern formation is still too limited to be included, but more details on the model might become available. Furthermore, to speed up the creation of the sequential segmentation module, it might be more productive to first develop it in a 2D system like in figure 2C.

Discussion

The main goal of this review was to explore what available tools are lacking to start creating tissues *de novo*. I used the construction of an elongated segmented tissue as a means to put this question into context. In the introduction, I put forth three subgoals of synthetic development in its mission to create tissues *de novo*. I will now discuss these three subgoals in the context of this review.

The first subgoal is expanding the toolbox of synthetic development, both for patterning and morphogenesis. In the section on elongation, it became clear that we are still lacking control over many morphogenesis processes. Especially, the lack of control over extracellular matrix depositions was a frequently reoccurring theme and will likely have to be addressed if we move forward towards designing larger tissues. In the section on sequential segmented patterning, it became clear that there are still many avenues to explore in the temporal patterning of tissues. Expanding the toolbox will allow for more mix and matching of these synthetic modules and enable the creation of more complex tissues.

The second subgoal is increasing the number of studies that link multiple synthetic developmental modules together. In essence, this review proposes that we do precisely that in creating a synthetic elongated segmented tissue. I identified two patterning modules and at least one morphogenesis module that will need to come together to form this tissue. The difference in the number of developmental modules needed between this hypothetical *in vitro* system and the actual *in vivo* system is striking to me. This gap is especially noticeable in the number of elongation modules required. The overlapping functionality of the different elongation modules *in vivo* will likely enhance the natural system's robustness (Kitano, 2004). This would also suggest that our synthetic elongated segmented tissue is significantly less robust. It would be interesting to explore if robustness improves when we increase the number of morphogenesis modules that contribute to the synthetic elongation.

The third subgoal is the development of dedicated computational tools for synthetic development. In many instances in this review, the information from computational studies has been key to help imagine and validate how individual components come together to form a developmental module. The use of computational studies was especially vital in the section on sequential segmented patterning. This shows the power of a computational approach in system-level studies and its usefulness in synthetic development. While this review focuses on mimicking a natural developmental program and has many computational models available to learn from, other completely novel tissue designs will not have this luxury. This is a problem, and I am currently only aware of two studies that have begun addressing the issue. One study uses the Cellular Potts Model (Lam et al., 2019), and another uses the Agent-Based Model (Sivakumar et al., 2021). Moreover, both studies incorporate only a very limited number of parameterized tools from the developmental's toolbox in their model. Additionally, both computational tools do not yet make any suggestions to the user on how to obtain the final desired outcome. These studies are thus still far away from the ideal case, where you would input the desired tissue morphology and expression pattern, and a computational program would create a viable developmental model given all the synthetic tools available.

In conclusion, it has become clear that it is likely possible to construct an elongated segmented tissue using the available tools in the synthetic development's toolbox. Of course, putting everything together *in vitro* will reveal flaws in the assumptions that were made in this review, but resolving these will push the frontier.

Acknowledgments

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