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HYPOTHESIS

Mathematically guided approaches to distinguish models of periodic patterning

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ABSTRACT

How periodic patterns are generated is an open question. A number of mechanisms have been proposed – most famously, Turing's reaction-diffusion model. However, many theoretical and experimental studies focus on the Turing mechanism while ignoring other possible mechanisms. Here, we use a general model of periodic patterning to show that different types of mechanism (molecular, cellular, mechanical) can generate qualitatively similar final patterns. Observation of final patterns is therefore not sufficient to favour one mechanism over others. However, we propose that a mathematical approach can help to guide the design of experiments that can distinguish between different mechanisms, and illustrate the potential value of this approach with specific biological examples.

KEY WORDS: Periodic patterning, Reaction-diffusion, Turing, Mathematical biology, Pattern formation, Pigment pattern

Introduction

Periodic patterns – most commonly stripes or spots – arise in a variety of organisms and tissues across a wide range of length scales (Marcon and Sharpe, 2012). Well-studied examples include hair follicle distribution in mouse epidermis (Sick et al., 2006; Stark et al., 2007), animal coat patterns (Frohnhöfer et al., 2013; Nakamasu et al., 2009; Yamaguchi et al., 2007) and digit/non-digit patterning during limb chondrogenesis (Badugu et al., 2012; Raspopovic et al., 2014; Sheth et al., 2012). There are multiple mechanisms by which periodic patterns can be generated, which have been exploited in different contexts in developing organisms. For example, apparently periodic stripes can be positioned independently, as in the case of stripes of pair-rule gene expression in the *Drosophila* embryo, which are controlled by separate enhancers (Jaeger, 2011; Stanojevic et al., 1991). Another way to generate stripes is to use an oscillator that introduces periodicity temporally, such as the 'clock and wavefront' model that has been proposed to explain the periodic appearance of somites (Cooke and Zeeman, 1976; Oates et al., 2012). Alternatively, a 'switch and template' patterning mechanism has been proposed to pattern photoreceptors in the *Drosophila* eye (Lubensky et al., 2011). In this article, we focus on a fourth, and commonly used, way to generate periodic patterns by regulation of pattern spacing.

In this case, an initially homogeneous tissue self-organizes into a periodically repeated pattern with a stereotyped distance between neighbouring stripes or spots. Recent studies suggest that this mechanism is at play in a variety of systems. One indication that stripes are generated via this mechanism, as opposed to each stripe having an independent identity or being established sequentially by a moving oscillator, is the presence of pattern bifurcations – the splitting of a stripe into two (Doelman and van der Ploeg, 2002). Such bifurcations have been observed in a number of tissues, including angelfish and zebrafish pigment stripes (Kondo and Asai, 1995; Yamaguchi et al., 2007), ridges on the hard palate (Economou et al., 2012) and digits of perturbed mouse limbs (Sheth et al., 2012). Various mechanisms have been proposed to explain the apparently spontaneous generation of regularly spaced stripes, which will be discussed in detail below.

In the 1950s, Alan Turing devised the reaction-diffusion model to explain how periodic patterning could be achieved (Turing, 1952). This model consists of a fast-diffusing inhibitor molecule and a slow-diffusing activator molecule. Interactions between these two molecules can generate periodic patterns, with a spacing determined by the diffusivity of the activator and inhibitor. Reaction-diffusion models have been used to explain a number of periodic patterns in biological systems, ranging from the spontaneous organization of bacterial populations using synthetic biology approaches (Liu et al., 2011) to developmental patterning events such as feather formation (Jung et al., 1998; Michon et al., 2008), lung branching (Menshykau et al., 2012; Miura and Shiota, 2002) and left/right asymmetry (Nakamura et al., 2006; Nonaka et al., 2002; for a recent review see Marcon and Sharpe, 2012). Moreover, mathematical simulations of different reaction-diffusion schemes can successfully reproduce a variety of natural periodic patterns *in silico* (Asai et al., 1999; Kondo and Miura, 2010; Miura and Maini, 2004; Miura et al., 2006; Murray, 1982).

However, Turing-like reaction-diffusion models are not the only way of generating periodic patterns *in silico*. Cell-based mechanisms generate periodic patterns using cell-cell interactions, with the spacing controlled by both the length scale of these interactions and by cell motility (Nakamasu et al., 2009; Zeng et al., 2004). Mechanical mechanisms can produce periodicity via mechanical instabilities, in which case the pattern spacing depends on the material properties of the tissue (Milinkovitch et al., 2013; Painter et al., 2012; Savin et al., 2011). Furthermore, there is experimental evidence to suggest that these alternatives might be relevant *in vivo*. For example, cell-cell interactions are necessary for zebrafish pigment cell patterning (Nakamasu et al., 2009) and tissue mechanics are important for patterning villi in the gut (Shyer et al., 2013). This raises the question: for a particular periodic pattern, how do we determine whether the mechanism is reaction-diffusion (molecular) versus cellular or mechanical?

A major challenge in answering this question is that periodic patterning involves complex, interacting, spatiotemporal processes, which might not be intuitively accessible. It can therefore be challenging to design experiments that (1) have different predicted outcomes for different hypotheses and (2) rigorously test different hypotheses. Similarly, it can be difficult to interpret existing experimental data from the literature, and the extent to which it favours one mechanism over another.

In this article, we explore a mathematical approach to address these challenges. First, we provide some examples of periodic

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patterning mechanisms and identify features that are common to many mechanisms. We then formalize these similarities using a mathematical model, and show that different mechanisms can generate similar final patterns. Therefore, agreement of *in vivo* and *in silico* final patterns is not a good test of the different hypotheses. To address this limitation, we develop mathematical tools to describe differences between the mechanisms. Using these tools, we discuss several experimental approaches with the aim of either (1) classifying a given mechanism as molecular, cellular or mechanical in nature or (2) rigorously testing a particular hypothesis for periodic patterning.

These mathematical tools rely on simplified descriptions of the biology and cannot substitute for a detailed experimental characterization of a system. Instead, we suggest that the mathematics can help to abstract a complicated biological mechanism and to develop an intuition when designing experiments and interpreting results. We discuss the potential utility of these tools as applied to several experimental systems.

Periodic patterning mechanisms in biological systems

Previous studies have identified a common feature in many periodic patterning mechanisms: 'local activation, long-range inhibition' (Gierer and Meinhardt, 1972; Meinhardt and Gierer, 1974, 2000; Oster and Murray, 1989). Local activation creates areas of increased pattern density throughout space; long-range inhibition ensures that these areas of increased density form at a defined distance from one another separated by areas of low pattern density, thus generating a periodic pattern. This principle applies to multiple periodic patterning mechanisms, as outlined below.

Here we consider several models of periodic patterning, which include molecular, cellular and mechanical processes (see Fig. 1 and Table 1). It is important to note that these mechanisms are not mutually exclusive, nor do they represent all possible means of generating a periodic pattern. However, they serve to illustrate our approach and we believe that they may often form the core of more complex mechanisms *in vivo*.

Molecular mechanisms

As described above, Turing's reaction-diffusion model uses two interacting molecules – a fast-diffusing inhibitor and a slow-diffusing activator – to generate periodic patterns spontaneously (Turing, 1952) (Fig. 1). Local activation is achieved by the slowly diffusing activator molecule and long-range inhibition is achieved by the rapidly diffusing inhibitor molecule. Variants of Turing's original model can also generate periodic patterns, and include models with different interaction logic, e.g. the substrate-depletion model (Gierer and Meinhardt, 1972) or models with more than two molecular species (Satnoianu et al., 2000).

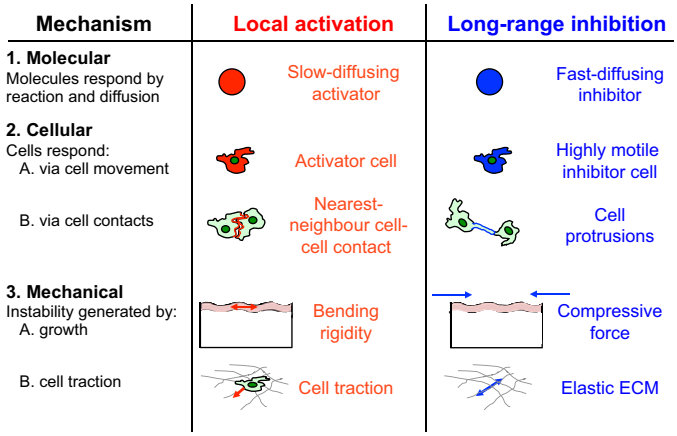

Fig. 1. Periodic patterning mechanisms. Molecular, cellular and mechanical mechanisms can produce periodic patterns. In each case, there are 'local activation, long-range inhibition' interactions that control pattern spacing.

Many periodic patterns have been interpreted, at least in part, using the reaction-diffusion model, although validation of putative activator/inhibitor pairs – the so-called 'Turing molecules' – is often lacking (Kondo and Miura, 2010). Candidate activator/inhibitor pairs include Nodal/Lefty to pattern left/right asymmetry (Nakamura et al., 2006), Wnt/Dkk or Eda/BMP to pattern hair follicles (Mou et al., 2006; Sick et al., 2006) and Wnt/BMP to pattern the digits in the mouse limb (Raspopovic et al., 2014).

Cell-based mechanisms

Instead of passively responding to a molecular prepattern as described above, cells themselves can be active participants during patterning (Fig. 1). For example, zebrafish pigment patterns are formed by the interactions and movement of three different cell types (as discussed further below) (Frohnhöfer et al., 2013; Nakamasu et al., 2009). Here, we consider two example mechanisms of cell-based patterning. In the first case, two different cell types move to form a periodic pattern, and proliferate according to their contact with neighbouring cells. This type of mechanism could be conceptually similar to a reaction-diffusion mechanism, if we replace diffusing activator/inhibitor molecules with the undirected migration of activator/inhibitor cell types. However, unlike diffusing molecules, cells can also undergo directional movement – being either attracted to or repelled by neighbouring cells – which can generate many more possibilities for pattern formation (see supplementary material Section 1B).

In the second case, cells are static but communicate via direct contact to regulate cell fate choices. An example would be where nearest-neighbour cell contacts give local activation and longer-distance contacts via cell protrusions give long-range inhibition. This is illustrated by the formation of regularly spaced hair cells in the fly notum, which is controlled by far-reaching, dynamic filopodia that send out inhibitory Delta-Notch signals to distant neighbours (Cohen et al., 2010).

Mechanical mechanisms

Mechanics alone can produce periodic patterns by generating mechanical instabilities (Milinkovitch et al., 2013; Murray, 2003; Murray and Oster, 1984b; Shyer et al., 2013). We consider two types of instability (Fig. 1). First, periodic buckling can be induced by growth of an epithelial tissue. As a tissue grows within a confined space, it is effectively compressed and a mechanical instability develops. This compression provides long-range inhibition, since growth at any location can be felt at a distance. Local activation is controlled by resistance of the tissue to bending ('bending rigidity'), which prevents high tissue curvature. Thus, if a certain point in the tissue is moved up, nearby tissue sections will also tend to move up, providing an analogous local activation. Together, these interactions result in buckling of the tissue once growth exceeds a critical value, thereby generating periodic tissue displacements. An example of growth-induced buckling is in the formation of regularly spaced villi in the gut, in which several stages of constrained growth cause the gut epithelial sheet to spontaneously bend into periodic undulations (Shyer et al., 2013).

Second, cells migrating over and interacting with extracellular matrix (ECM) can generate periodic patterns when a mechanical instability develops as the result of cell movement. *In vitro* studies show that migrating cells cause the ECM to contract by exerting traction forces on it as they move (local activation) (Klumpers et al.,

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Table 1. Parameter constraints on patterning mechanisms

Patterning mechanism	Parameters	Constraints on parameters
1. Molecular (reaction-diffusion) *A*, activator; *I*, inhibitor	• Diffusion constants: D_A, D_I • Molecular half-lives: τ_A, τ_I • Reaction sensitivities: **h**	Inhibitor diffuses faster than activator: $D_I > D_A$ (Murray, 1982, 2008) Autoactivation is sufficiently sensitive: $h_{AA} > 1$
2A. Cellular via cell movement *A*, activating cell; *I*, inhibiting cell	• Cell motility: η_A, η_I • Lifetime of a cell: τ_A, τ_I • Sensitivity of cell proliferation to other cells: **h**	Inhibitor cell is more motile: $\eta_I > \eta_A$; or there is directional cell movement, e.g. activators aggregate: $\eta_A < 0$
2B. Cellular via cell contact signals	• Sensitivity of cells to signalling: h • Length of protrusion: $L_{protrusion}$	Constraint not calculated*
3A. Mechanical with cell growth	• Thickness of tissue: h • % growth of tissue: g • Stiffness of tissue: E_{sheet} • Stiffness of underlying substrate: E	Sufficient growth of tissue: $g > \frac{1}{4}\left(\frac{3E}{E_{sheet}}\right)^{2/3}$ (Groenewold, 2001; Khang et al., 2008)
3B. Mechanical with cell movement (assume small viscosities)	• Thickness of tissue: h • Stiffness of tissue: E • Poisson ratio of tissue: ν • Traction force/area exerted by cells: $\sigma_{traction}$	Cell traction forces exceed ECM forces: $\sigma_{traction} > 0.5E$ (Murray and Oster, 1984a)

Shown are parameter constraints that, for a given mechanism, guarantee periodic patterns will form. See supplementary material Section 1 for additional information and further constraints.

*In contrast to many molecular signals, cell protrusions can have a very well-defined length scale, meaning that pattern formation can occur for a wide range of parameters; see supplementary material Section 1 and Lopez (2006) for more details.

2013). These forces are opposed by the long-range elasticity of the ECM (long-range inhibition). Short-range contractility and long-range rigidity can generate periodic ECM displacements and cell condensations *in silico*, and have been proposed to generate periodic condensations in an *in vitro* model of chondrogenesis (Murray, 2003; Murray and Oster, 1984a,b; Newman et al., 2008).

We now focus on the guiding question of this article: how can different periodic patterning mechanisms be distinguished?

Different mechanisms can generate similar periodic patterns *in silico*

A variety of periodic patterns have been modelled based on a particular mechanism, often a Turing-like reaction-diffusion mechanism (Kondo and Miura, 2010). Fig. 2 summarizes some of these different patterns. However, different mechanisms can produce patterns that are qualitatively similar. The reason for this lies in the shared 'local activation, long-range inhibition' property, which means that each type of mechanism described above (see also Fig. 1) falls into the same general class and can be approximated by a common mathematical model (Cross and Hohenberg, 1993). This common model is detailed in Box 1. Importantly, this model is sufficient to predict several key features of periodic patterns (see supplementary material Section 2 for further details). First, the model predicts that periodic patterns can arise spontaneously from an initially homogeneous state provided that some form of 'local activation, long-range inhibition' is applied. Second, the final pattern can be stripes, spots or zigzags, with the transition determined by the specific parameters in the model. This transition between stripes and spots has been observed in several genetic mutants of fish pigment patterning (Asai et al., 1999), while zigzags have been observed during stages of villi patterning in the gut (Shyer et al., 2013). Third, in the absence of any factors that introduce bias into the system, the orientation of the pattern can be random, or labyrinthine, as seen for example in the zebrafish *choker* mutant (Frohnhöfer et al., 2013) (see Fig. 5A). However, initial conditions can bias the orientation of the pattern. For example, in zebrafish pigment patterning it is thought that the first stripe is specified as an initial condition and that further stripes form sequentially, parallel to the first (Nakamasu et al., 2009). Finally, boundary conditions can affect both the orientation of the pattern and the selection of stripes versus spots in the final pattern. For example, boundary conditions may explain why animals can have spotted coat patterns but striped tail patterns, but not vice versa (Murray, 1988, 2008).

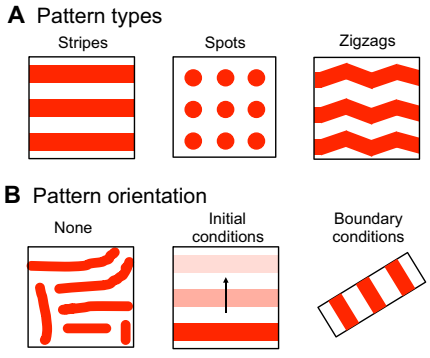

Fig. 2. Different mechanisms produce qualitatively similar final patterns. A simple and generic model of periodic patterning can generate a variety of patterns. (A) Stripes, spots and zigzags, which are common periodic patterns, can be generated. (B) Stripes can either be randomly oriented (labyrinthine) or oriented by initial conditions and/or boundary conditions. See Box 1 and supplementary material Section 2 for a description of the model.

Since these features are common to many mechanisms (molecular, cellular and mechanical) they cannot be used to favour one mechanism over another. Therefore, the mechanism underlying any particular periodic pattern cannot be determined from its final appearance, and similarity of *in vivo* and *in silico* patterns is not a rigorous test of a given hypothesis. In the remainder

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Box 1. A mathematical description of a generic periodic patterning mechanism

Periodic patterns formed by molecular, cellular and mechanical mechanisms have been analyzed using partial differential equations (PDEs). For each of the example mechanisms in Fig. 1, we adapt existing PDE models to write a mathematical model for patterning. The assumptions and validity of each of these example models is discussed in supplementary material Section 1.

In the following, we use a linearized reaction-diffusion PDE to illustrate our approach:

$$\frac{\partial}{\partial t}\begin{pmatrix} A \\ I \end{pmatrix} = \underbrace{\begin{pmatrix} D_A & 0 \\ 0 & D_I \end{pmatrix}\nabla^2\begin{pmatrix} A \\ I \end{pmatrix}}_{\text{Diffusion}} + \underbrace{\begin{pmatrix} h_{AA}/\tau_A & h_{AI}/\tau_A \\ h_{IA}/\tau_I & h_{II}/\tau_I \end{pmatrix}\begin{pmatrix} A \\ I \end{pmatrix}}_{\text{Reactions}} - \underbrace{\begin{pmatrix} \tau_A^{-1} & 0 \\ 0 & \tau_I^{-1} \end{pmatrix}\begin{pmatrix} A \\ I \end{pmatrix}}_{\text{Degradation}}. \quad (1)$$

Here, D_A, D_I are diffusion constants of activator/inhibitor molecules; τ_A, τ_I are molecular half-lives; A, I are normalized concentrations of activator and inhibitor molecules; and the reaction matrix **h** represents normalized sensitivities. For example, the autoactivation parameter, h_{AA}, describes how sensitively the production rate of A, f_A, increases as a function of the concentration of A. Sensitivity has a clear biological interpretation – if activator concentration increases by 1%, then the production of activator increases by h_{AA}% (Paulsson, 2004).

In order to describe similarities between mechanisms, we recast them into a common framework, as formulated by Cross and Hohenberg (1993). These authors showed that patterns form provided periodic disturbances grow over time, which is calculated by Fourier transforming Eq. 1 and looking for solutions of the form:

$$\begin{pmatrix} A \\ I \end{pmatrix} = \begin{pmatrix} A_0 \\ I_0 \end{pmatrix}\exp[\mu_q t]. \quad (2)$$

Here, μ_q is the rate at which a periodic pattern with wavelength $\lambda=2\pi/q$ grows over time, commonly referred to as a 'dispersion relation'. Substituting Eq. (2) into Eq. (1) gives:

$$\begin{vmatrix} -D_A q^2 + (h_{AA}-1)/\tau_A - \mu_q & h_{AI}/\tau_A \\ h_{IA}/\tau_I & -D_I q^2 + (h_{II}-1)/\tau_I - \mu_q \end{vmatrix} = 0. \quad (3)$$

For periodic patterning, μ_q must have a maximum value greater than zero. In most cases, we may then approximate any μ_q by a general form that specifies the position, q_0, height, a, and width, κ, of the peak near its maximum. By calculating μ_q for different mechanisms, we can transform the different model PDEs into a common equation that uses a single variable $\phi(\boldsymbol{x}, t)$ to describe the pattern:

$$\frac{\partial\phi}{\partial t} = a(1-\kappa)\phi - 2a\kappa\frac{\nabla^2\phi}{q_0^2} - a\kappa\frac{\nabla^4\phi}{q_0^4} - c\phi^2 - d\phi^3. \quad (4)$$

The ∇^2 term favours the periodic instability, whereas the ∇^4 term stabilizes it; with c, d representing nonlinearities. This equation is an example of a generalized Swift–Hohenberg (SH) equation, which can generate many types of periodic patterns (Burke and Knobloch, 2006; Cross and Hohenberg, 1993) (see supplementary material Section 2). Since many periodic patterning mechanisms can be faithfully represented by an SH equation in the parameter regime $\max_q \mu_q \to 0^+$, we conclude that the patterns in Fig. 2 can be produced by many periodic patterning mechanisms, and thus observation of these patterns does not constrain the mechanism.

of this article, we describe several mathematically inspired approaches that can better distinguish mechanisms. These approaches include two types of experiment: (1) those that can broadly categorize a mechanism (e.g. as molecular, cellular or mechanical); and (2) those that start from a hypothetical mechanism and rigorously test the assumptions of that mechanism.

Experiments to distinguish broad categories of mechanism

One approach to identify the mechanism underlying a particular patterning process is to design experiments that place the mechanism into a broad category, e.g. molecular, cellular or mechanical, without specifying a hypothesis *a priori*. Common experimental designs include (1) observation and (2) perturbation of pattern formation. Sometimes the interpretation of these experiments will be straightforward. Careful observation of pattern formation can 'rule in' hypotheses; for example, if extensive cell movement is observed concomitant with patterning, this suggests a role for a cellular mechanism. Perturbation, on the other hand, can 'rule out' hypotheses; if, for example, perturbing tissue stiffness experimentally has no effect on the pattern, a mechanical mechanism can likely be ruled out. However, in many cases, the results will not be as definitive and will be consistent with several categories of mechanism. Distinguishing between categories therefore requires a more rigorous experimental design, which we explore using mathematics.

The main challenge in designing this type of experiment is in making the experimental predictions sufficiently generic to encompass different mechanisms within a category, but sufficiently specific to distinguish between categories. One way to achieve this is by experiments in which different mechanisms have qualitatively different outcomes. By focusing on qualitative outcomes, the results are sufficiently generic to encompass different model parameterizations in a single category. However, as discussed previously, periodic patterning mechanisms share many qualitative features and therefore we must choose features that are specific to each category. We describe two such approaches below.

Response to broad perturbations

Our first approach is to identify perturbations that affect the periodic pattern in the same way for multiple mechanisms within the same broad category, but have no effect on mechanisms from other categories. We have identified a set of such perturbations using dimensional analysis, which uses the fact that any equation that describes the final pattern spacing must have units of length. For example, a molecular reaction-diffusion model can be parameterized by diffusion constants, D (unit: $m^2\ s^{-1}$), molecular half-lives, τ (unit: s), and reaction sensitivities (dimensionless). In order to write an equation for the pattern spacing, λ (unit: m), we must have $\lambda \propto (D\tau)^{0.5}$ [since the units of $(D\tau)^{0.5}$ are $(m^2\ s^{-1}\times s)^{0.5} = m$]. Therefore, for a reaction-diffusion model, the pattern spacing is predicted to increase with changes in diffusion constants and/or molecular half-lives. This prediction holds for all mechanisms within the reaction-diffusion category, i.e. for mechanisms with different parameter values, different numbers of components and different types of molecular interactions. Dimensional analysis makes assumptions about the units of the important parameters in the system, but no assumptions on the model specifics.

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Box 2. Mathematical tools to identify differences between mechanisms

Parameter constraints

Final periodic patterns are described by the equilibrium solutions of Eq. 4 and depend strongly on the nonlinear terms c, d (Ermentrout, 1991), which are difficult to measure. However, Cross and Hohenberg (1993) show that a sufficient condition for periodic pattern formation is instability of the homogeneous state, which is independent of c, d. This can be computed for a set of PDEs by deriving the linear dispersion relation, μ_q (Box 1), and requiring its real part, $\mathrm{Re}(\mu_{\mathbf{q}})$, to be positive for some non-zero $\mathbf{q}$, which is the actual pattern wave vector (Cross and Hohenberg, 1993; Murray, 2008). This is equivalent to $a>0$ in Eq. 4. This constraint can also be interpreted as a necessary condition for patterning, valid within the limit of small stochastic fluctuations and homogeneous initial conditions. In addition, it is often assumed that the homogeneous state is stable in the absence of spatial processes, implying $\mathrm{Re}(\mu_{\mathbf{q}=0})\leq 0$. These inequalities constrain the underlying parameters differently for each of our example models, and are summarized in Table 1 (see also supplementary material Section 1).

Response to perturbation

How the final pattern responds to changes in the underlying parameters can be difficult to predict and depends on nonlinearities in the PDEs (Ermentrout, 1991). However, we may use dimensional analysis, via Buckingham's H-theorem (Buckingham, 1914), to constrain an equation for the final pattern spacing, λ, which has units of length. To apply this method to a particular model, one writes down all the parameters in the model, combines them into dimensionless groups, and rewrites any equations in terms of these dimensionless groups.

For example, in the reaction-diffusion model (Eq. 1), a dimensionless group for pattern spacing is $\lambda/\sqrt{D_A\tau_A}$, which gives an equation:

$$\frac{\lambda}{\sqrt{D_A\tau_A}} = f\left(\frac{D_I}{D_A}, \frac{\tau_I}{\tau_A}, h_{ij}\right), \tag{5}$$

where $f(x, y)$ is an arbitrary function. Each example mechanism that we consider has a different dimensional scaling, as listed in Table 2 (see also supplementary material Section 3).

Pattern dynamics

Initial pattern dynamics are described by equations in the form of Eq. 2, equivalently $\partial\phi_{\mathbf{q}}/\partial t=\mu_{\mathbf{q}}\phi_{\mathbf{q}}$. In real space, this becomes:

$$\frac{\partial\phi(\boldsymbol{x},t)}{\partial t} = \int d\boldsymbol{x}' K(\boldsymbol{x}-\boldsymbol{x}')\phi(\boldsymbol{x}',t). \tag{6}$$

$K(\boldsymbol{x})$ is the inverse Fourier transform of $K(\mathbf{x}) = \int\frac{d\mathbf{q}}{2\pi}\mu_{\mathbf{q}}\exp[-i\mathbf{q}.\mathbf{x}]$ (and can be generalized to include a time component). This function, $K(\boldsymbol{x})$, is interpreted as an interaction function – describing how two components interact with each other when separated by a given distance – and can be derived for different mechanisms from the model's PDE description (see supplementary material Section 4). As an example, if cells interact with one another by secreting a molecule A, with diffusion constant D_A and lifetime τ_A, then the interaction function is (Wartlick et al., 2009):

$$K(\boldsymbol{x}) \propto \exp\left[\frac{-|\boldsymbol{x}|}{\sqrt{D_A\tau_A}}\right]. \tag{7}$$

In Fig. 4B, we illustrate that different basic interactions – diffusible (molecular), direct contact (cellular) or material (mechanical) – have qualitatively different interaction functions. These interaction functions can be inferred by measuring the pattern, $\phi(\boldsymbol{x}, t)$, and its dynamics, $\partial\phi/\partial t$, over time. We can fit the data to Eq. 6 to estimate $K(\boldsymbol{x})$ (see supplementary material Section 5 for the algorithm). We show the feasibility of this approach by applying the algorithm to simulated data in Fig. 4C.

In Box 2 we outline this approach more generally and in Table 2 we give analogous results for cellular and mechanical mechanisms (see also supplementary material Section 3). The suggested perturbations change the final pattern spacing by modifying the characteristic length scale in the system – for molecular mechanisms, this length scale is set by diffusion; for cellular mechanisms, either by cell motility or by the length of signalling protrusions; and for mechanical mechanisms, by tissue stiffness. Therefore, provided we can apply broad perturbations – e.g. indiscriminately hinder the diffusion of all secreted molecules – and subsequently observe whether pattern spacing increases or decreases, then it is possible to classify the mechanisms into broad categories.

Table 2. Predictable responses to pattern perturbations

Patterning mechanism	An equation for pattern spacing, λ	Pattern spacing is predictably changed by perturbing:
1. Molecular (reaction-diffusion)	$\lambda \sim \sqrt{D_A\tau_A} f\left(\frac{D_I}{D_A}, \frac{\tau_I}{\tau_A}, \mathbf{h}\right)$	Diffusion constants; molecular half-lives
2A. Cellular via cell movement	$\lambda \sim \sqrt{\eta_A\tau_A} f\left(\frac{\eta_I}{\eta_A}, \frac{\tau_I}{\tau_A}, \mathbf{h}\right)$	Cell motility; cell lifetime
2B. Cellular via cell contact signals	$\lambda \sim \mathrm{L}_{\mathrm{protrusion}} f(h)$	Length of cell contacts
3A. Mechanical with cell growth	$\lambda \sim h\left(\frac{E_{\mathrm{sheet}}}{\mathrm{E}}\right)^{1/3} f(g)$	Tissue thickness; tissue stiffness
3B. Mechanical with cell movement	$\lambda \sim hf\left(\frac{\sigma_{\mathrm{traction}}}{E}, \nu\right)$	Tissue thickness; tissue stiffness

Shown are parameters that affect pattern spacing in a predictable way, identified by dimensional analysis. $f(x, y)$ is an unknown function of the dimensionless variables x, y. See supplementary material Section 3 for a more detailed description.

Although challenging, making broad perturbations may be feasible using *in vitro* models of patterning (Miura and Shiota, 2000a,b; Paulsen and Solursh, 1988; Yamanaka and Kondo, 2014), together with careful tuning of the extracellular environment. One way to control the external environment is to use hydrogels (Cushing and Anseth, 2007; Elisseeff, 2008), in which one can independently modulate the diffusion constants of secreted molecules, the motility of different cell types and the material properties of the underlying ECM (Forget et al., 2013; Kyburz and Anseth, 2013; Lin and Metters, 2006; Weber et al., 2009). Here, we consider limb patterning as an example where this approach can help to distinguish potential mechanisms.

Digit/non-digit patterning in the vertebrate limb

Patterning of the vertebrate limb is regulated by several morphogen gradients (Tickle, 2006; Zeller et al., 2009). A separate periodic patterning mechanism has also been hypothesized to generate the digit/non-digit pattern (Newman and Frisch, 1979), a notion supported by a recent study by Sheth et al. (2012). The authors of this study show that perturbed mouse limbs exhibit altered digit spacing and digit bifurcations, both being characteristics of periodic patterning (see Fig. 3).

Reaction-diffusion models can replicate this periodicity *in silico*, including several patterns seen in mouse mutants (Miura et al., 2006). Furthermore, candidate Turing molecules have been suggested, with Wnts acting as short-range activators and BMPs as long-range inhibitors (Raspopovic et al., 2014). Raspopovic and colleagues used a mathematical approach to provide evidence in favour of a Wnt-BMP reaction-diffusion mechanism for digit patterning. Computer simulations predicted that digit spacing would increase when the secretion of Wnt ligands was reduced and BMP receptors were inhibited. These outcomes were indeed seen when Wnt/BMP signalling was experimentally perturbed in developing mouse limbs.

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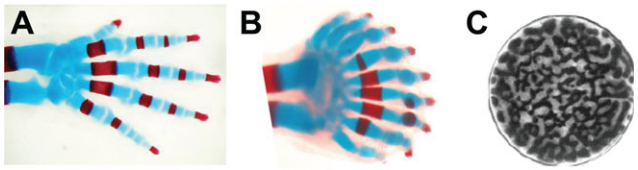

Fig. 3. Periodic digit/non-digit patterning. (A) Digits of a wild-type mouse limb and (B) increased digit number and reduced digit spacing in Hox mutants [reproduced with permission from Sheth et al. (2012)]. (C) Labyrinthine periodic patterns are observed in limb micromass culture [reproduced with permission from Miura and Shiota (2000b)].

Although the agreement between *in silico* and *in vivo* digit spacing provides excellent support to a molecular hypothesis, these data do not yet rule out cell-based and mechanical mechanisms, which, like reaction-diffusion models, also recapitulate patterning *in silico* (Miura and Shiota, 2000a; Murray and Oster, 1984a). For example, it is possible that the effect of the Wnt/BMP perturbations is to alter cell motility in a dose-dependent fashion. Indeed, Wnt/BMP-regulated cell motility has been observed in a number of other systems (Endo et al., 2005; Veerkamp et al., 2013).

As a complement to the experiments of Raspopovic et al. (2014), we suggest that perturbations such as those described in Table 2 can help rule out these other broad classes of mechanism. For example, the analysis predicts that for reaction-diffusion systems the pattern spacing will increase as one increases the diffusion constants in the system. By contrast, a model based on cell movement will be affected by perturbing cell motility, and a mechanical model by perturbing the stiffness of the surrounding ECM. A particularly attractive system in which to perform these experiments is an *in vitro* model of chondrogenesis (Miura and Shiota, 2000a; Paulsen and Solursh, 1988; Raspopovic et al., 2014), where, as described above, there are methods for independently perturbing extracellular diffusion, cell motility and the mechanical properties of the ECM.

Dynamics of pattern formation

Although any interaction that has 'local activation, long-range inhibition' can generate periodic patterns, the distance dependence of the interaction is qualitatively different for different mechanisms. Molecular signals tend to be graded, smoothly changing signals, whereas cellular protrusions often have a well-defined signalling range, and mechanical forces can transmit periodic instabilities over large distances (Fig. 4B; supplementary material Section 4). By measuring this interaction, we can broadly describe the type of interaction that is occurring, an important step in distinguishing mechanisms.

In Box 2, we describe how these qualitative differences can be inferred by measuring pattern dynamics. Pattern dynamics could be molecular (e.g. translation rates), cellular (e.g. proliferation rates) or mechanical (e.g. strain rates). An advantage of this approach is that it can describe a 'local activation, long-range inhibition' interaction without knowing its specific components and in a way that is independent of other parameters in the system. A corresponding limitation of this approach is that, without also measuring or perturbing specific components, the classification into molecular, cellular or mechanical interactions is based on qualitative similarity to an expected interaction function, and therefore can only be suggestive and not definitive. Measurement of the interaction function following known perturbations can overcome this limitation. In the following section, we illustrate how to implement this approach using a model of pigment patterning in the zebrafish skin.

Zebrafish pigment patterning

Zebrafish genetic mutants reveal a variety of possible final patterns of pigmentation in the skin, some of which are shown in Fig. 5. Many of these patterns have been successfully simulated using reaction-diffusion equations (Watanabe and Kondo, 2012). However, recent experiments favour a cellular mechanism. Removing particular cell types (melanophores, xanthophores and iridophores), either completely via genetic mutants (Frohnhöfer et al., 2013) or at certain times and positions by laser ablation (Nakamasu et al., 2009; Yamaguchi et al., 2007), has revealed a number of cell-cell interactions that are necessary for correct pattern formation, consistent with 'local activation, long-range inhibition' (Nakamasu et al., 2009). Two of these interactions have been characterized molecularly. Contact-dependent depolarization of membrane potential controls repulsion between melanophores and xanthophores (Inaba et al., 2012; Iwashita et al., 2006). Meanwhile, melanophores receive Notch-dependent survival signals by extending long cell protrusions to distant xanthophores (Hamada et al., 2014), controlling melanophore stripe width.

Several key components of this system are yet to be determined. In particular, we highlight a role for directed cell movement, which is important during initial patterning in juveniles (Singh et al., 2014) and during pattern regeneration (Yamaguchi et al., 2007) (Fig. 5B). What signals control directed cell movements? One hypothesis is that Notch-Delta signalling via direct contact or via longer cell protrusions regulates the movement of nearby cells (a cell-based interaction). Other possibilities include a role for (unknown) molecular signals that guide cell movement, or mechanical influences from interactions between migrating cells and the underlying ECM. We suggest that these different hypotheses can be distinguished using time-lapse imaging, which might be most feasible in regenerating patterns (Yamaguchi et al., 2007) or *in vitro* models (Yamanaka and Kondo, 2014).

Specifically, by tracking individual cells as they migrate, and measuring the positions of their neighbouring cells, we can measure cell velocities (pattern dynamics) to determine how the cells interact with one another. To illustrate this principle, imagine that you isolate two cells and place them a distance, x, apart from one another, and then measure how fast and in which direction they move (see Fig. 4A). By repeating this measurement at different distances, one can measure how the cell response depends on the distance between the cells. In practice, however, it can be difficult to isolate pairs of cells to perform this experiment. In Box 2, we describe an algorithm to infer the distance dependence of the interaction function by measuring dynamics when there are many cells in the system.

Using this analysis, if no interaction is observed, this might suggest that the cells are passively migrating towards an existing prepattern. If the interaction is very short ranged, it points to communication via direct cell contact. If, instead, there are longer-range interactions, we can tentatively classify them into one of three categories – molecular, cell-based or mechanical – by comparing them with the expectations outlined in Fig. 4B.

Analysis of data from simulations shows that the interaction function can be estimated from noisy data using a limited number of data points (Fig. 4C; supplementary material Section 5), suggesting that this approach is feasible for biological data that are limited in time points and often noisy.

Experiments to rigorously test hypothetical mechanisms

Although the discussion above suggests ways in which a mathematically guided approach can help to distinguish between broad categories of mechanism, it is more usual to start with a

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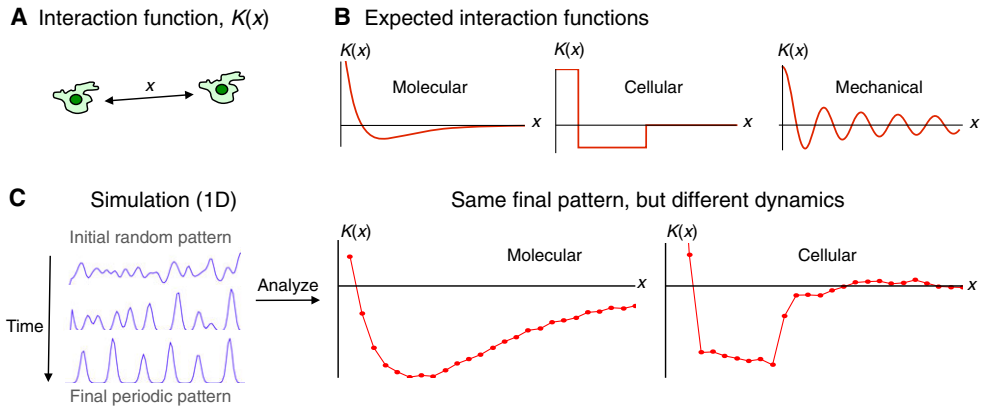

Fig. 4. Dynamics of pattern formation. The dynamics of pattern formation can be used to distinguish between mechanisms. Here, we consider a case in which cells move to form a periodic pattern, but with unknown cell-cell interactions. (A) A cell responds to nearby cells differently depending on how far away the 'sender cell' is. This distance dependence defines an interaction function, $K(x)$. Cells respond 'positively' to close cells (local activation) and 'negatively' to more distant cells (long-range inhibition), i.e. the interaction function is positive for short distances and negative for long distances. (B) Expected interaction functions are different for molecular, cellular and mechanical interactions (see supplementary material Section 4 for details). (C) Simulation of dynamics of pattern formation. We create synthetic data by simulating a periodic pattern formed by cells moving both randomly and according to interactions described by $K(x)=K_A\exp[-x/L_A]-K_I\exp[-x/L_I]$ (molecular) or by $K(x)=K_A(1-H(x-L_A))-K_I(1-H(x-L_I))$ (cellular), with H denoting step functions. (Left) Plots of cell density over time as the pattern evolves. (Right) Analyzing the data from these simple simulations shows that $K(x)$ can be estimated from pattern dynamics using a reasonable number of time point measurements (see supplementary material Section 5 for algorithm and example code).

specific hypothesis in mind. Typically, one would start by identifying key patterning components by studying mutants with aberrant patterning, or by observing the expression of particular genes (or positions of particular cell types) over time. These data are necessary to define the components in the system and to generate hypotheses, but different types of experiment are needed to rigorously test these hypotheses – we require experimental designs that generate falsifiable predictions (see also Box 2). Below, we propose several such experiments and illustrate how they may help to determine the patterning mechanism underlying specific biological examples.

Parameter constraints

Multiple periodic patterning mechanisms have been simulated by mathematical models (Maini et al., 1991; Miura and Maini, 2004; Murray and Oster, 1984a,b). As we have argued, qualitative features of these simulations cannot rigorously test a given hypothesis. However, quantitative predictions can be used provided that we can measure the relevant parameters. A major challenge is that, for many features of periodic patterns (e.g. the selection of stripes versus spots), the relevant parameters include nonlinear effects (Ermentrout, 1991).

An example of such a nonlinear effect is the interactions between activator and inhibitor molecules in a reaction-diffusion model. Accurately predicting a reaction-diffusion pattern *in silico* would require quantitative measurements of these interactions, including full dose-response curves for how molecular reaction rates depend on the concentrations of the activator and inhibitor molecules present. Given that this type of data is difficult to acquire, a common strategy is to simulate the system using a 'guess' for the nonlinearities. Such an approach is liable to overfitting and the results of the simulations may depend on the choice of nonlinearity, making it difficult to falsify a particular hypothesis.

Instead, we have identified an experimental prediction that is independent of these nonlinear effects. Specifically, the ability of a mechanism to generate any pattern, other than a spatially uniform pattern, is a feature that is easy to predict without fully characterizing the nonlinearities. We can derive a set of parameter constraints that a hypothetical mechanism must satisfy in order that periodic patterns can form. In Table 1, we list some of the parameter constraints for the mechanisms considered in this article, and describe how to obtain these results for any mechanism in Box 2 (see supplementary material Section 6 for additional parameter constraints).

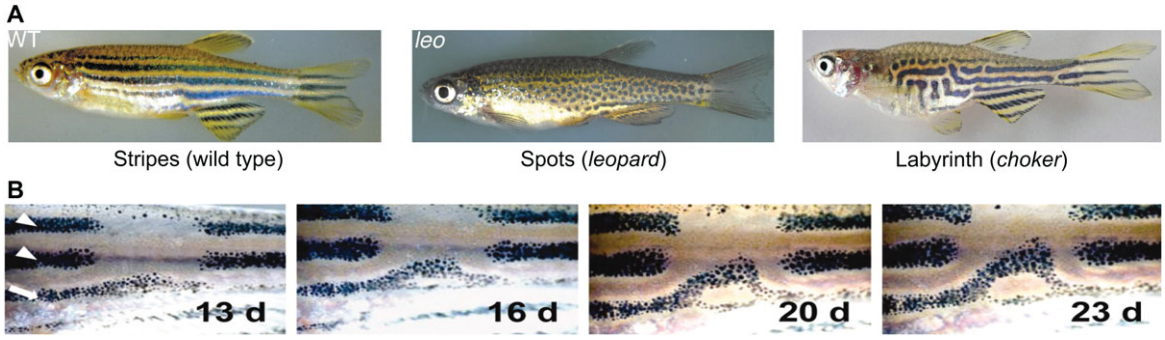

Fig. 5. Zebrafish pigment patterns. (A) Compared with wild-type stripes (left), particular mutants display spots (middle) or labyrinthine (right) final patterns. Reproduced with permission from Rawls et al. (2001) for wild type and *leopard* and from Frohnhöfer et al. (2013) for *choker*. (B) Cell movement is important during regenerative patterning: following laser ablation of melanophores within the central region, cells migrate into the ablated region to regenerate (aberrant) patterning. d, days after ablation; arrow and arrowheads indicate stripes. Reproduced with permission from Yamaguchi et al. (2007).

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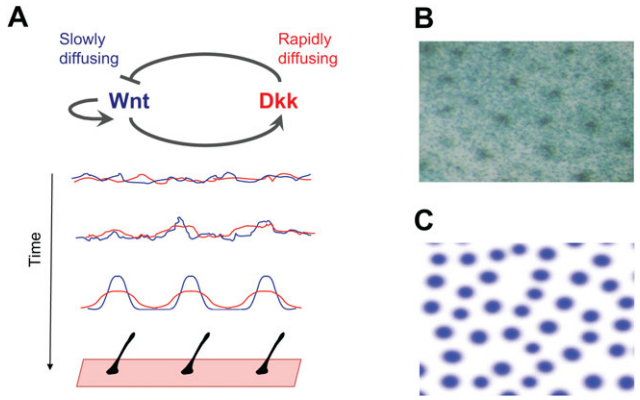

Fig. 6. Hair follicle patterning by a reaction-diffusion system. (A) Reaction-diffusion for a putative activator/inhibitor pair: Wnt/Dkk. Over time, fluctuations in Wnt/Dkk concentrations are amplified by 'local activation, long-range inhibition' until the concentration profile has regularly spaced peaks (Meinhardt and Gierer, 2000). These peaks define the position of future hair follicles. (B) *In vivo* pattern of murine hair follicles. Blue staining shows a *lacZ*-based Wnt reporter that marks the position of the hair follicles. (C) *In silico* pattern of hair follicles. (B,C) Reproduced with permission from Sick et al. (2006).

Several of the parameter constraints in Table 1 are completely independent of nonlinear effects. In other cases, the nonlinear effect is aggregated into a single parameter that is more easily measured, which we refer to as the 'sensitivity' (see Box 1 for a mathematical definition). Instead of measuring an entire input-output dose-response curve, the sensitivity parameterizes how much the output changes in response to a fold change in input; for example, a sensitivity of 2 means that a 1% change in input produces a 2% change in output. The inputs/outputs can be molecular, cellular or mechanical; for example, the input could be the concentration of a molecule and the output could be cell proliferation rate. Sensitivity is measured by observing the change in output corresponding to a single change in input and, importantly, requires only relative and not absolute levels to be measured.

It is therefore possible to falsify hypothetical mechanisms by measuring the parameter values in a given mechanism and determining whether they obey the appropriate parameter constraints. If the parameter constraints are not met, this implies that the hypothesis must be revised. This could include minor revisions – for example, incorporating stochastic effects can slightly relax the parameter constraint – but could also include major changes, such as implementing a three-component, as opposed a two-component, reaction diffusion model or even considering cellular and mechanical models of patterning. Recent experimental advances suggest that the relevant parameters, such as diffusion constants, molecular degradation rates and tissue material properties, along with sensitivities can be measured, or at least estimated, with reasonable precision *in vivo* (Akhtar et al., 2011; Campas et al., 2014; Müller et al., 2012). We illustrate this approach by considering the patterning of hair follicles in mammals.

Hair follicle patterning

The regular spacing between hair follicles is an example of periodic patterning and has been attributed to a reaction-diffusion mechanism in the epidermis (Huh et al., 2013; Nagorcka, 1983; Nagorcka and Mooney, 1985). Simulations show qualitative similarities between reaction-diffusion models and a final 'spotted' pattern of hair follicles, although, as we have argued in this article, this type of observation is not unique to a reaction-diffusion mechanism (see Fig. 6B).

Recent experimental studies in mouse have placed the reaction-diffusion model on a molecular footing by proposing Wnt and Dkk as the activator and inhibitor molecules (Fig. 6A) (Mou et al., 2006; Sick et al., 2006; Stark et al., 2007). Sick et al. (2006) proposed a Wnt/Dkk-based reaction-diffusion mechanism, showing that hair follicle density is reduced following overexpression of the putative inhibitor Dkk. In addition, Wnt signalling is necessary and sufficient for inducing hair follicles (Zhang et al., 2008) and is expressed in a periodic pattern prior to early morphological indicators of follicle specification, again consistent with a Wnt/Dkk reaction-diffusion model. However, these results do not test whether Wnt/Dkk can generate periodic patterns alone, as Wnt/Dkk might be cooperating with, or acting downstream of, other patterning mechanisms (including cellular or mechanical mechanisms). To test the reaction-diffusion hypothesis, we suggest that key properties of the system should be measured to determine if they are consistent with the parameter constraints in Table 1.

The first parameter constraint requires that the putative inhibitor Dkk diffuses more rapidly than the putative activator Wnt, thereby guaranteeing 'local activation, long-range inhibition'. Müller et al. (2012) successfully performed this type of experiment in zebrafish embryos by measuring diffusion coefficients of fluorescently tagged Nodal/Lefty (another activator/inhibitor pair), confirming that $D_{Lefty} > D_{Nodal}$.

Wnt/Dkk reaction parameters are also constrained. In particular, we suggest that the key parameter to measure is the sensitivity of Wnt autoactivation. In order for the Wnt/Dkk system to generate periodic patterns, the net production rate of Wnt must increase by more than 1% when extracellular levels of Wnt increase by 1%, which is a fairly stringent requirement. Such biophysical measurements and calculated parameter constraints would test the sufficiency of Wnt/Dkk reaction-diffusion to produce regularly spaced hair follicles; if the measured parameters are inconsistent with the model then other mechanisms must presumably be at play, which might, for example, involve other proposed activator/inhibitor pairs, such as Eda/BMP (Mou et al., 2006), or possibly include cellular and/or mechanical processes.

Response to perturbation

In addition to distinguishing broad classes of mechanism, as described above, perturbation experiments can be used to rigorously test a particular hypothesis. For example, if perturbation of a particular component has no effect on the pattern then we can conclude that it is dispensable in the patterning process, thus falsifying the hypothetical mechanism. However, if the perturbation does have an effect, its interpretation can be challenging.

The first challenge is that many perturbations commonly applied to periodic patterning mechanisms affect nonlinear processes. For instance, many perturbations to putative reaction-diffusion systems involve overexpressing or inhibiting a particular molecular pathway i.e. altering the nonlinear dose-response curve. As discussed previously, these nonlinearities are difficult both to measure and to interpret. It can therefore be challenging to (1) determine how the perturbation will affect the nonlinearity and consequently the final pattern and (2) to determine the extent to which this prediction relies on the particular form of nonlinearity that is assumed.

To address this limitation, we can use the dimensional analysis from the previous section, which identified perturbations that have effects that are independent of model non-linearities. For example, the prediction $\lambda \propto (D\tau)^{0.5}$ for reaction-diffusion models is independent of the specific assumptions of how the component molecules interact with one another. Therefore, observing an

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increase in pattern spacing when molecular diffusivities are increased is a good test of a reaction-diffusion model. By contrast, changes to dimensionless quantities, such as reaction sensitivities (e.g. by overexpressing activator/inhibitor molecules), are more difficult to interpret and could have multiple explanations (see supplementary material Section 7 for an expanded discussion).

As discussed previously, the suggested perturbations change the final pattern spacing by modifying the characteristic length scale in the system – for molecular mechanisms, this length scale is set by diffusion; for cellular mechanisms, either by cell motility or by the length of signalling protrusions; and for mechanical mechanisms, by tissue stiffness. Although easier to interpret, these perturbations are more difficult to apply than traditional knockdown or overexpression approaches, but are nonetheless feasible given recent experimental advances. For example, diffusion constants can be perturbed by adding extracellular diffusion modulators and, presumably, through the use of different fusion tags (Müller et al., 2012; Sarrazin et al., 2011; Wartlick et al., 2009).

A second challenge in designing perturbation experiments is that, in real biological systems, the periodic patterning mechanism receives input from a number of secondary processes, so that a given perturbation will often change multiple parameters in the system. For example, a common strategy is to overexpress a particular molecule or to knock it out genetically. In a complex biological system, these perturbations can often have pleiotropic effects on parameters other than the molecule that is directly perturbed. In addition, since many mechanisms fall into the same 'local activation, long-range inhibition' class, the same change in pattern can be caused by a number of different underlying parameter changes. For example, a change from stripes to spots may be due to changes in molecular production rates, molecular diffusion constants or mechanical properties of the material (Ermentrout, 1991; Miyazawa et al., 2010; Watanabe and Kondo, 2012; Zhu and Murray, 1995). Therefore, it is hard to rule out the possibility that the change from stripes to spots is due to the direct effect of the perturbation, as opposed an indirect, pleiotropic effect, and thus hard to rule out alternative mechanisms using this experiment alone.

One approach to overcome this difficulty is to design clean perturbations and to make control measurements to ensure that other components in the system are not perturbed. A second (and potentially more feasible) approach is motivated by our dimensional analysis. If it is possible to quantitatively measure how pattern spacing scales in response to perturbation, then it is also possible to test hypotheses in a parameter-independent way, ruling out the possibility that the perturbation is having an indirect effect on other parameters in the system. For example, in a diffusion-based system, we expect $\lambda \propto \sqrt{D}$. If square root scaling is observed (i.e. if we measure a scaling exponent n=0.5), this strongly supports a role for diffusion during patterning. If instead we find that pattern spacing does not scale this way (but is still affected by changes in diffusion constant), there are two possibilities. First, it is possible that the mechanism is molecular in nature but the transport of this molecule is not predominantly diffusive; for example, if advection (or flow) is also present, a scaling exponent n=1 is expected. Alternatively, if the pattern spacing does not scale but instead changes in an irregular way with diffusion constants, this could be due to indirect effects of the perturbation (e.g. changes in cell motility or ECM density), and therefore additional controls explicitly examining these indirect effects are necessary.

Conclusions

Over 60 years ago, Turing showed that a deceptively simple system comprising two interacting and diffusing molecules could generate a wide variety of periodic patterns. Since then, a series of extensions, adaptations and alternatives to the reaction-diffusion model have been proposed, all of which are capable of producing periodic patterns. How, then, do we determine which of these many mechanisms is responsible for a particular periodic pattern?

In this article, we have outlined the reasons that make this a challenging question to answer. Our analysis suggests that the observation of a final periodic pattern cannot distinguish between mechanisms, since many mechanisms are qualitatively similar. Instead, we sought experiments that either (1) are designed such that the basic classes of mechanism have different expected outcomes or (2) rigorously test assumptions in the patterning mechanisms. We have explored several mathematically inspired approaches to guide the design of such experiments.

These approaches rely on measuring, perturbing and/or imaging the processes of pattern formation, and not simply defining the genes involved. These types of experiment are technically challenging. However, we propose that they may now be achievable given recent technological advances, which include *in vivo* biophysical measurements (Spiller et al., 2010), *in vivo* genome editing (Cong et al., 2013), quantitative perturbations to the tissue microenvironment (Cushing and Anseth, 2007), continuous time-lapse imaging (Amat and Keller, 2013; Garcia et al., 2011; Megason, 2009) and *in vitro* models of periodic patterning (Yamanaka and Kondo, 2014). We hope that these techniques, combined with the approaches outlined in this article, will allow us not only to generate hypotheses for periodic pattern formation, but also to rigorously test them.

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Competing interests

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Supplementary material

Supplementary material available online at
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