On the principles of multicellular organism development

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Summary. Non-equilibrium physics has traditionally dealt mostly with inanimate matter. Yet, in the last decades there has been increasing interest in understanding living systems from this perspective. One example is using the framework and tools of non-equilibrium statistical mechanics and nonlinear physics to study how living organisms composed of many differentiated cells develop from a single initial cell. The dynamic process of multicellular organism development is out of equilibrium, in that it consumes and dissipates energy. It also involves the formation of many precise and complex structures. Herein we review some of the paradigms being used that focus on how these multicellular structures initially emerge at the molecular level. [**Contrib Sci** 11(2):215-223 (2015)]
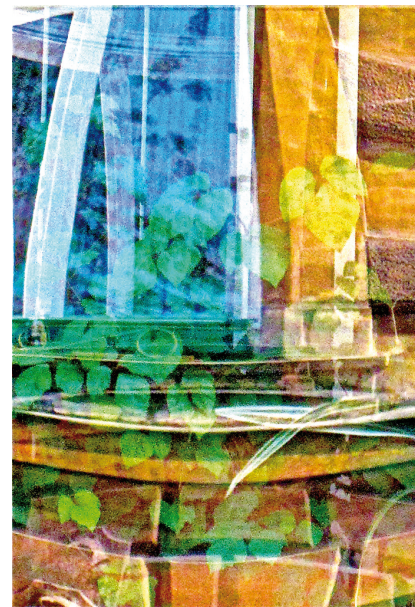
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A historical overview

An example of the beauty and complexity of Nature is the development of multicellular organisms. Animals and plants develop from a single cell, which through division gives rise to all the cells of the organism. During development, these cells become distinct in an organized and precise manner to robustly form complex structures such as organs. How does this occur? What are the principles behind it? Many physicists are now engaged in investigations of multicellular organism development, with the aim of understanding how it proceeds and finding its fundamental principles. Resolving these questions is expected to help shed light on more applied challenges ranging from biomedical issues, such as embryonic malformations and cancer, to agricultural issues, such as the optimization of crop growth. However, the quest for underlying principles is still in its own early developmental stage, and an immense universe of knowledge lies ahead. In the following, we consider some of the ideas and insights that appeared early on and that have influenced current research.

Over 70 years ago, Conrad H. Waddington used the metaphor that during development cells roll down through valleys that bifurcate [25], having to choose what to become at each bifurcation. This metaphor for cell differentiation is now commonly used, with a free energy landscape of which Waddington's valleys are the minima.

Alan Turing, very well known for his contributions to computer science, proposed, in a seminal work published in 1952, that patterns arising during development might be the natural output of chemical reactions between molecules that diffuse with different diffusion coefficients across space [23]. That chemical systems can form spatiotemporal patterns, in which the concentrations of molecules are organized in space and time, was proven later in well-controlled chemical and physical assays. However, these were not linked to multicellular organism development, but instead drove intense research in the field of nonlinear dynamics. The relevance of this mechanism, known as Turing's instability, in the context of development is now appreciated but still debated [15,18,19].

In 1970, the Nobel Laureate Francis Crick proposed that the diffusion of molecules could create gradients across developing tissues [4]. These gradients could convey to the cells the positional information that Lewis Wolpert had already proposed [26], guiding them in their further development. This is the morphogen gradient paradigm, which has dominated research on patterning in developmental biology. The finding that numerous molecules form gradients during development and that the gradients themselves are relevant for the development of different tissues has led to many other complex questions: How does the gradient form? How is it sensed? And what information from the gradient does the cell use?

Stuart Kauffmann showed that the interactions between genes strongly restrict the possible cell types [14]. In this case, cell types are understood as the attractors of the dynamics of genetic interactions. At present, deciphering the large gene regulatory and signaling networks and their dynamics in a developing cell is an intense field of research.

These conceptual frameworks, i.e., bifurcations to produce changes of cell types, self-organization out of equilibrium and cell types as attractors, were mathematically formulated and developed. However, in the last decades of the 20th century, the use of mathematical formulations to understand development became unpopular because they failed at describing and predicting patterns. The result was a split between developmental biologists and physicists/ mathematicians [16]. More recently, however, knowledge of

which biological molecules participate in development, the ability to manipulate them, and their spatial and temporal resolution, have increased dramatically. At the same time, important progress has been made in non-equilibrium statistical mechanics, dissipative systems, complex systems, nonlinear dynamics, and networks, accompanied by an extraordinary increase in computational power. As a result, interdisciplinary research involving both physicists and biologists has become more common and the advantages to this approach are now acknowledged [20]. Thus, we are in an exceptional position to embrace the challenge to understand development and the principles behind it.

Patterning the embryo

A crucial step in understanding how multicellular organisms develop is to unravel how cells become distinct in a coordinated and organized manner. In the language of developmental biology, this can be rephrased as how a cell attains a specific fate into which it ultimately differentiates. Two main mechanisms have been proposed for coordinated cell differentiation in tissues. One mechanism is through positional information, proposed by Lewis Wolpert as mentioned above [26]: the fate of a cell is a readout of its spatial localization from a reference system (Fig. 1A). Cells read the information of where they are located and differentiate accordingly. Gradients of molecules, previously referred to as morphogens (we retain this term here for convenience), have been proposed to confer such positional information. The origin of the reference system is the source where the morphogen is produced. The amount or concentration of the morphogen decays as the distance from the source increases and thereby conveys positional information to the cell. This information can be conferred to cells through molecules that become activated at distinct thresholds of morphogen concentrations (Fig. 1A). There are multiple proteins that have been shown to be distributed along gradients in different developing embryos and that seem to convey positional information. Specifically, if the gradient is altered, the fate of the cells changes accordingly (Fig. 1B). This is the case, for instance, for the protein Bicoid, which forms a gradient along the anterior-posterior axis of the embryo during the very early stages of insect development, including that of the fruit fly *Drosophila* [10]. The region where Bicoid is at high concentration becomes the head of the fly.

The other proposed mechanism is that cells become distinct only because of coupling. This is an example of
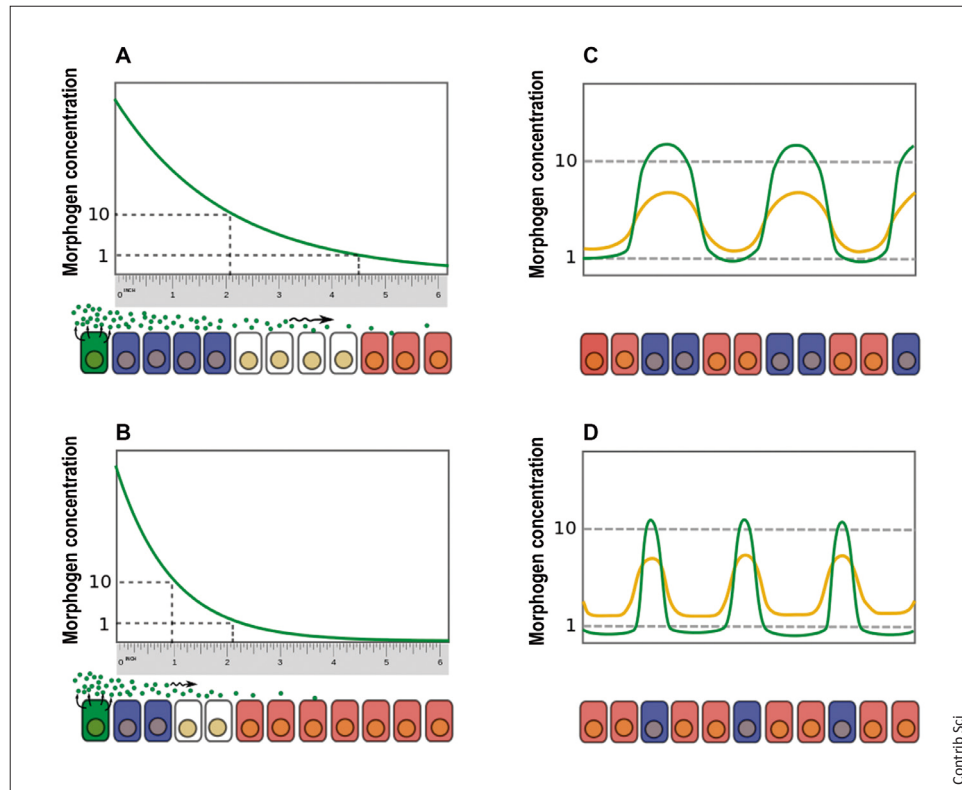
Fig. 1. Pattern formation mechanisms that rely on diffusion along an extracellular medium. (**A–B**) *Morphogen gradient mechanism.* The leftmost, green cell generates a molecule that acts as a morphogen. The molecule diffuses to the right and generates a gradient, as shown in the curve above the cells. The amount of morphogen sensed by each cell conveys positional information to it. There are two thresholds, one at concentration 1 and another at concentration 10, and cells differentiate depending on whether the concentration is above or below these thresholds. In **A** there is extensive diffusion, as indicated by the larger curvy arrow. In **B**, there is less diffusion, altering the gradient and the position of cell types accordingly. (**C–D**) *Turing pattern mechanism.* Two or more chemicals that diffuse and react are needed to establish a pattern. In **C** the pattern for certain values of the parameters is shown. In **D**, when diffusion is modified so is the pattern and the corresponding cell fates. (Note that the term "morphogen" is no longer used with the mechanism shown in C and D. We use the term here because it was introduced by Turing precisely in this context).

self-organization in which a structure or order emerges spontaneously because of the interactions between elements. Coupled dynamics enable the emergence of robust proportions and periodic distributions of cell types. In contrast with the positional information mechanism, coupling does not drive a specific cell type in a given spatial position. In a developing organism this self-organization can happen in different ways. The first one corresponds to the dynamics Alan Turing studied [23]. When chemicals initially distributed homogeneously throughout a given space react and diffuse, they form heterogeneous distributions. Because the reactants diffuse with different diffusion coefficients, tiny small random fluctuations in the reactant concentrations become amplified, such that the homogeneous state becomes destabilized. This happens for a wide range of diffusion coefficients and reaction kinetics. It is an example of a non-equilibrium pattern formation process, in which the

balance between antagonistic processes, such as driving and dissipation, results in the formation of non-homogeneous structures [5]. Thus, for instance, periodic stationary distributions of the molecules can emerge. Cells produce proteins, which react and diffuse in the extracellular space. Accordingly, when a periodic pattern of protein distributions emerges from these dynamics, some cells end up producing or sensing large amounts of proteins while others do not. Therefore, cells become distinct (Fig. 1C,D). A change in the spatial interactions, as in the diffusion coefficient, results in relevant changes of the molecular pattern being formed. Accordingly, the pattern, if periodic and stationary, can change its periodicity (Fig. 1D). Empirical evidence that such a mechanism can drive the formation of the digits in vertebrates has recently been provided [21]. The digits form from an initial rather two-dimensional round palette. In this palette a stripe-like pattern emerges that divides it into two

intercalating regions: interdigital and digital regions. In this specific case the reaction-diffusion mechanism does not act in isolation but it is coupled to a positional information mechanism.

Another way that could drive the differentiation of cells in a self-organized manner but does not require the transport of a molecule is through direct cell-to-cell contact. In this case, cells interact through molecules present on the cell membrane that, upon binding, send signals to the cell nucleus. An example of this is lateral inhibition with feedback [3]. In this case, the signal a cell receives arises from protein ligands in adjacent cells and it decreases the amount of ligand in the cell. Thus, a cell that has more ligand than its neighboring cells, even if the difference is very small, will reduce their amount of ligand and, at the same time, increase its own ligand production by preventing inhibition by those neighbors. Ultimately, the cell with an initially very small excess of ligand will end up with a relatively large amount of that ligand, while ligand in neighboring cells will be almost completely eliminated. This type of interaction underlies the specification of neurons, for instance.

In the 1970s, Meinhardt and Gierer proposed a theory for biological pattern formation based on two elements: (1) self-activation and (2) long-range inhibition [9]. Turing-like reaction-diffusion dynamics and lateral inhibition with feedback can both be understood in terms of these two elements. Moreover, self-activation evidences a key aspect in the dynamics of coupled elements that drive patterning: nonlinearities. All these self-organizing interacting dynamics drive the emergence of robust proportions and periodic distributions of cell types. In this mechanism based on coupling, the cell types arise in a coordinated manner but, unlike in the positional information mechanism, it does not enable the robust specification of a cell type in a given spatial position. Nevertheless, if spatial asymmetric cues are added to interacting dynamics, then spatial precision can arise as well.

It is worth noting that how the pattern will be modified when the elements driving it are altered can be predicted by constructing mathematical and computational models of the dynamics. The resulting predictions can then be used to test whether assumptions regarding the mechanism of patterning are correct, by comparing the predicted results with the empirically derived data. This task is nowadays common routinely done but it has not always been so easily possible. Now we can propose which specific molecules are acting and, in several cases, we can experimentally see how their distribution changes over time and space with detailed resolution. Manipulations of the interactions and reactions and how the molecular distribution changes accordingly can now be done and the results measured.

The mechanisms described herein assume that, in terms of their patterning, cells can be described by only a few relevant molecules. The role of cell dynamics and the particular mechanical forces that are active are not taken into account. This simplification is valid in some circumstances, especially when the dynamics that control the molecular concentrations are much faster than those of the cell. Many efforts are being done on the role of mechanical forces in shaping developing multicellular organisms, which are not reviewed herein. A challenge that remains is to determine how mechanical forces and the dynamics of the molecular components that direct cell signaling or impinge on gene regulation are coupled to each other.

Nonlinear responses

We have discussed how molecular gradients can confer positional information, in which each cell type is dictated by a threshold, cell-type-dependent, morphogen concentration. In Fig. 1A, cell type "blue" is induced above a morphogen concentration of 10 (arbitrary units), whereas cell type "white" is induced above a morphogen concentration of 1. Yet, is this type of threshold response possible in biological systems? It is, thanks to ultrasensitivity. As opposed to a gradual or linear response, in which the relative changes in input (signal) and output (response) are equal, an ultrasensitive response is that in which a small relative change in the signal generates a very large (relative) response. Since a cellular response usually saturates (i.e., when the input signal is large enough, the response no longer changes), an ultrasensitive response in cells can translate to a threshold or "all-or-nothing" response (Fig. 2A).

But how is this ultrasensitivity achieved by cells? A variety of mechanisms have been elucidated through mathematics and then experimentally demonstrated [27]. A few of them are summarized in Fig. 2 and reviewed in [27]. "Zero-order ultrasensitivity" was the first of these mechanisms to be proposed, in 1981 [11]. In this mechanism, an enzyme covalently modifies a protein (covalent modification is a common regulatory mechanism in which a molecule such as a phosphate or methyl group is bound to a protein by an enzyme), and an opposing enzyme restores the protein to its unmodified state. When both enzymes are working at saturation, a small change in the amount of one of them can

produce a large change in the proportion of the modified and unmodified proteins, thus enabling an ultrasensitive response (Fig. 2B).

Another, very common mechanism is multistep signaling, in which an element representing the signal, or proportional to the signal intensity, acts on two or more elements that independently affect the strength of the response. An example is a signal that acts on two different steps of the modification of a protein that will ultimately turn it into its active form. The multiplied effect elicits an ultrasensitive behavior. Mathematically, the repeated effect of the signal is represented as multiplicative terms that can raise it up to the power of the number of points at which the signal affects the system independently (Fig. 2D).

Direct or indirect self-activation, also known as positive feedback, can drive ultrasensitive responses as well. Positive feedback occurs, for instance, when a protein binds to its own DNA promoter to boost its own transcription (auto-activation), or when a protein inhibits the production of its inhibitor (mutual inhibition) (Fig. 2F).

Bistability

A positive feedback loop can also enable bistability, i.e., two different responses to the same input (mathematically, the equation that represents the system has two stable solutions instead of one). In other words, genetically identical cells exposed to the same environmental conditions can be in two different states and hence become two distinct cell types. An example of bistability in development occurs in the vulval development of the hermaphroditic nematode worm *Caenorhabditis elegans* [10,12]. Before this egg-laying organ is formed, two adjacent cells, which can be labeled 1 and 2, for instance, become distinct from each other based on their position in the embryo. One becomes an anchor cell (AC) and the other a ventral uterine (VU) cell. Each cell has a 50% probability of becoming an AC. Hence, under the same conditions two states can arise, with 50% probability each: (AC,VU) or (VU,AC), in which the first term within the parentheses denotes the type acquired by cell 1, and the second term refers to cell 2. In this case, the bistability of these two states arises through a positive feedback that involves the above-described lateral inhibition with feedback. Nonlinearities are essential for this bistability. Figure 3 provides an example of this case and shows how a mathematical model of the interactions can help us to understand and visualize this process.
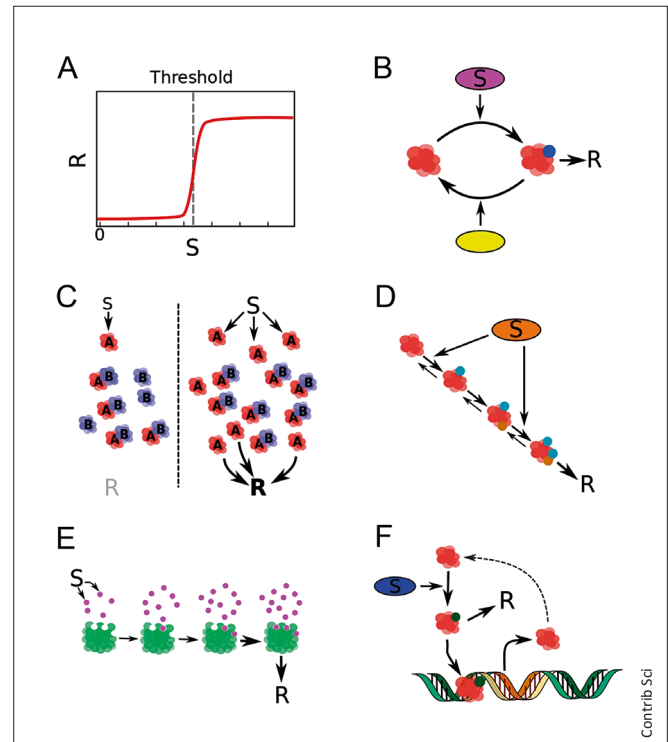

Fig. 2. Mechanisms that generate ultrasensitivity. (**A**) A signal-response function showing ultrasensitivity and an all-or-nothing response, as shown in panels **B**, **D**, **E**, **F**. (**B**) *Zero-order ultrasensitivity.* As explained in the text, the purple enzyme, corresponding to signal S, enhances the covalent modification of the red protein, while the yellow enzyme mediates its de-modification. The modified protein amount corresponds to the response R. (**C**) *Molecular titration.* Free molecule A (corresponding to or activating a response R) can be sequestered by B, which is present in very large amounts. Molecule A exhibits an ultrasensitive response to changes in its production. There are free A molecules only when their production level surpasses the sequestering effect. At this threshold, the amount of A suddenly increases. This behavior is not like that shown in A, because its response does not saturate. (**D**) *Multistep signaling.* The signal S, or some element proportional to it, aids in two different steps of the modification of a protein that will ultimately assume its active form, which then enacts response R. Its effect is multiplied and can elicit an ultrasensitive response. (**E**) *Cooperative binding.* A receptor, in green, has several binding sites for the same ligand, the amount of which corresponds to signal strength S. If full occupancy of the receptor's binding sites is needed to elicit a response R, or if each occupied site increases the chance that a new ligand will bind (thicker arrows indicate larger amounts of bound ligand), ultrasensitivity arises. (**F**) *Positive feedback loop.* A signal S (here a blue enzyme) activates a protein (in red). This active protein elicits response R, but it can also bind to DNA and enhance the production of its own unmodified form. This increases the amount of substrate upon which the signal can act, multiplying its effect and making the response ultrasensitive. These mechanisms are reviewed in [27].

Fluctuations

As we have seen, cells have mechanisms to process signals coming from neighboring cells and from their surroundings that can yield precise results. However, these signals cannot
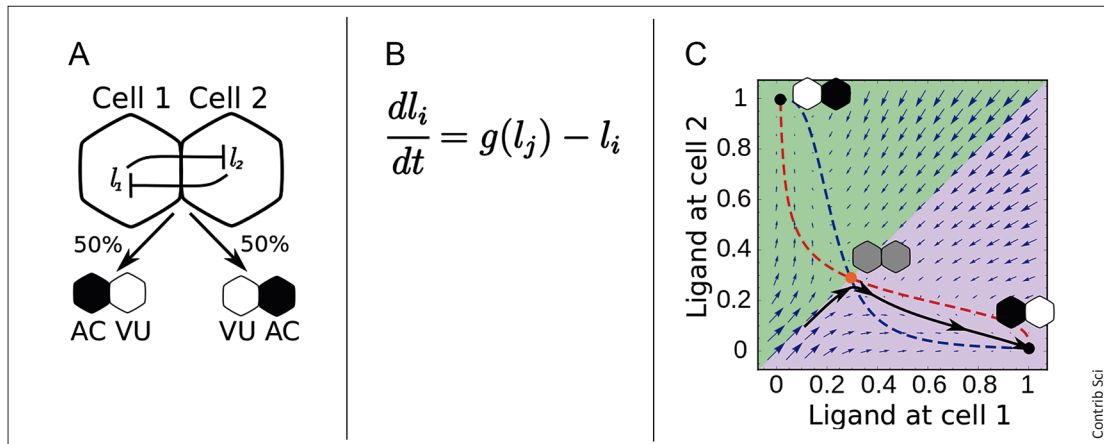
Fig. 3. Bistability. (**A**) Lateral inhibition with feedback. The ligand in cell 1 inhibits the ligand in neighboring cell 2 and vice versa, establishing positive feedback. Inhibition is represented by the blunt arrows. There is a 50% probability for the (AC,VU) outcome and 50% for the (VU,CA) outcome, determined by which cell achieves a high or low amount of ligand. (**B**) The equation that governs the temporal evolution of a ligand in cell i (1 or 2). dl_i/dt is the time derivative of concentration l_i and represents its changes over time. The production term $g(l_j)$ decreases nonlinearly when l_j (the ligand in the other cell) increases. (**C**) Phase diagram of this two-cell system. Each point corresponds to a unique pair of $l_1 - l_2$ values. The evolution of either one is fully determined and shown by the blue arrows of the vector field. The red and blue dashed lines are called nullclines and correspond to the points at which the time derivative, i.e., the rate of change, for the ligand at one of the cells (blue for cell 1 and red for 2) is zero. At the points where the nullclines cross both derivatives have the values of zero, so the system, if unperturbed, will not move away from them. Because of the nonlinearity of the nullclines, there are three of these points; if they were not nonlinear, there would only be one such point. Of these, the black points are stable states: when the system is at one of them, it will return to it after a small perturbation (this state is therefore also called an attractor). Indeed, all trajectories starting in the purple half of the portrait (called the basin of attraction) will evolve towards the (AC,VU) stable state at the bottom left (one such trajectory is shown in black). Similarly, the green area is the basin of attraction for the (VU,AC) stable state. The orange point represents a state with intermediate values of ligand for both cells, as shown in gray, that is not stable. A small perturbation from this state can lead the system away from it and to one of the stable solutions. The scenario in **B** was obtained from simulations performed by Juan Camilo Luna-Escalante, Dept. of Condensed Matter Physics, University of Barcelona). The data are used with permission.

be sensed with perfect precision due to the physical laws that govern molecular dynamics [2]. These signals, and the proteins that process them, consist of discrete molecules that jiggle around, embedded in the thermal bath of the cytoplasm. This aqueous medium is crowded with many moving molecules such as proteins. Some molecules move stochastically without a preferred direction, because of thermal forces coming from collisions with water molecules. Others, such as molecular motors, move directionally using electrochemical forces. Several of these electrochemical reactions have associated energies (such as the energy required for some reactions to start, or the energy required to break specific chemical bonds) comparable to the thermal energy of the medium. Therefore the stochastic "jiggling" of molecules can spontaneously activate reactions or break chemical bonds.

These fluctuations also affect the production and degradation of different proteins in the cell, which stochastically vary in time. This could not be directly observed until the very recent advances in the spatial and temporal resolution of fluorescence microscopy techniques. Before that (but also only recently), temporal fluctuations in the

amount of specific molecules could only be inferred from the heterogeneous amounts found among genetically identical cells in the same environment. Even though fluctuations are a common object of study in non-equilibrium and statistical physics, our direct knowledge of the motion and fluctuations of particles embedded in the crowded medium of a cell is still incipient. Yet, with the advent of nanotechnologies we are entering a new era in which it will be possible to characterize the motions of and fluctuations in cellular components.

Fluctuations and cell decisions

Because fluctuations are ubiquitous in the cell, they must somehow be relevant to an understanding of all cellular processes, including those in the previously mentioned examples of morphogen diffusion, cellular sensing of these molecules, and the related signaling processes. The exquisite precision and regularity of developmental processes indicates that cells can cope with this variability, or perhaps even profit from it.

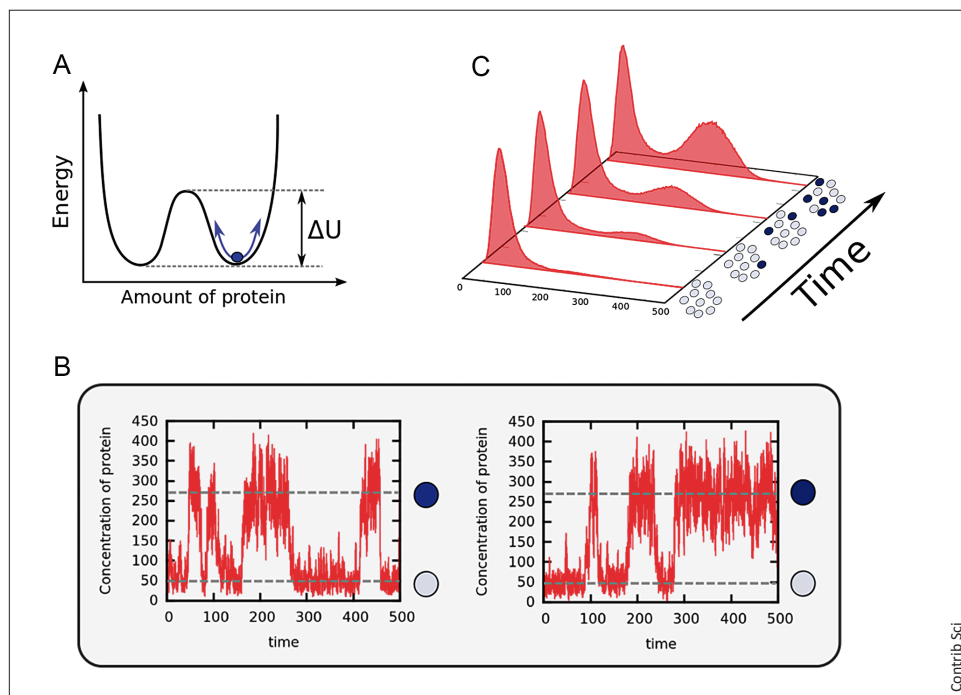One obvious way of avoiding the effect of fluctuations is by producing large amounts of molecules to minimize

Fig. 4. Stochastic switching. (**A**) A model of a bistable system. The black continuous line is the energy landscape of the system; the bottoms of the two wells are the stable states. The blue circle represents the system at one of these states, and the blue arrows the fluctuations, which can drive the system to higher energies. If the fluctuations are large enough, or the energy barrier (ΔU) low enough, the system can jump to the leftmost well and switch states. (**B**) Time evolution of the amounts of a protein for a single cell in two different cases. There are two clearly defined states, a high concentration state at 270 protein copies and a low concentration state at 50 copies. The cells switch from one state to the other. Note that the transitions are very fast and that the system spends most of its time around one of the two stable states. (**C**) Evolution over time of the concentrations of a protein of interest in a cell culture, as shown in a histogram. When a subpopulation in one of the states from an originally bistable population is separated and left to evolve over time, stochastic switching allows the recovery of the two states. The cells in a population are shown on the right. Note how one cell may switch states more than one time. Panels **B** and **C** are simulations of a mutual inhibition system, simulated through the Gillespie algorithm, which allows exact simulations based on the theoretical description of discrete stochastic systems in the form of master equations.

their effects. This is not always worthwhile, or possible. For instance, when a cell receives a fluctuating signal that it cannot control, how can it cope with the fluctuations? One way to buffer fluctuations is to respond to the amount of signaling molecules received only during an interval of time [2]. This corresponds to an integration over time of the number of molecules, the result of which is much less variable than the number of molecules at any given time. Therefore, the input to which the cell responds is not the highly fluctuating number of molecules but the much more constant total number of molecules received per unit time. This time integration is performed, for instance, by bacteria to sense the level of nutrients in their environment [2]. It also is the mechanism proposed for developing embryos, in the cellular response to morphogen gradients [6]. In cases in which cells respond too rapidly compared to the time interval that would be required for integration to filter out fluctuations, the additional interactions of neighboring

cells may reinforce the correct cell decision and increase its robustness [13].

There are several examples of biological systems that profit from fluctuations [7]. Most of them are in unicellular rather than multicellular developing organisms, but their existence can suggest that fluctuations may also be used during development. For instance, fluctuations enable wide-ranging heterogeneity between genetically identical cells in the same environment. This heterogeneity can be beneficial when the environment changes rapidly and the cellular response is heterogeneous. If this heterogeneous population of cells comprises different cell types that respond differently, then when the environment changes some of the cell types may die while others will prevail. Because of this heterogeneous response to environmental change, the cell population persists, providing a benefit. This is known as bet-hedging (the colony of cells hedges its bets instead of putting "all of its eggs in one basket") and has been described in

different types of bacteria [24]. Fluctuations in the number of molecules can drive large heterogeneities among cells in different ways. One is through positive feedback, which can drive the molecule to be present at either high or low concentrations. These two concentration states can be understood, at least conceptually, as free energy minima and are separated by an energy barrier [1]. Dissipation drives the molecular concentration to reach one of these two states and remain there forever after. Which concentration is achieved depends on the initial state; that is, on which concentration was present initially. This scenario changes when we take into account that there are fluctuations. They provide the energy required to surpass the energy barrier that separates the states, allowing a switch from a low to a high concentration or vice versa (Fig. 4).

An example of heterogeneous cell populations comes from experiments using mouse embryonic stem cells (ESC), which in culture express pluripotency factor NANOG in a highly stochastic manner [8]. NANOG allows ESCs to self-renew and to maintain their pluripotency. When NANOG levels of individual ESCs in a culture are measured, the distribution of values is very broad. If cells with, for instance, low NANOG expression are selected, separated from the others, and allowed to divide over time, measurements show that the very broad distribution of NANOG concentrations is eventually recovered. Hence, some cells, despite initially being in the low NANOG concentration state, have clearly switched and now express very high concentrations of NANOG. Whether this stochastic switching corresponds to bistable or other type of dynamics is a current topic of research.

A role of fluctuations in multicellular development has been proposed for cells that need to establish a pattern that is not spatially ordered but, instead, only needs to preserve certain proportions of different types of cells, randomly spaced around the tissue. A stochastic decision mechanism has been proposed for processes such as the differentiation of different photoreceptors in the retina of humans and flies, or of olfactory cells in the mouse [17]. Mice have 1000 olfactory proteins, with only one expressed in any given cell to avoid sensory confusion. Hence, initially equivalent cells become distinct, reaching one of 1000 different states. This has been proposed to be accomplished by the activation of one olfactory protein type stochastically and subsequent inhibition of all the other remaining types of olfactory proteins. In addition, fluctuations of molecular components can be expected to trigger patterns arising from interacting self-organizing dynamics such as reaction-diffusion and lateral inhibition.

Our knowledge on the effect and role of fluctuations in developmental processes is still limited. However, research in physics over the last few decades has evidenced that nonlinear systems can take advantage of fluctuations [22]. Thus, it is to be expected that developing organisms, which exhibit highly nonlinear dynamics and are subject to fluctuations, profit from them as well. The concepts and tools to study this topic have already been developed by physicists and biologists, and the results should soon be available.

Conclusions

The development of multicellular organisms is subject to the physical laws that govern Nature. It is indeed because cells live out of equilibrium that they are able to create the myriad of rich and complex structures that form multicellular organisms. Insights have been gained into some of the molecular gene regulatory and signaling mechanisms used by cells in the spatially and temporally coordinated processes that allow them to become distinct in an organized and reproducible manner. These processes require nonlinear responses and dynamics. Previously, development was mostly understood as a succession of stationary states and many aspects were described through averages over many cells. However, we now have strong evidence that development is a highly dynamic process and that cellular dynamics are strongly stochastic. Although many technical limitations to advancing our knowledge remain, new data are expected that will reveal the highly complex and dynamic nature of developing organisms. As physicists, we expect to continue to work together with biologists to define the principles that govern multicellular organism development.

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