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REVIEW ARTICLE

# From single cells to tissue self-organization

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Self-organization is a process by which interacting cells organize and arrange themselves in higher order structures and patterns. To achieve this, cells must have molecular mechanisms to sense their complex local environment and interpret it to respond accordingly. A combination of cell-intrinsic and cell-extrinsic cues are decoded by the single cells dictating their behaviour, their differentiation and symmetry-breaking potential driving development, tissue remodeling and regenerative processes. A unifying property of these self-organized pattern-forming systems is the importance of fluctuations, cell-to-cell variability, or noise. Cell-to-cell variability is an inherent and emergent property of populations of cells that maximize the population performance instead of the individual cell, providing tissues the flexibility to develop and maintain homeostasis in diverse environments. In this review, we will explore the role of self-organization and cell-to-cell variability as fundamental properties of multicellularity—and the requisite of single-cell resolution for its understanding. Moreover, we will analyze how single cells generate emergent multicellular dynamics observed at the tissue level 'travelling' across different scales: spatial, temporal and functional.

## Self-organization during tissue formation, homeostasis and regeneration

Multicellular organisms are composed of cells and tissues with identical genomes but different properties and functions. They all develop from one cell toward multicellular structures of great complexity. On a series of carefully organized steps in space and time, different cell types, architectures, and functions are formed during embryogenesis and development. In adult life, maintaining tissue homeostasis, via periodical tissue renewal and regenerative processes, also requires spatio-temporal coordination of cells to ensure tissue function and integrity. Moreover, the malfunction of these coordinated behaviours during embryogenesis is the cause of many congenital disorders and their deregulation during adult life in actively proliferating and regenerating tissues, such as the intestine, is the basis of many cancers [1,2].

These spatio-temporally organized processes in multicellular organisms are known as collective behaviours. In the early steps of embryogenesis, cells in a seemingly symmetric embryo reorganize themselves collectively into a patterned arrangement giving rise to primitive tissue specification [3,4]. Later into organogenesis, the majority of multipotent dividing cells commits to differentiation and acquires specific functionalities, while only a fraction retains stemness [5]. Once development is complete, cells in a tissue must be able to sense organ size and functionality to stop proliferating, and yet, in some cases, retain a minimal level of stemness for homeostasis and a potential to regenerate upon injury [4,6].

**Abbreviations**
ESCs, embryonic stem cells; PI, phosphatidylinositol; PSM, presomatic mesoderm.

Although the molecular machineries governing these processes are determined genetically, thus making them precise and reproducible, the genome alone does not encode for the complex cellular interactions required to keep them robust and contextual in dynamic environments. To understand biological processes such as development and regeneration, we must understand how a group of individual cells organize themselves into patterns and tissues [4,7,8]. In many developing organisms, the patterns are driven and maintained by concentration gradients of signals, named morphogens, and each individual cell in the tissue senses its position along the morphogen gradient and responds accordingly [8]. Generally, morphogens are released from a local, but dynamic source, and the gradient shape is determined by the flux from the source, the spreading of the morphogen (e.g., composition of the extracellular matrix and transcytosis) [9] and its degradation in the target tissue. Interestingly, morphogen gradients, downstream signaling, and the activity of cell-intrinsic gene networks respond dynamically to the local environment by sensing complex extracellular cues [8]. This means that the precision and robustness of pattern-forming systems requires not only pre-existing morphogens but also spatio-temporally coordinated self-organized processes (Fig. 1A).

Self-organization is a process in which interacting entities organize and order themselves in global and larger scale patterns [10,11]. Order appears not because it has been planned by a central controller but because local interactions between individual cells generate complex functional patterns such as tissues and organisms. At the individual level, no cell knows the complexity of the overall structure. Self-organization is not restricted to developmental processes. In adult organisms, the regeneration of tissues is also an emergent self-organized property of cells. After an injury, local interactions between different cells drive the healing and repair of the tissue without any single cell knowing how the final tissue should look like at the global scale. To achieve these coordinated and self-organized process, each individual cell has molecular mechanisms to sense its local environment and respond correctly to injuries, recreating a healthy tissue [10].

In this review, we explore the role of self-organization and cell-to-cell variability as fundamental properties of multicellularity. We discuss cellular mechanisms by which single cells sense their local environment in a multicellular system driving collective behaviours during

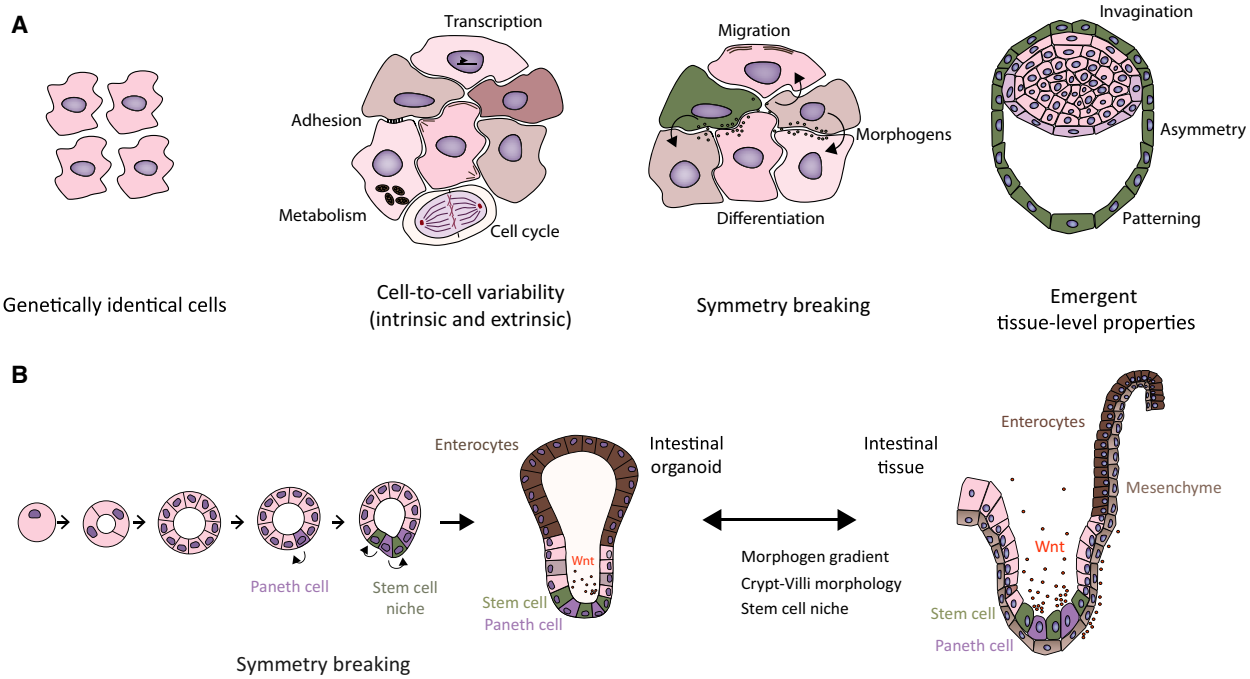


**Fig. 1.** From a population of single cells to tissues. (A) Heterogeneous population of cells where each single cell senses a combination of cell-intrinsic and cell-extrinsic cues, ultimately driving tissue patterning. (B) Spatio-temporal variability and symmetry breaking in intestinal organoids. An intestinal stem cell develops into a symmetrical cyst and undergoes symmetry breaking with the appearance of Paneth cell. This Paneth cell defines the position of the nascent crypt where the stem cell niche will reside. The intestinal organoid develops into a self-organized structure containing different cell types distributed in a zonated manner recapitulating part of intestinal patterning.

developmental and regenerative processes. Finally, we provide an overview on current technologies that cross-scales at the spatial, temporal, and functional level to bridge the gap between single cells and organized tissues.

## Sensing mechanisms in a population of interacting single cells

An important question during development is how does a single cell in a tissue sense its complex environmental cues and take individual cellular decisions generating robust and reproducible emergent properties at the population and tissue level?

### Number of cells and cell-packing effects

Eukaryotes evolved many different ways to sense the number of individual cells in a population including global and local mechanisms. One mechanism to generate a defined number of cells in a population is to count the number of cell divisions, as described in Mid Blastula transition in *Xenopus laevis* [12] and in mammalian hematopoietic stem cells [13]. Another way is to rely on chemical information, such as a signal that is secreted locally and sensed globally by other individuals in the population. Morphogen gradient is a conserved strategy in different animals: from the simple counting peptide in *Dictyostelium discoideum* (secreting a 'cell-counting' factor) [14], toward Dpp gradient in wing tissue development in flies [15,16] and Wnt3a gradient along the mouse intestinal stem cell niche [17]. In addition, a classical environment sensing mechanism that operates at local scale is contact inhibition. MDCK cells, *in vitro*, have been shown to compute local information on cell density, motility, and cell division rates to trigger contact inhibition [18]. Many other mechanisms have been identified in regulating contact inhibition such as increased Clusterin secretion [19] and E-cadherin-mediated control of cell proliferation via cell-cell contact [20].

Cells can also sense and transduce extrinsic physical cues from the microenvironment such as cell-packing effects. This mechanosensing capability relies on membrane tension sensing pathways such as Yap1 [21,22], Piezo [23,24] and Misshapen-Yorkie pathway [25]. Understanding how single cells perceive tissue size and function is also essential for growth termination and for successful completion of developmental and regenerative processes. Tissue damage triggers activation of stem cell division and differentiation to replenish lost cells, but this activation must be timely repressed once tissue integrity is restored to limit tissue hyperplasia [26,27].

### Localized signals

Lumen formation is also an important mechanism that cells use to measure their environment by locally restricting and, thereby, coordinating communication between selected groups of cells. In zebrafish lateral line development, the formation of microlumens in a population of migrating cells restricts and enhances FGF signaling only in cells limiting the lumen [28]. This increased signaling halts migration and leads to the formation of stable organ precursors [28].

The environmental sensing machineries exemplified above are subjected to cell-intrinsic cellular states. For example, some cells may present maximal responsiveness to extracellular signals depending on their cell cycle position, rather than to an increased exposure to the signals [29]. This regulates how the cell responds to extrinsic cues determining individual cellular behaviours such as secretion of molecules [30], apical constriction [5], counting proliferation rounds [13], symmetric and asymmetric cell divisions [31], adhesion [32], migration [33], and differentiation[34]. The combination of intrinsic and extrinsic cues establishes positive and negative feedback loops that move the entire population to a new state generating complex architectures. For example, neuronal development requires a period of extensive proliferation of progenitor cells followed by a switch to asymmetric division and differentiation when the population of progenitors has reached the correct size [31,35,36]. The balance between these two processes in different regions of the nervous system and in different organisms gives rise to differential growth, cellular diversification, and diverse brain structures during evolution [37]. Interestingly, in mammals, cell cycle length [38] (particularly the length of G1 phase [39–41]), influences the decision to terminally differentiate. However, it is still unclear if this could be a mechanism of sensing the population size and how progenitor cells would regulate their cell cycle length. Also, during regeneration, especially in Hydra and Planarians, a minimum tissue size and a certain minimum cell number is shown to be required for regeneration and patterning [42,43].

The mechanisms by which single cells sense their local environments and implement it at the population level are the driving forces of self-organization and collective behaviours during development, tissue remodeling and regenerative processes.

## Symmetry breaking

A defining step during self-organization and pattern formation is the first moment when initially identical cells in a developing organism or tissue differentiate,

and lineage segregation is established. For the mechanistic understanding of this step, symmetry breaking is a key concept. Precisely, the symmetry-breaking event occurs when, despite all cells being exposed to a uniform growth-promoting environment, only a fraction of cells becomes activated, differentiates and acquires new functions. This process is called symmetry 'breaking' because the transitions usually bring the system from a symmetric, but disordered and variable state, into one or more defined, less variable and asymmetric states (e.g. differentiated states) [44]. Symmetry breaking correlates with functional specialization [45] and diversification across different scales: from molecular assemblies, to cell type specification, tissue organization and whole body axis formation.

At the single-cell level in a tissue, a multitude of reactions and signaling pathways takes place continuously. Each step (e.g., cell cycle progression, metabolism, adhesion and migration), is decided based on an integrated response of computed signals (Fig. 1A). Once a given combinatorial threshold of cell-intrinsic and cell-extrinsic signals is reached, a substantial change in behaviour is observed. This change in behaviour can be defined as a newly acquired functionality: a cell which differentiates, starts secreting a molecule or changes its shape, triggers a cascade of effects which moves the entire population to a new state.

Often, cell polarity is the initial building block determining asymmetries at the tissue and body levels. At early stages of mouse embryogenesis, for example, it was demonstrated that the trophectoderm fate is based on differential inheritance of a cell's apical domain [46,47]. Also, the left-right axis in vertebrates is determined by the polarization and orientation of nodal cilia and molecularly dictated by the chiral nature of molecular motor [48]. The directionality of the cilia rotation induces a specific flow of extracellular fluid that, in turn, determines the left-right body axis properties such as the positioning of the heart [49], a conserved feature of fish [50], frog [51], mouse [52] and humans [53]. Similarly, an epithelial cell undergoing apical constriction acts as a nucleator for a pattern of negative straining at the tissue surface driving invagination in embryogenesis or development [5,54,55]. An essential step during Drosophila development is the ventral furrow formation. It results from apical constriction of a few cells along the ventral side of the embryo leading to invagination and movement of mesoderm into the embryo during gastrulation [3,56]. The initial polarity events occur at the molecular and cellular level and trigger the upcoming patterns at the whole tissue level.

Another example of symmetry breaking happens during intestinal organoid formation, where a single intestinal LGR5+ stem cell cultured in Matrigel in the presence of Wnt3a, EGF, Noggin and R-spondin [57] is able to generate a fully grown organoid (Fig. 1B). First, a symmetrical cyst-like structure is formed. Then, the first Paneth cell emerges showing hallmarks of active Wnt signaling and determining the future crypt budding sites [58]. The emergence of a Paneth cell is the first symmetry-breaking event. After that, Wnt3a removal from the medium generates local gradients of Wnt3a around the activated Paneth cells within the cyst, inducing the formation of the stem cell niche and, later on, of the intestinal crypt that maintains itself due to positive feedback mechanisms. The paradoxical initial stage, where all cells in the growing cyst are exposed to a uniform growth-promoting environment but only a fraction becomes activated and differentiates into Paneth cells is still poorly explored [59].

Defining which combination of signals determines the behaviour and interactions of individual cells is important to understand how self-organized patterns emerge. To model and built theoretical frameworks of such complex mechanisms we need to have access to multivariate information of single cells, including cell cycle phase, signaling pathways, metabolic status, and mechanical properties. The complexity of such multidimensional molecular and cellular interactions has made it difficult to explain, which signals underlie symmetry breaking in a given cell and how complex behaviours emerge.

## Cell-to-cell variability

Given the fundamental importance of self-organization, symmetry breaking, and pattern formation in multicellular systems [11,60], several experimental and theoretical frameworks have been used such as Turing's reaction-diffusion systems [61,62], Notch/Delta lateral inhibition and agent-based models [63,64]. A unifying property of these pattern-forming theories is the importance of fluctuations, cell-to-cell variability and feedback loops [65–69]. In all these systems, an initial cell-to-cell variability in a population of cells is then amplified and stabilized by positive and negative feedback loops. The extent of the initial variability and the boundary conditions can confer different steady-state patterns and robustness to the system. In this review, we will not discuss in length the different pattern-forming theories, but we will explore the role of the initial heterogeneity in the system.

Cell-to-cell variability is an inherent and emergent property of populations of cells. It refers to the

phenomenon that no two genetically identical cells behave and look identical [70–72]. This difference may arise from the inherently stochastic and discrete nature of intracellular biochemical reactions, especially when these reactions involve low numbers of molecules. Generally though, robustness in molecular mechanisms [73–76] can buffer the intrinsic stochasticity of molecular processes [77] while other factors, such as the cell cycle and the microenvironment, can explain the cellular heterogeneity, especially in eukaryotes [78,79]. One major extrinsic factor determining cell-to-cell variability is the microenvironment of individual cells. Even in environmentally controlled cell culture conditions, a growing population of adherent cells will continuously experience changing microenvironments as a consequence of an increase in cell number combined with cell adhesion and migration [79]. Another significant source of cell-to-cell variability in an unsynchronized population of cells is cell cycle phase [29]. In fact, when considering cell cycle and microenvironment, much of the unexplained variability in different molecular readouts can be deconvoluted and correlated with local population contexts such as cells being in same cell cycle phases or whether a cell is in a more or less crowded environment [79–82].

Extensive cell-to-cell variability has been shown for key molecular components involved in embryogenesis in the early mouse embryo [83] and in embryonic stem cells [84]. For example, *Nanog*, a key transcription factor for the maintenance of pluripotency, exhibits large variability between cells in the early mouse embryo [85] and populations of undifferentiated embryonic stem cells [86]. This variability in Nanog expression has been linked to cell cycle phase, reaching highest expression during G1/S transition [87]. Another example is the extensive heterogeneity in expression of Oct4 target genes at the 4-cell embryonic stage [88,89]. This variability might confer an initial metastable state to a subpopulation of cells with a fluctuating transcriptome that drives the reversible priming of pluripotent cells toward different cell fate decisions. In fact, if populations of pluripotent cells would be uniform in cellular activities, we would expect an 'all-or-none' response in a homogenous environment, with a single critical threshold below which all cells remain undifferentiated and above which all cells differentiate. A graded response is conceivable only in the presence of initial cell-to-cell variability making it possible to control the rate of differentiation at low homogenous stimuli concentrations [67,90] (Fig. 2). Cell-to-cell variability in key molecular components confers to a small fraction of cells an increased probability to break the symmetry and transition to an activated or differentiated state, making stemness and pluripotency not a property of a single cell but a global and statistical property of a population of cells that are able to self-renew and differentiate [91–93]. This is a particularly important concept to understand the dynamics of stem cell populations [94]. A heterogeneous population of cells with different potencies to perform as stem cell is clearly advantageous, as it provides flexibility and easier adaptability to changing environmental conditions. Variability helps to maximize the population performance instead of that of the individual cell. And finally, it is the control of the stemness potential of a given population which provides tissues the flexibility to maintain homeostasis [92].

Measuring single-cell behaviour beyond high abundant genes in transcriptomics for whole cell populations *in vivo* is still challenging. Hence, comprehensive understanding of the extent and sources of cell-to-cell variability for different cellular processes and how variability affects *in vivo* self-organization, patterning and multicellular programming of cells is sparse [39,95,96]. One important question is: what is the minimal amount of information required at the single-cell level to understand molecularly an emergent pattern at the tissue level? It is probably not necessary to follow every single molecular player of every cell over the course of hours or days to describe emergent properties at a higher scale such as development or regeneration processes. With sufficient single cell data of key signaling pathways, gene regulatory networks and positional information, we might be able to predict interactions and infer causal relations between fluctuating cellular activities and the emergence of a pattern over time [44,79–81,89,97–99]. Ultimately, understanding self-organization and symmetry breaking in multicellular systems is a problem across scales. To explain with sufficient detail the multicellular dynamic interactions that govern a self-organized process, the field is moving into developing technologies across scales which combine three essential elements: single cell resolution, temporal resolution, and tissue functionality.

## Scale-crossing technologies

To quantitate and model the population-level properties of a large group of interacting cells, such as in organogenesis and tissue regeneration, and understand how such properties arise from single cells, we need an experimental framework combining multivariate single-cell techniques and traceability of spatio-temporally dynamical problems. Therefore, to explain with sufficient details the multicellular dynamic interactions that govern a self-organized process, we need scale-crossing technologies linking three essential elements: multiple

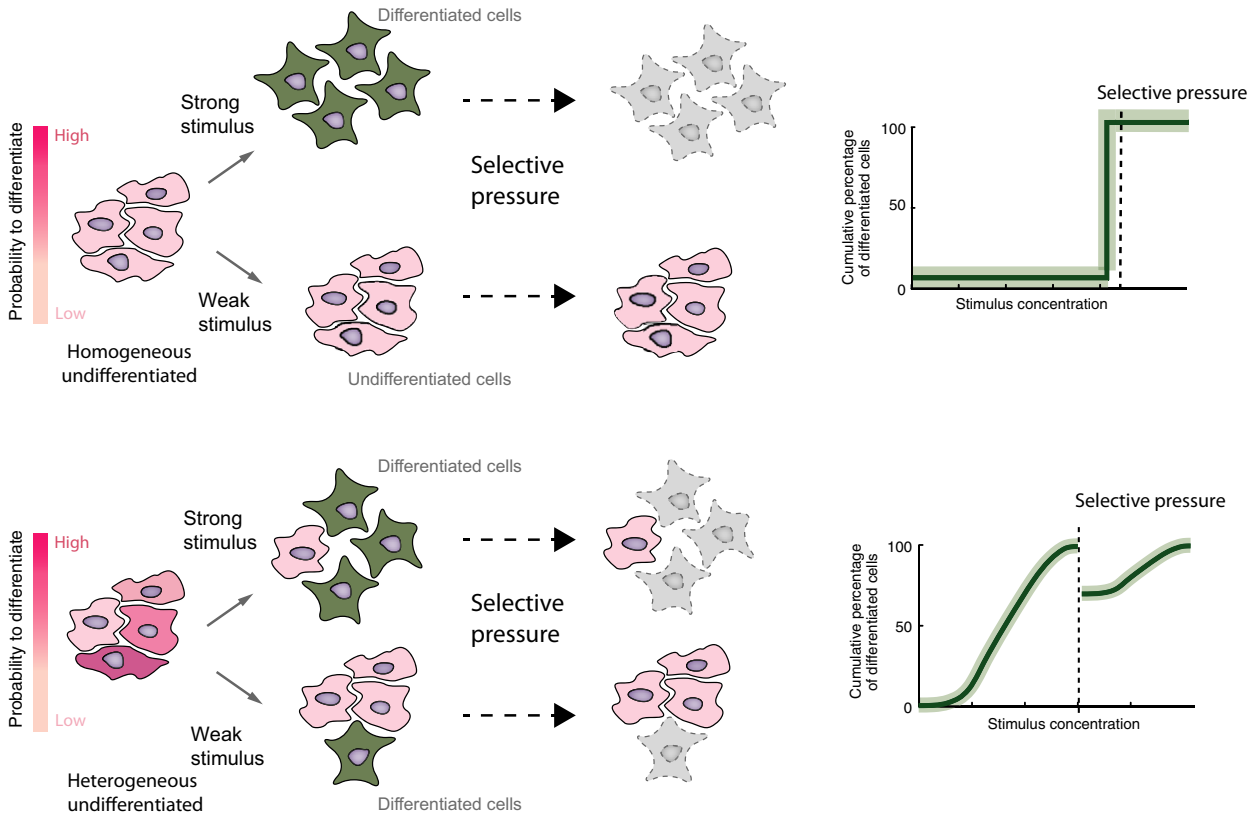


**Fig. 2.** Cell-to-cell variability is an advantageous property of a population of stem cells. A population of stem cells which are uniform in their cellular activities, respond to a stimuli in an 'all-or-none' manner, with a critical threshold below which all cells remain undifferentiated and above which all cells differentiate (upper panel). A graded response is observed in the presence of cell-to-cell variability making it possible to control the rate of differentiation according to stimuli concentrations (lower panel). Variability provides adaptability to selective pressure (right side). In a homogeneous scenario, an environmental challenge results in poor population performance, while a heterogeneous population is more robust to the selective pressure, allowing the survival of some individual cells.

simultaneous measurements at single-cell resolution, temporal resolution accommodating short and long responses, and distinctive quantifiable emergent tissue functionalities (Fig. 3).

An all-inclusive tool capable of multiplexing single-cell measurements on a spatio-temporally resolved scale is still unavailable. We must rely on combinations of advanced imaging, single-cell 'omics' and functional assays as complementary approaches for describing population dynamics at the cellular level. In this final section, we present the available technologies to gain quantitative understanding on the pursuit of self-organization and emergent properties in multicellular arrangements.

## Spatial scale

Spatially, the scales that need to be bridged are from the subcellular resolution (low micrometer range of organelles and cells) to the tissue organization (ranging from millimeters to centimeters) combining multivariate measurements at both scales. Ideally, we would need information on the genome accessibility, mRNA and protein abundance and localization, combined with the phenotypic state of each single cell (such as cell size and shape, cell cycle, signaling, and metabolic state) with spatial localization. At the tissue level, informative measurements of morphological features (size, shape, and curvature), mechanical forces (compactness, pressure, tension, and traction) and functional readouts (morphogen secretion in a niche, organ-like structures such as hair-follicle or intestinal crypts) are required as a final outcome of the self-organized process. Among the different available techniques to obtain spatial information from a tissue at single-cell resolution, fluorescent light microscopy is the most versatile. With optical sectioning methods such as confocal and light sheet imaging [100] cellular details and general architecture of complex structures can be visualized across the spatial scale: from differential expression of transcripts in neighboring cells

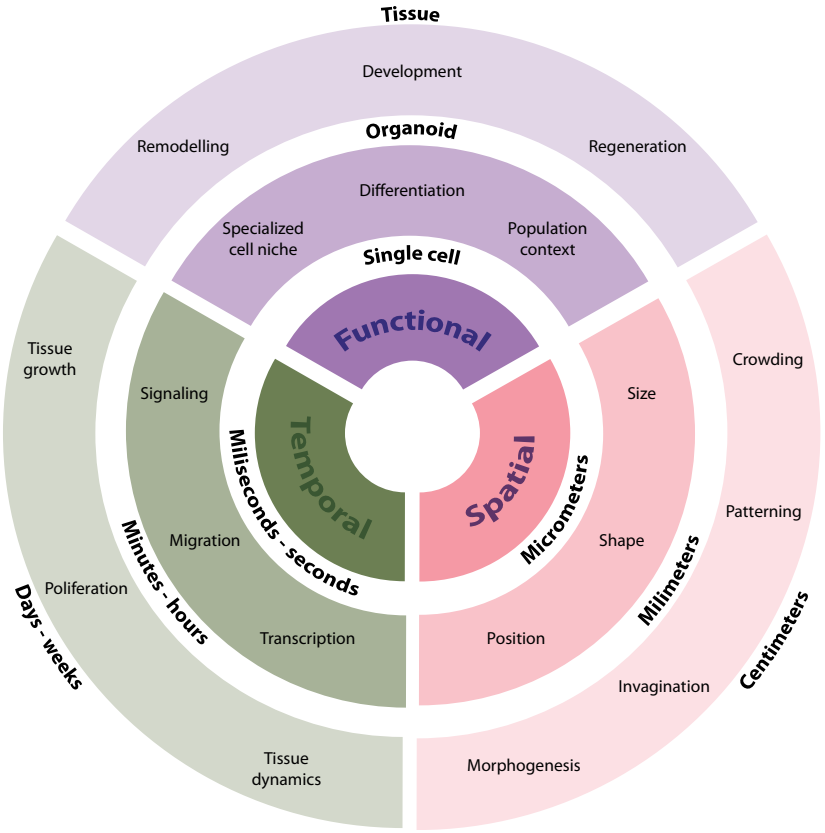


**Fig. 3.** Scale-crossing technologies required for understanding self-organization. Different experimental frameworks are required to quantitate and model the population-level properties of a large group of interacting cells during self-organized processes. Scale-crossing technologies described in the text are able to link functional, spatial and temporal scales. Detailed information at each level of these scales, from single cells to tissues, will help to explain the multicellular dynamic interactions that govern a self-organized process.

[101], toward proteins abundances and specification of different cell types in different organs [102–104], up to mechanics of tissue folding in development [3,105,106]. One of the major limitations in tissue and whole animal imaging is sample opacity. Several approaches have been used to overcome it known as tissue clearing methods (for an overview, see [107,108]) and recent developments have enabled whole tissue and animal imaging at the single cell resolution[104,109]. Visualizing specific subcellular structures and compartments with fluorescence microscopy has been historically limited by the diffraction limit, the phototoxicity and the number of different fluorophores that can be imaged at once. Now advances on image analysis and antibody multiplexing have recently broadened the spectrum of detection. One of the possibilities is to minimize fluorescence spectral overlap by integrating high-resolution confocal microscopy with an imaging analysis pipeline which separates up to six fluorophores simultaneously [110]. This approach has been applied to mapping dynamic inter-organelle interactions in live cells with six different organelle-specific fusion proteins, representing an important tool for investigating organelle spatial organization during different cellular processes. When applied to multicellular 3D structures, it will be necessary to contextualize multiple subcellular readouts with tissue organization. Other alternatives to increase number of readouts in imaging have been developed based on cyclic rounds of antibody staining with chemical inactivation of fluorescence [111,112] or more recently with sequential antibody elution and stripping, a method called iterative indirect immunofluorescence imaging (4i) [113]. The latest allows multiplexing of up to 40 fluorescent molecular readouts inside every single cell in fixed samples with complete sample preservation and unprecedented high spatial resolution. With such an approach, it is possible to monitor different molecular activities on a 3D tissue at single cell level resolution combining, for example, cell cycle reporters, cell type markers, signaling pathways and the cellular microenvironment.

Another method for phenotypic characterization with spatial resolution is imaging mass cytometry [114,115]. It is based on antibodies tagged with metal isotopes allowing quantitation of dozens of proteins simultaneously in individual cells *in situ*. Because of its high parameterization, it identified heterogeneities at single-cell level in subpopulations of cells in cancer samples [114,116]. This method, however, relies on tissue sectioning and laser ablation of the sample, and is currently not compatible with a full 3D characterization of the tissue sample.

Spatially resolved data can also be obtained for gene transcript levels by fluorescence *in situ* hybridization (FISH) and its multiplexed versions such as seqFISH [117,118] and MERFISH [101]. They provide subcellular localization of thousands of RNA species in single cells simultaneously while preserving spatial population context [119]. These RNA imaging techniques have identified transcriptionally distinct cells *in situ* with important applications for characterizing the expression signature of tissues. In the intestine, for example, it has been used to map endogenous markers of intestinal stem cells like Lgr5, Bmi1, and mTert [120] and follow them under different physiological conditions. And by combining spatial information of selected transcripts with whole transcriptome measurements of dissociated cells, it is possible to spatially reconstruct the expression profiles of cells in a tissue coinciding with metabolic cascades and functional zonation as shown in liver [103] and intestine [102].

Besides gene and protein expression, an important aspect to consider during multicellular organization is the metabolic state of cells. Different metabolic identities are adopted during tissue development, homeostasis or disease progression [121–124]. The transition of embryonic stem cells from naïve to primed, for example, is accompanied by a metabolic shift toward a predominantly glycolytic state, and as differentiation progresses, toward a highly respiring mitochondrial state [125]. Exploiting a shift in metabolic activities is also observed during organogenesis, where a gradient of glycolytic pattern in the presomitic mesoderm is responsible for coordinating FGF and Wnt signaling during body elongation [126]. Later in homeostasis, this has been observed in the intestine, where neighboring cells at the proliferative niche present metabolically distinct identities (yet, complementary functions) with Paneth cells being more glycolytic and providing lactate as a fuel for the oxidative stem cells [127]. The relationship between metabolic transitions and morphogenesis seems to hold true also during cancer development, where a zonated glycolytic signature is adopted upon low oxygen input, being a target for therapies [124]. High coverage techniques to assess metabolites, like lipidomics and metabolomics, share the same limitations of transcriptomics and proteomics: insufficient spatial resolution and endpoint measurements. An exception being MALDI imaging, where it is possible to identify multiple analytes, including proteins, lipids, and small metabolites, while keeping positional information. However, spatial resolution of MALDI imaging is still limited at the micrometer range [128]. Transitions in cellular metabolism are emerging as determinants of cell differentiation and tissue development [122,129–132], and learning about the mechanisms driving the onset of these transitions at the single-cell level certainly will contribute to the understanding of symmetry breaking and self-organization in multicellularity.

## Temporal scale

As discussed before, imaging has the great advantage of combining functional and structural information simultaneously. Self-organized events leading to multicellular patterns are long-term dynamic molecular processes occurring over hours, and observing them in living cells *in situ* requires a temporal layer of information. Again, the different scales are important because we need to record short-term events such as signaling activation, protein translocations and cell cycle dynamics with long-term patterning events at tissue scale such as tissue invagination and crypt development (ranging from milliseconds to days). This power to observe dynamical processes over scales enables us to infer causal relationships between molecular mechanisms. Moreover, the ability to use light-induced manipulation allows to challenge the system and to test experimentally the inferred causal interactions. High-resolution live cell imaging and optogenetics tools are becoming fundamental in understanding the dynamics of several biological processes and is the current frontier of imaging development [133–135]. Despite number of readouts still being limited by reporters, lasers and filters, the constant advances on temporal and spatial resolution of live fluorescent imaging represents a promising platform for the study of self-organizing events [136,137].

## Real time

*In toto* imaging techniques, such as light-sheet microscopy, enable long-term live imaging of developing or regenerating tissues and animals with single-cell resolution [133,136,138–141], opening an extraordinary window to the complexity of living systems. However,

observing full development of embryos or having access to complete tissues is not universally applicable to all specimens. Human development, for example, cannot be experimentally studied beyond pre-implantation stages *in vivo* [97,142–144], an alternative being *in vitro* culturing in the absence of maternal tissues [97], the study of foetuses [145] or explanted organs [146]. Adult model systems, such as mouse, can be fully genetically manipulated but cannot be immobilized for imaging longer than a couple of hours. For the moment, studying self-organization with *in toto* imaging during development is performed on embryonic bodies [89,123], gastruloids [147], organoids [148] or small-scale animals. Whole animals such as Hydra [42,149] or *C. elegans* [150] can be imaged during their entire development, while larger organisms such as *D. rerio* [151] or *D. melanogaster* [152] provide fundamental insights into embryonic development. These model systems are highly informative for studying evolutionary conserved mechanisms, despite limitations in their multicellular complexity and tissue functionality. Another aspect to consider is that light sheet microscopy, just as any other fluorescence imaging techniques, suffers from effects of scattering and absorption, which poses technical challenges in deep tissue imaging [133].

Imaging whole animals thorough development is not an absolute requirement for understanding self-organization in multicellular systems and much can be learned about the molecular mechanisms behind tissue growth and organization, for example, by monitoring the dynamics of live tissue segments. Drosophila wing disc formation and ventral furrow invagination are great models for studying epithelial morphogenesis, where mechanical factors determining cell shape, division rates, and intercellular tension can be assessed with high temporal resolution [3,16]. Similarly, oscillations in the mouse presomatic mesoderm (PSM) can be visualized as wave-like patterns of signaling and manipulated on the embryos' tail bud [98,123,126,153] or with PSM-like tissues [154].

With a high spatio-temporal resolution and fluorescent reporters, time-lapse microscopy is a powerful technique to infer causal links between cellular events leading to patterning. In a lineage tracing analysis of stem cell dynamics during epidermal homeostasis [155], a highly coordinated collective behaviour of stem cells during hair-follicle formation has been shown. Similarly, in intestinal crypts continuous intravital imaging following short-term dynamics of intestinal stem cells [156]confirmed that stemness is a function of a heterogeneous cell population rather than of a single stem cell [157,158]. On the mechanical side, real-time imaging is an important tool to understand how variability in physical properties of cells in a tissue might drive multicellular patterning [159] and how tissues which are normally robust in their architectures can also be remodelled during regeneration [32,137].

One important aspect in understanding temporal scales is the ability to manipulate the system in a spatially and temporally controlled manner. Light-induced manipulations have been largely improved in spatial and temporal control with the advancements of optogenetics [54,160]. This allows, for example, to understand the importance of timing in signaling pathways involved in cell fate specification during tissue formation. Using a light-induced regulation of phosphatidylinositol(4,5)$P_2$ levels at the plasma membrane of Drosophila embryonic cells, Guglielmi *et al*. [54] showed that local modulation of cell contractility interferes with tissue contraction and invagination. Also by manipulating the duration of Nodal signaling during zebrafish embryogenesis using a photoactivatable receptor, it has been shown that extended Nodal signaling drives prechordal plate specification at the expense of endoderm differentiation [161] in a process that depends on long-lasting cell-to-cell contacts [162]. In tissue morphogenesis, temporal manipulation of signaling pathways which promote cellular contractility, such as RhoA [163], or phosphatidylinositol(4,5)$P_2$ levels at the plasma membrane [54], allows local modulation of mechanical forces at the cellular level.

## Pseudotime inference of molecular events

Time-lapse imaging is currently the main approach by which spatial and temporal scales can be monitored. However, number of samples that can simultaneously be acquired is limited. Temporal information can also be inferred with computational methods using thousands of samples simultaneously. Resolving molecular and cellular processes pseudotemporally can be achieved by reconstructing time-series of events based on information from numerous fixed time points [80,164–166]. With single cell transcriptomics, for example, it is possible to resolve differentiation from stem cells to functionally committed progenies [167–169]. The temporal cascade of events can be inferred computationally based on gradual differences within the population capturing a pseudotemporal trajectory. Recently, a whole embryo developmental landscape has been reconstructed based on scRNAseq data [170,171], describing cells transitioning from pluripotency to different cell types during early zebrafish embryogenesis. This is a useful way to explore high

content expression information overcoming the current technical limitations on temporal scale. While differentiation trajectories from transcriptomics can provide a high content molecular picture, imaging and mass cytometry data from fixed samples can also be used to reconstruct trajectories both spatially and temporally [80,172].

## Functional scale

Besides being able to follow single cells in space and time, we require an adequate model system that considers the functional 3D organization of cells in a tissue and has the ability to replicate some of the *in vivo* environment. Without easy accessibility into live tissues or the possibility of following their long-term development, scientists over the last decade mostly exploited immortalized cell cultures [18,173–175], embryos [4,5,97,98,144,176], explanted tissues [16,98], cocultures [177], as well as whole animal model systems, like Hydra [149], Axolotl [178] and planarians [43]. And while much has been learned on fundamental processes, it is clear that each of these systems has a trade-off between physiological relevance and experimental amenability.

A powerful model system assessing these limitations are organoids. Organoids are 3D organ-like structures derived *in vitro* from primary tissues and adult stem cells, embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) [179–187]. These complex multicellular structures arrange through a self-organized mechanism requiring no external guidance, only appropriate niche factors and, importantly, a 3D extracellular scaffolding [55,188,189]. Organoids develop into multicellular structures resembling key aspects of the native organ with differentiated cell types and tissue-like architecture [190,191]. The cells self-organize into complex structures from a range of organs such as optic cups [33], neuro-rosettes [192,193], cerebral cortex layers [181], intestinal crypts and villi [57], liver [194], lung [195], and kidney [190,191,196–198]. Moreover, other powerful systems starting from ES cells have been developed that recapitulate embryo formation [5,44,199] and gastrulation [200]. Clearly, organoids are simplified models of the complexity of tissue architecture and function. Intestinal organoids, for example, lack important aspects of tissue structure such as stromal cells [201,202], vascularization and enteric nervous system [177]. Nonetheless, they deliver powerful means for *ex-vivo* modeling of tissue morphogenesis and organogenesis and also represent an opportunity to understand fundamental principles and molecular mechanisms of self-organized processes.

Organoid cultures combine: (a) advantages of *in vitro* culture (controlled growth conditions and multi-parallelized assays possible)[203], (b) amenability to chemical and genetic manipulations, (c) single cell accessibility with imaging and genomics techniques in both live and fixed samples [17,32,37,179,204], (d) temporal resolution, as organoid development can be followed in real time for monitoring short and long-term events [32,204–207], (e) tissue functionality, which ultimately provides cellular information in physiologically unique contexts (such as organ-specific development, homeostasis, regeneration or disease progression) [208,209] and finally, (f) organoid cultures are expandable offering the opportunity to reach sample sizes of hundreds or thousands, which is not feasible with explanted tissues.

Organoid cultures allow to question how single cells exposed to a uniform growth-promoting environment can generate asymmetric structures and how local interactions between single cells give rise to self-organized patterns visible at the organoid level. Using embryonic kidney cells, for example, it has been shown that after enzymatic dissociation and re-aggregation *in vitro*, cells spontaneously recreate the morphological arrangement between epithelial and mesenchymal cells, without prior spatial information [179]. This re-aggregation relies solely on movement of cells and differential cadherin-based cell-cell adhesion providing molecular evidence and evolutionary conservation of classical dissociation/re-aggregation experiments in sponges and differential adhesion hypothesis of Steinberg [210]. In an adult intestinal epithelia, the patterning of intermingled progenitors and differentiated cells in the stem cell niche is driven by the higher propensity of elongated cells to intersperse during interkinetic nuclear migration and cell division [207], showing that mechanical properties of cell division are driving forces in tissue patterning [105].

Finally, a recent paper exalted the power of understanding tissue self-organization for clinical applications. Using hair-bearing skin organoids from new-born mice, the authors not only showed successful transplantation and further hair growth in adult nude mice [32], but also dissected the molecular mechanisms and morphological transitions during organoid formation. By combining time-course transcriptome analysis and immunostaining, they described spatio-temporally zonated patterns of expression of adhesion molecules and signaling pathways, which allowed experimental manipulation and hair growth restoration. It is therefore clear that understanding self-organized processes that initiate and propagate regenerative and pathological conditions has also a therapeutic potential in

medicine [196]. Sensing the tissue environment is essential for a healthy regenerating tissue. In the recent years, the mechanosensors YAP and TAZ have been described as primary sensors of a tissue's physical context [211,212] and master regulator of tissue regeneration. Moreover, it has been shown previously that engraftment of organoids into a damaged epithelium has a potential regenerative application, such as in ulcerative colitis [213]. Because organoids can be derived from human samples, either healthy or diseased, it is now possible to envision personalized strategies [214,215].

## Outcome

Multicellular tissue self-organization events can now be studied in a quantitative way at single cell resolution. The mechanisms by which single cells sense their local environments and implement it at the population level are the driving forces of self-organization and collective behaviours during development, tissue remodeling, and regenerative processes. The rapid development of imaging and genomics techniques combined with powerful modeling tools, now, enables us to bridge scales of complexity: spatially, temporally and functionally. With subcellular resolution, we can better understand fundamental concepts of how symmetry-breaking events occur, which roles cell-to-cell variability plays in a biological process and how cellular patterns emerge. From that, we can then move forward to describe the mechanisms behind complex collective behaviours in development and tissue homeostasis, with immediate application in development and regenerative medicine [20,173].

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