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Review Article

To be more precise: the role of intracellular trafficking in development and pattern formation

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Living cells interpret a variety of signals in different contexts to elucidate functional responses. While the understanding of signalling molecules, their respective receptors and response at the gene transcription level have been relatively well-explored, how exactly does a single cell interpret a plethora of time-varying signals? Furthermore, how their subsequent responses at the single cell level manifest in the larger context of a developing tissue is unknown. At the same time, the biophysics and chemistry of how receptors are trafficked through the complex dynamic transport network between the plasma membrane–endosome–lysosome–Golgi–endoplasmic reticulum are much more well-studied. How the intracellular organisation of the cell and inter-organellar contacts aid in orchestrating trafficking, as well as signal interpretation and modulation by the cells are beginning to be uncovered. In this review, we highlight the significant developments that have strived to integrate endosomal trafficking, signal interpretation in the context of developmental biology and relevant open questions with a few chosen examples. Furthermore, we will discuss the imaging technologies that have been developed in the recent past that have the potential to tremendously accelerate knowledge gain in this direction while shedding light on some of the many challenges.

Why endosomal trafficking?

When studying development, of particular interest is cell-fate determination, which is crucial in the development of organised and functional architecture of tissues. This specialised developmental process is a reproducible emergent phenomenon that is able to overcome the stochasticity of receptor activation and signalling cascades. Intracellular trafficking is central to cells' processing of receptor activation, influencing how they 'read' and 'respond' to their surrounding environments. By constantly controlling receptor and ligand; concentration, location, and environment, cells are able to subtly modulate the activation and signalling of receptors. This precise modulation enables receptors to differentially respond to external cues depending on their nature, concentration, and time-dependent characteristics. How does endosomal trafficking provide the finesse to modulate the response of cells to their surrounding environments, and how can their history and cellular context be integrated into their decision making? It should be noted that these examples we have chosen in the review are by no means a complete list of modulation of receptor activation and signal processing by endosomes. Investigating an inherently noisy system such as endosomes and signal modulation by endosomes is extremely challenging, and an increasing number of studies are providing more insight on this. Here, we have chosen a handful of such developmentally pertinent examples that hopefully give an insight into some of the common and more explored mechanisms employed.

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The logistics of endocytic modulation of signalling

Endocytic vesicles mediate the constant exchange between the intracellular and extracellular environments by trafficking receptors and intraluminal material, such as ligands, between the plasma membrane and endoplasmic reticulum (ER)–Golgi apparatus. Transmembrane receptors are rapidly internalised into endocytic vesicles following their activation by extracellular ligands. The concentration of receptors into endocytic vesicles has been shown to be critical for the full activation of many receptor families [1]. It has been shown for some specific receptors that endocytosis leads to the concentration of the receptor above a critical level, thereby allowing low rate constants of effectors to be overcome and promoting signal propagation [2,3]. This has also been suggested to reduce spurious signalling from stochastic activation of individual receptors. Indeed, it has been suggested that the concentration of receptors is tightly controlled. A key study by the Zerial laboratory showed that following activation, phosphorylated epidermal growth factor receptors (pEGFRs) were internalised into early endosomes; and that the number of pEGFR-bearing endosomes but not activated receptors per endosome was linked to epidermal growth factor (EGF) concentration [4]. The group postulated that these clusters of pEGFR per endosome may be tightly regulated via key positive and negative feedback loops. They further demonstrated that modulating the number of receptors contained within each endosome, by perturbing endocytic fusion, led to altered signalling outcomes. How such control is maintained over receptor number and whether such a mechanism exists for other systems and receptors is yet to be determined. However, modelling of these receptor-signalling 'quanta' on endosomes suggested they may be critical in overcoming the inherent stochasticity of receptor activation [4,5]. Viewed in light of a subsequent study, endosomes are emerging as a potential mechanism to improve the robustness of receptor signalling and overcome the inherently noisy system [6].

It is critical to note that receptors are not deactivated immediately after internalisation. In fact, it is increasingly apparent that endocytic vesicles can provide the spatial arrangement for the formation of distinct signalling platforms and promote distinct interactions and signalling outcomes. This concept of signalling endosomes was first proposed in neurons, as reviewed by Howe and Mobley [7]. Post-internalisation in neuronal cells, receptor-bearing vesicles associate with the motor proteins dynein and kinesin to traffic the vesicles along microtubules towards the perinuclear region (PNR) [8]. Because of the extreme geometry of neurons and the inefficiency of diffusion over large distances, it was observed that receptors were required to be actively transported via vesicles away from their site of activation to activate downstream effectors [9–11]. Furthermore, due to the non-homogenous nature of the cytoplasm, trafficking of these receptors allows for the interaction within distinct cellular compartments which can produce distinct effects [12]. Interestingly, it has been evidenced that whilst the spatially separated intracellular environments play a fundamental role in controlling the physiology of neurons, these same concepts are at play within all eukaryotic cells, and across many receptor families including tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs) [5,12–15].

As receptors are trafficked towards the PNR they are exposed to an increase in phosphatase activity as well as other secondary messengers [16,17]. This is critical as the intracellular tails of transmembrane receptors are still exposed to the cytoplasmic environment and govern their interaction with endosomal adaptor proteins and downstream effectors [18]. These interactions and signalling cascades can be further tuned and modulated by post-translational modifications such as phosphorylation and ubiquitination [19,20]. To add a layer of further detail that highlights the highly organised intracellular environment; throughout the cell exist regulated inter-organellar contact sites, specifically between the ER and endosomes. Several studies have suggested that maturing endosomes form contact sites with the ER [21–24], and that the ER may harbour specific enzymes to modulate receptor activity, also reviewed elsewhere [25,26].

It has been appreciated for many years how internalisation of activated receptors via receptor-mediated endocytosis is the first step in the attenuation of many signalling pathways and receptors' deactivation. For instance, GPCRs are ubiquitinated and phosphorylated to deactivate the receptor and inhibit the interaction with downstream effectors [27]. Similarly, the canonical EGFR signalling pathway involves secondary messenger activation by tyrosine residues on the intracellular tail of the EGFR dimer, and is attenuated following phosphatase dephosphorylation of these residues upon perinuclear accumulation [28]. A recent study by Stanoev et al. [6] demonstrated that there are two spatially distinct populations of dephosphorylating enzymes that control the kinetics of EGFR activation (Figure 1i). They show that the plasma membrane-localising protein tyrosine phosphatase RJ/G (PTPRJ/G) and the ER-localising protein tyrosine phosphatase N2 (PTPN2) phosphatases have distinct rates of dephosphorylation for EGFR. Since the PTPN2 only can act on the EGFR following the PNR accumulation of the receptor-bearing endosome, this provides a mechanistic link between the

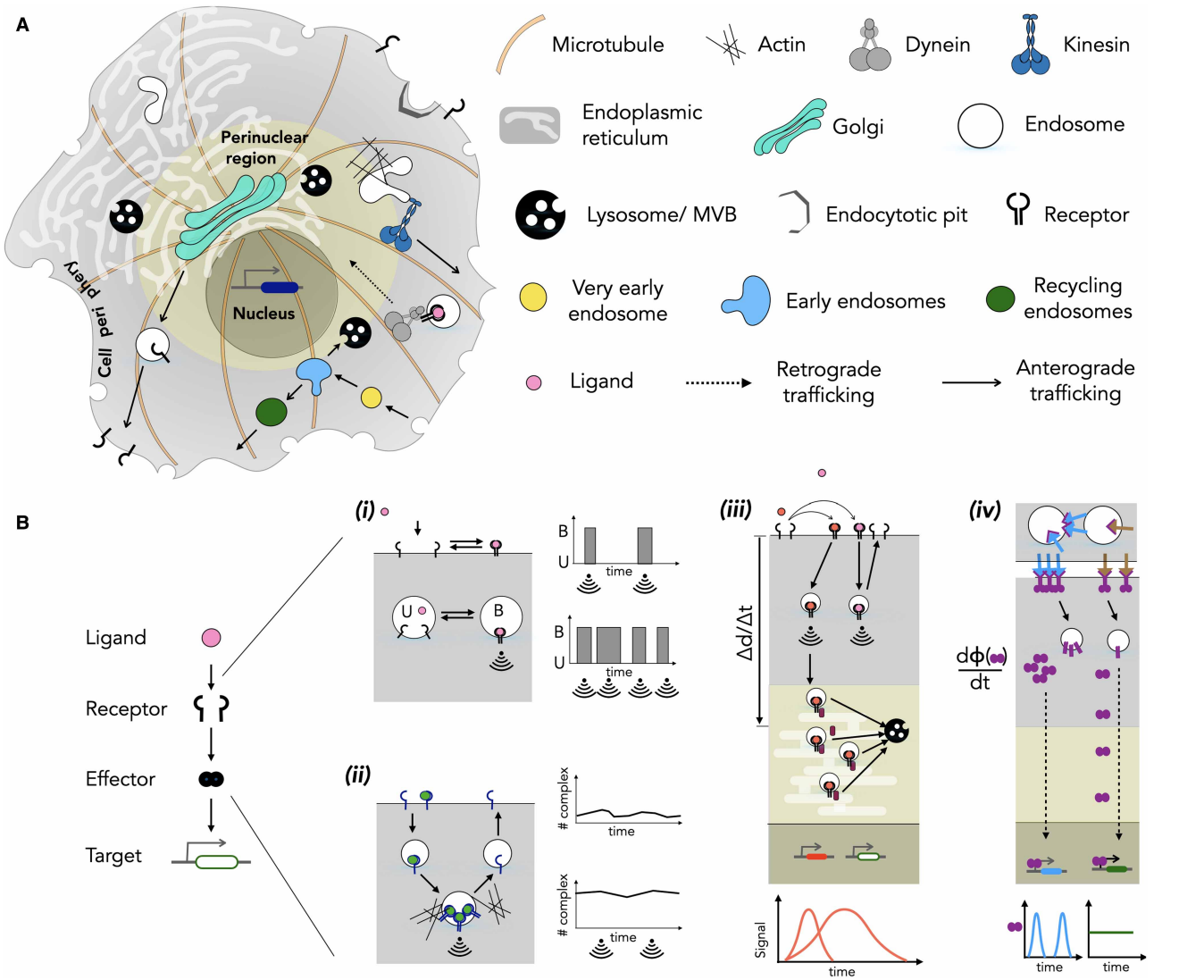

Figure 1. A schematic representation of the endocytic logistics of signal modulation.

(**A**) Activated transmembrane receptors show biased trafficking towards the perinuclear region of the cell where they are deactivated via ER-associated enzymes and potentially degraded in lysosomes. Modulation of the dynamics of this transport by motor proteins or tethers can tune the signalling response. (**B**) Schematic illustrations of the corresponding signalling responses of endosomal modulation. (i) Activated receptor internalisation can promote increased and distinct signalling outcomes, shown as increased time spent and frequency in the unbound (no signal) (U) as compared with bound (B) signalling state. (ii) The actomyosin cortex can tether signalling endosomes leading to prolonged signalling outcomes sensitive to endosomal quanta of receptors, by producing endosomes with an increased number of signalling complexes. (iii) Modulating the timing of trafficking to the ER-containing PNR ($\Delta d/\Delta t$) can lead to distinct differences in signalling lengths by modulating the rate of receptor-signalling attenuation. This is seen as different lengths of signalling of the same gene. (iv) Dll1 and Dll4 produce distinct signalling dynamics due to the differences in receptor clustering, which in-turn produce differences in the rate of NICD release ($d\phi/dt$) which are subsequently decoded by the transcriptional machinery to produce different outcomes. This is seen as a difference in the transcriptional dynamics as well as genes transcribed.

rate of perinuclear accumulation (and subsequent dephosphorylation of the receptor) and the signalling lifetime of EGFR receptors.

Many receptors have been shown to require degradation in lysosomes to completely relinquish the ability to signal, including EGFR and members of the GPCR superfamily [18,29,30]. A regulation of receptor-mediated activation and receptor deactivation is crucial to cell signalling homeostasis. Non-attenuated signalling of these receptors has been implicated in a diverse range of pathologies, including cancer [31–33]. In a similar manner,

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intracellular trafficking can also govern the sensitisation of cells by controlling the balance of recycling versus degradation of receptors and thus transmembrane concentration. In this manner, the responsiveness of receptors [34] can be modulated depending on the cell state [35,36]. For example, there is a shift in the balance of recycling versus degradation of EGFR receptors towards increased lysosomal degradation following high EGF stimulation [37,38]. This leads to a critical desensitisation of cells following high EGFR activation due to the reduction in receptor number.

Given the distinctions between the PNR and the periphery of the cell with respect to enzymes that act on the receptors as well as secondary messengers, one can appreciate that mechanisms which control the time endosomes spend in these distinct compartments may play a large role in modulating the signal processing of the cell (Figure 1ii). A relevant example is in EGF bearing endosomes where myosin-mediated tethering to the actin cell cortex was shown to selectively prolong the phosphorylated state of receptors [39]. Whilst the recruitment of motor proteins to direct different cargo bearing vesicles is well established, their role in modulating signal processing has been less extensively studied [8]. However, a recent study has shown an Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1)-Dynein interaction that is responsive to EGF stimulation and mediates a concentration-dependent recruitment to EGF-containing very early endosome (VEE), thereby promoting the cohort trafficking of these newly formed receptor-bearing vesicles towards the PNR [40,41].

The ability of the cell to control these various parameters of signal processing is critical to its ability to interpret and distinguish distinct external signals. The previous mechanisms of modulation enable cells to sense the extent of receptor activation by controlling the 'area under the curve' of secondary messenger activation. However, how cells interpret the dynamics of receptor and downstream effector activation can also have important consequences for developmental processes, i.e. cells respond to signalling dynamics such as pulse width and frequency as well as amplitude variation. A striking example of this was recently demonstrated in notch signalling [42]. In chick neural crest cells, myogenesis is either promoted or inhibited by the Delta-Like Ligand (Dll) 1 and 4, respectively. However, these two ligands signal through the same receptor notch 1, and the same downstream release of notch intracellular domain (NICD) (Figure 1iii). Nandagopal et al. show that the intracellular tails of Dll1/4 lead to distinct patterns of endocytic clustering and that the subsequent transcytosis of the notch extracellular domain (NECD) results in distinct patterns of NICD release and signalling. The authors go on to show that the pulsed versus sustained NICD activity is decoded differently by the cell, leading to distinct gene expression and physiological outcomes. How mechanistically these distinct dynamics of NICD production are decoded by the cell to produce distinct transcriptional programs remains to be identified *in vivo*.

In the context of exocytosis, there has been a recent study showing that cells can similarly use pulsatile dynamics as signal sending cells. Decapentaplegic (Dpp) is released by exocytosis during *Drosophila* wing disk development, and it has been found to follow naturally oscillating calcium waves present during development. In general, calcium levels have been extensively linked to exocytosis in excitable cells through modulation of the cell polarisation [43]. Rather than Dpp being released in a gradual manner, Dpp is believed to be released in an oscillatory/pulsed manner in line with calcium oscillations. The pulsatile release is believed to allow for Dpp to fully fill vesicles and cause the release of a large concentration of Dpp, rather than a constant gradual release of Dpp signalling [44]. What is more remarkable is that when these potassium channels are mutated, developmental defects consistent with low Dpp signalling are observed, such as thickened veins [45].

These few examples show that the dynamics of secondary messenger activation may have an underexplored role in maintaining the fidelity of extracellular sensing, and may play a role in many yet unidentified, key signalling pathways. How cells utilise the temporal characteristics of signalling to distinguish between pleiotropic pathways is an active area of research that still requires much attention [46,47].

Notch signalling — juxtacrine and biased endosomal division

Up till now we have considered cell signalling in isolation; however, intracellular trafficking plays a role in modulating signal sending pathways as well as signal receiving. This can be demonstrated by a two-cell system undergoing juxtacrine signalling. Juxtacrine signalling involves direct communication between adjacent cells when transmembrane receptors on the signal-receiving cell are activated by ligands expressed on the membrane of an adjacent cell (Figure 1iv). By far the most studied example in development is the activation of the Notch receptor by its various cognate ligands. This highly conserved pathway is integral in coordinating tissue

patterning and morphogenesis through a variety of outcomes including differentiation, proliferation, and apoptosis, as reviewed by Artavanis-Tsakonas and Matsuno [48] and Bray [49]. In this review, we will cover some of the key mechanisms in which this pathway can be modulated by the trafficking machinery. However, we direct interested readers to the recent and excellent review by Henrique and Schweisguth [50] for a detailed coverage of the molecular mechanisms of this pathway.

The role of trafficking in the ligand presenting cell is relatively well established in Notch signalling. Firstly, there is evidence to suggest that endocytosis of the ligand-bound NECD may contribute a physical force that helps to induce the conformational change of the ligand-bound receptor. This conformational change enables the subsequent s2 cleavage by ADAM metalloprotease/TNF-a converting enzyme, releasing the ECD [51,52]. Another mechanism that is suggested to control notch activation is the post-translational modification of the Delta (Dl)/Serrate/Lag2 (DSL) ligands, which is mediated by their movement through endosomes. It has been shown in some systems that the ligands must be first internalised by the signal sending cell, allowing for ubiquitination by modulating enzymes such as Neuralized [53,54] and Mindbomb [55], before being recycled back to the plasma membrane [56,57]. Furthermore, by using hybrid delta ligands with modified intracellular tails, it was shown that the spatial arrangement of delta ligand can produce distinct transcytosis dynamics that are decoded by the cell, as mentioned previously (Figure 1iv) [42].

In the signal-receiving cell, the possible mechanisms by which endocytic mutants lead to effects in delta signalling are less well understood. It has been shown, however, that changing the number of receptors present on the membrane of the cell leads to changes in both cis- and trans-activation of Notch [58]. Given that it has been suggested that cis-activation of the notch receptor predominately produces a signalling incompetent state that potentially buffers cells against spurious activation, how the complexities of notch receptor number translate to signalling sensitivity remains to be explored. Furthermore, Notch receptors can be internalised without activation, potentially leading to a ligand-independent signalling state as reviewed by Steinbuck et al. [59]. This involves ligand-independent internalisation and release of NICD from the limited membranes of intracellular vesicles in a force and ADAM/TACE-independent mechanism [59,60]. However, the significance of this mechanism in living organisms has been suggested to be limited [59].

Where the s3 cleavage event occurs has been a source of much study; it has been reported to occur at both the plasma membrane and at the limiting membrane of endocytic vesicles following internalisation [61–63]. It has also been shown extensively that in Drosophila [64], and more recently in mammalian systems, some Notch receptors are required to be localised to an endocytic compartment in order for the s2 and/or s3 cleavage events to occur [65,66]. Furthermore, since γ-secretase has been shown to have increased activity in more acidic environments [67] and that this difference in activity can produce NICD fragments with distinct amino termini and with different predicted stabilities [68]. This suggests that the timing and positioning of the Notch receptor throughout the endocytic system may influence its signalling capabilities; however, this is yet to be assessed in an *in vivo* context.

It is evident that Notch signalling is influenced by the endocytic machinery within both the signal sending and receiving cells. However, manipulation of the endocytic machinery has been used for Notch-mediated asymmetrical division during *Drosophila* sensory organ precursor (SOP) cell fate determination. SOP precursor cells can divide to give rise to more precursor cells, or they can divide asymmetrically to give rise to different cell types. Notch plays a role in this cell fate determination by being present in larger numbers in one of the SOP daughter cells. Multiple rounds of this asymmetrical cell division leads to cells with different cell fates, and to the development of the sensory organ.

During SOP asymmetrical cell division, the precursor cell divides and produces two types of cells, Notch signal-receiving and signal-sending that are termed either pIIa or pIIb, respectively. During cytokinesis, Delta and Notch as well as key modulators are asymmetrically distributed between the two dividing cells to produce either signal sending or receiving cells [69–72]. In addition to Notch distribution, the asymmetrical distribution is maintained in multiple ways. Two of which are discussed below; detailed descriptions of these processes have been covered elsewhere [64,72,73].

In *Drosophila* SOP cells, a protein called Numb is asymmetrically located to only the pIIb cells. Numb's localisation causes Notch signalling to be inhibited in the pIIb cell and up-regulated in the pIIa cell. Numb is an inhibitor of Notch signalling, and it does so by associating with an adaptor protein (AP-2) involved with clathrin coated pit formation that causes the Notch receptor to be endocytosed and degraded in only the pIIb daughter cell, and not the pIIa daughter cell. Numb will also use the E3 ubiquitin ligase 'Itch' to target Notch containing endosomes to the lysosome for degradation in the pIIb cell [74,75]. As a result, Notch signalling is

Biochemical Society Transactions (2020) **0** 2051–2066
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mostly inhibited in the pIIb cell through Numb. Similarly, Numb promotes endocytosis of Sanpodo into the pIIb cell which further leads to Notch inhibition in the future signal-sending cell [76].

A further level of control on Notch localisation is mediated by Smad anchor for receptor activation (SARA)-bound endosomes. During asymmetric division, SARA endosomes are localised to the pIIa cell. Delta is a typical cargo of SARA endosomes, therefore, an increase in Notch containing endosomes will be seen in the pIIa cell. Notch signalling does not determine the asymmetric movement of SARA endosomes, but rather biased SARA endosomal localisation leads to biased Notch signalling in one of the cells. SARA endosomes traffic Notch to the pIIa cell where it is subsequently cleaved in a γ-secretase and delta-dependent manner [71]. SARA endosomes are directed to the pIIa cell by Notch via the association with Uninflated [77] and are trafficked by the kinesin Klp98A to the future signal-receiving cell [78]. To traffic towards the pIIa cell the endosomes associate with a central spindle polarised due to the action of the depolymerising kinesin Klp10A and its antagonist Patronin [78]

SARA asymmetric division of Notch has also been demonstrated to regulate differentiation in a variety of stem cell populations other than *Drosophila* SOPs. Including *Drosophila* stem cells in the gut [79] and central nervous system [71] as well as zebrafish spinal cord neural precursors [80].

Signal processing by single cells embedded in tissues

In its most simplistic form, cell signalling can be analysed by focusing on a single cell's response to a signal or ligand, and as it has been discussed, signalling with respect to single cells is well understood. However, when cell signalling is being considered in a multicellular environment, the story becomes a little more complicated. Cells must act in concert to produce different cell fates from the same set of precursor cells, and this requires careful modulation of the signals provided. Importantly, cells must be able to sense their location in a multicellular context and respond to non-uniform stimuli [81]. We have discussed the pertinent role of the endocytic machinery in single cell signalling, and its role is no less important in multicellular cell fate determination. Endocytosis can provide the basis for transporting long range signalling molecules and determines how the interpretation of the signals will be shaped in a multicellular context [82,83].

The term morphogen 'form-producer' was originally coined by Turing in 1952 [84] to describe the concept of released factors that can determine the differentiation and fate of surrounding cells by activating specific signalling pathways [85]. Morphogens were later postulated by Crick [86] to form gradients from high concentration at the 'source-cells' to low concentration at a distance, as the surrounding tissue takes up the secreted factors — the 'source-sink' model (Figure 2). This model relies on the assumption that morphogens are soluble, secreted extracellularly and diffuse from cell to cell [87]. One can intuit that the gradient steepness is a result of the rate of uptake by the surrounding tissue; increasing the rate of morphogen-binding or receptor-mediated endocytosis produces a steeper gradient as morphogens are taken up by cells close to the source [81]. A steepened gradient like this has been shown with fibroblast growth factor 8 (Fgf8) [88]. Fgf8 binds to its receptor, and they are both then trafficked with Rab5 early endosomes, leading it to two possible fates — either receptor recycling or receptor degradation [89]. Up-regulation of Rab5 leads to an increase in receptor endocytosis and hence, a steeper gradient is observed towards the source cell, as the morphogens have been taken up by cells close to the source [89,90]. The gradient steepness can also be influence by morphogen degradation, it was shown that a freely diffusing morphogen exposed to a constant rate of degradation in the extracellular space will produce a gradient that tends towards an exponential function [91,92].

How morphogens are transported from the source cell to their receiving cells has an impact on the formed gradient. Diffusion accounts for the transport of some morphogens, but more complex mechanisms of transport have been suggested such as cytonomes, hindered diffusion and transcytosis [93–95]. Their transport is modified by the integration of ligands that hinder their movement, such as heparan sulfate proteoglycans (HSPGs) [96]. The relevance of understanding these different transport mechanisms can be seen in relation to Dpp. Dpp has been highly implicated to move via transcytosis. With Dpp, it has been found that this molecule can only move ∼5 cells away from the source cell when endocytosis is inhibited, but its gradient implies that it should be capable of moving at least 25 cells away, which diffusion could not account for [97,98]. Multiple experiments have shown that inhibiting dynamin-mediated internalisation abolishes Dpp accumulation in distant cells, suggesting a transcytosis-dependent motility. Furthermore, experiments involving localised inhibition of dynamin showed that endocytosis inhibited cells acted as a barrier to Dpp transport [83]. Dpp's observed diffusion constant is slower than a constant predicted for diffusion, suggesting that another mechanism of transport is being utilised. Despite the evidence for transcytosis in Dpp movement, whether it genuinely

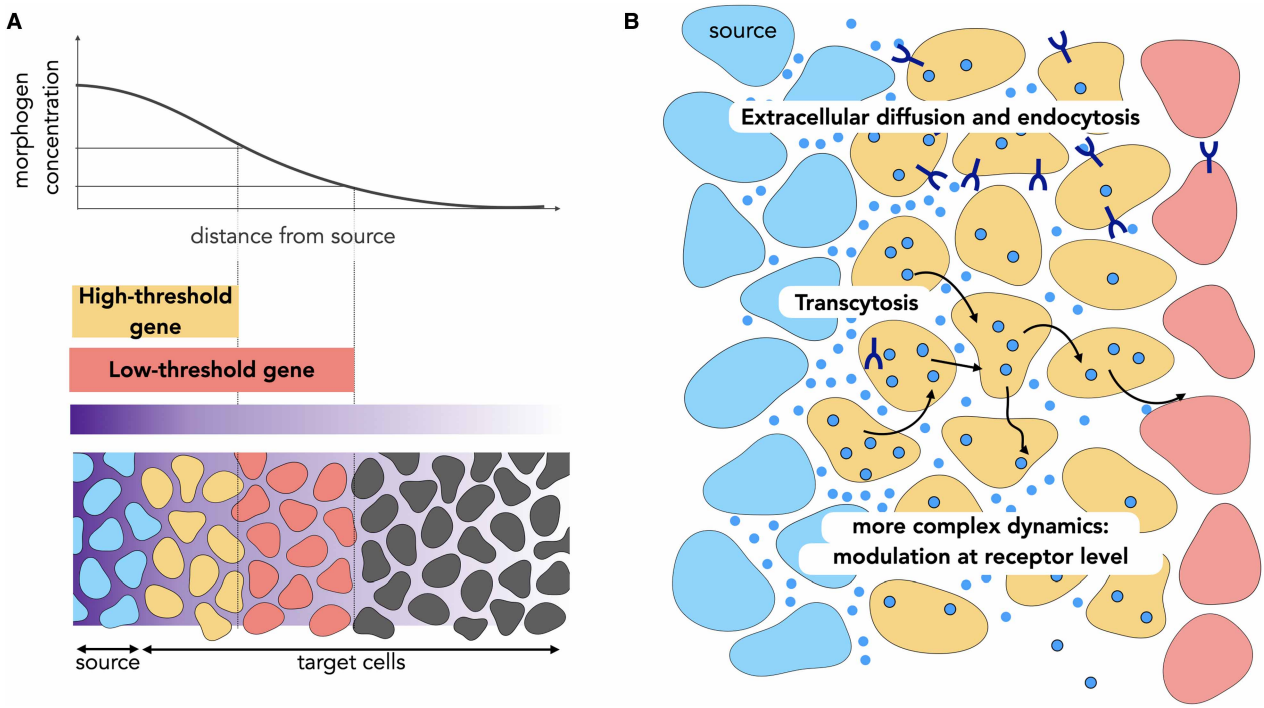

Figure 2. How morphogen gradients can give rise to developmental patterns.

(**A**) Schematic diagram of the concentration of a given morphogen (purple) as the distance from the source-cells (blue) increases. This can lead to morphological boundaries formed by different transcriptional programs, corresponding to the thresholds of morphogen-receptor activation. (**B**) A close up of the movement of the morphogen across a field of 'sink-cells' which can take-up the morphogen.

is trancytosed is still actively debated, with some laboratories postulating that restricted diffusion may account for Dpp movement [99,100].

How is a gradient of signalling molecules interpreted by the tissue? Each cell within a tissue experiences a different concentration of extracellular ligand, related to their distance from the source-cells. How such a concentration difference can give rise to morphological boundaries and distinct cell fates, was termed as the French Flag Problem by Wolpert in 1969 [101]. Wolpert postulated that a spatial gradient could create different cell fates, or different colours of the stripes in the flag, by introducing thresholds to the signalling molecule (Figure 2a). This implies that the cells have the ability to distinguish distinct changes in concentrations precisely and establish boundaries through a varying field of morphogen concentration [81,102]. This French Flag problem also introduces boundary conditions, whereby the left and right boundaries of the gradient must be kept constant to establish the correct scaling of the pattern. More complex patterns, such as repetitive stripes, could be formed by the introduction of a multitude of thresholds.

It is immediately apparent that in a system which follows the French Flag paradigm that the borders between two different populations of transcriptional activation or cell fates is the site of some interesting biology. A well-substantiated complication is that the gradient of a single morphogen should not be nearly steep enough to produce robust transcriptional changes [103]; given that experimental studies suggest that a 2-fold or greater change in signalling would be required between the two populations [104]. The mechanisms by which cells are able to accurately detect their positional information has been a site of much theoretical and experimental study and we direct interested readers to some key reviews [81,102,103]. Some of the suggested ways in which morphogen gradients are 'steepened' are by incorporating feedback loops; either positive feedback or bistability has the potential to produce large jumps in morphogen signalling at a specific point in the gradient [105]. Another way organisms have evolved to produce robust morphological boundaries is by utilising multiple morphogen gradients [106]. How cells robustly process these external signals in a manner that is sensitive to minute changes in ligand concentration, yet is insensitive to the stochasticity of receptor activation, as well as stoichiometric variations in the protein and genetic landscapes across a tissue, remains an area of much study.

Biochemical Society Transactions (2020) **0** 2051–2066
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Throughout this review we, and indeed the majority of the studies have, considered signalling pathways in isolation as a necessary simplification within the scope of available experimental strategies. However, in reality, cells sense and must process many concomitant signals. How this is achieved or how the interplay of signalling pathways effects the cellular outcomes is poorly understood. One of the best studied developmental pathways relies on the interpretation of multiple signalling pathways simultaneously — the Clock and Wavefront model of somitogenesis [107]. In the early embryo of vertebrates, blocks of presomitic mesoderm (PSM) differentiate into repeating segments of different cell types termed somites. The number of and timing of these segments differ in each species, but are tightly controlled within the same species [108]. The reproducible production of these segments requires individual cells to be able to sense both the position and timing of signals [109]

The clock and wavefront model employs morphogen gradient signalling of FGF/Wnt that travel throughout the PSM. In concert with interpreting FGF/Wnt and opposing retinoic acid (RA) gradient-sensing-based positional information, the Notch signalling pathway mediates the coupling of oscillatory gene expression across a field of cells (Figure 3). These oscillations arrest when a certain threshold is reached of the extracellular gradients [108]. In this manner, a cell in a developing organism simultaneously infers multiple signalling pathways and unravels a complex interplay.

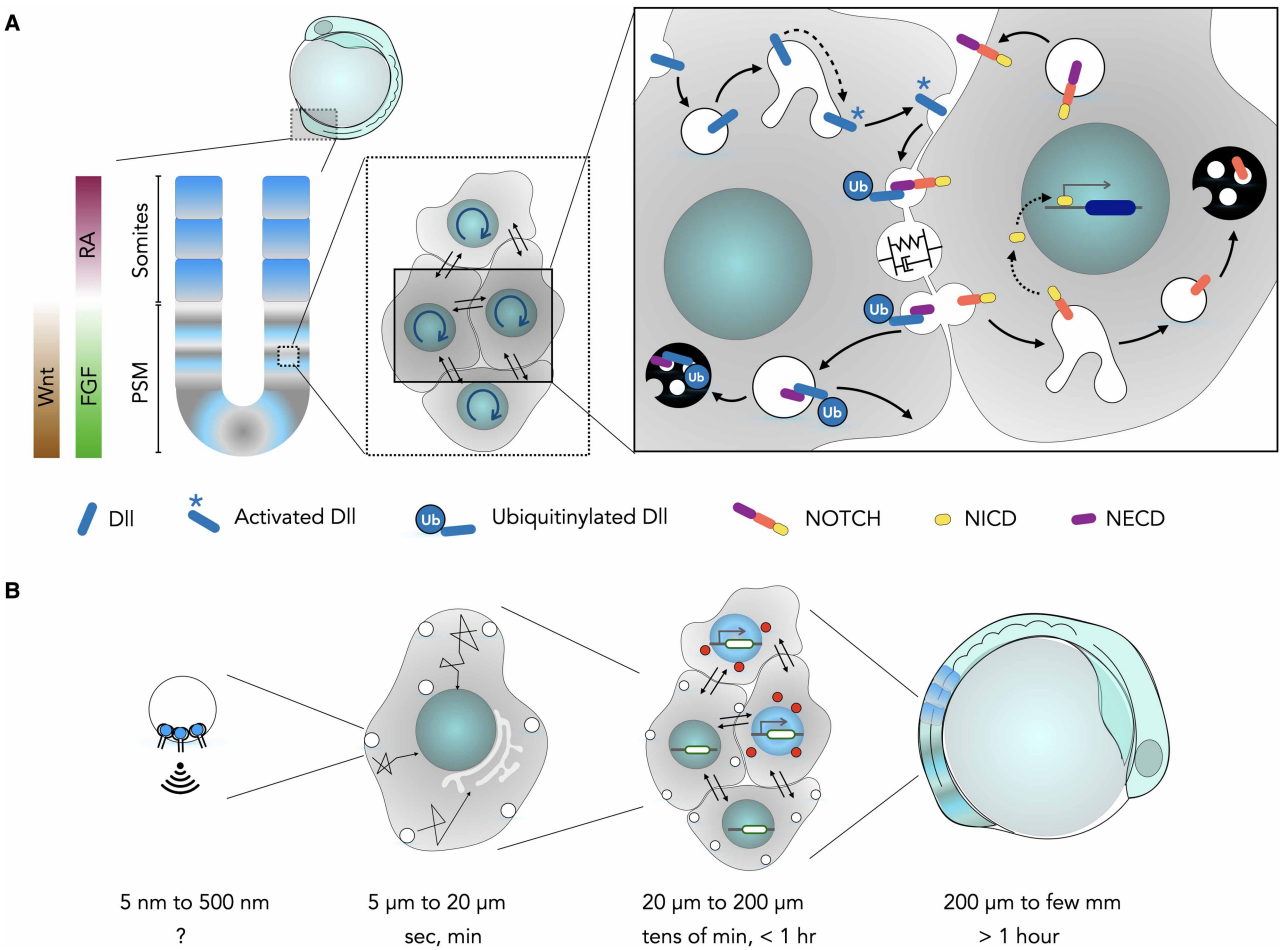

Figure 3. Somitogenesis and spatio-temporal scales of signaling processes.

Somitogenesis and spatio-temporal scales (**A**) A schematic representation of somitogenesis at the organ, multicellular and single cell levels. The tightly controlled periodic development of somites is underpinned by the intercellular coupling of transcription by Notch signalling. The coupling strength is in turn influenced by trafficking in both the signal sending and receiving cells. The process of somitogenesis also depends on reading the gradients of Wnt, FGF and opposing RA by single cells. (**B**) The time and length scales of endosome-based processes in a developing organism schematically represented to emphasise the scaling required in imaging techniques to simultaneously view local, high resolution interactions and dynamics as well as larger, emergent processes.

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Somitogenesis provides a developmentally critical, and incredibly robust system to study signal processing and pattern formation. However, the Delta/Notch system has also been recently used as a catalytic backbone to create a synthetic cell–cell communication system (synNotch), in which both the ligand binding domain and the intracellular signalling domain were replaced with heterologous protein domains [105]. This system presents the opportunity to construct reductionist and adaptable systems to understand cell–cell signalling and complex pattern formation using synthetic biology approaches [106,107].

Imaging approaches to study development across scales

Traditionally, studies at the single cell level (tissue specific endosomal regulations for example through alternative splicing or receptor signalling dynamics) and the consequence at larger spatial and longer timescales (e.g. pattern formation, tissue morphogenesis) have largely remained segregated. Imaging at the spatial resolutions adequate for resolving and capturing organelles with their dynamics, at a view volume of a population of cells, with imaging durations that are relevant for developmental processes is crucial to enable cell-biology level discoveries in the context of developing tissues. In a developing tissue, aberration free imaging of processes deep in tissues is an added requirement owing to aberrations caused by the layers of cells between the volume of interest and the imaging optics (Figure 4a). While many excellent reviews exist highlighting post-acquisition requirements for data handling and analysis from advanced imaging technologies [127], we focus here

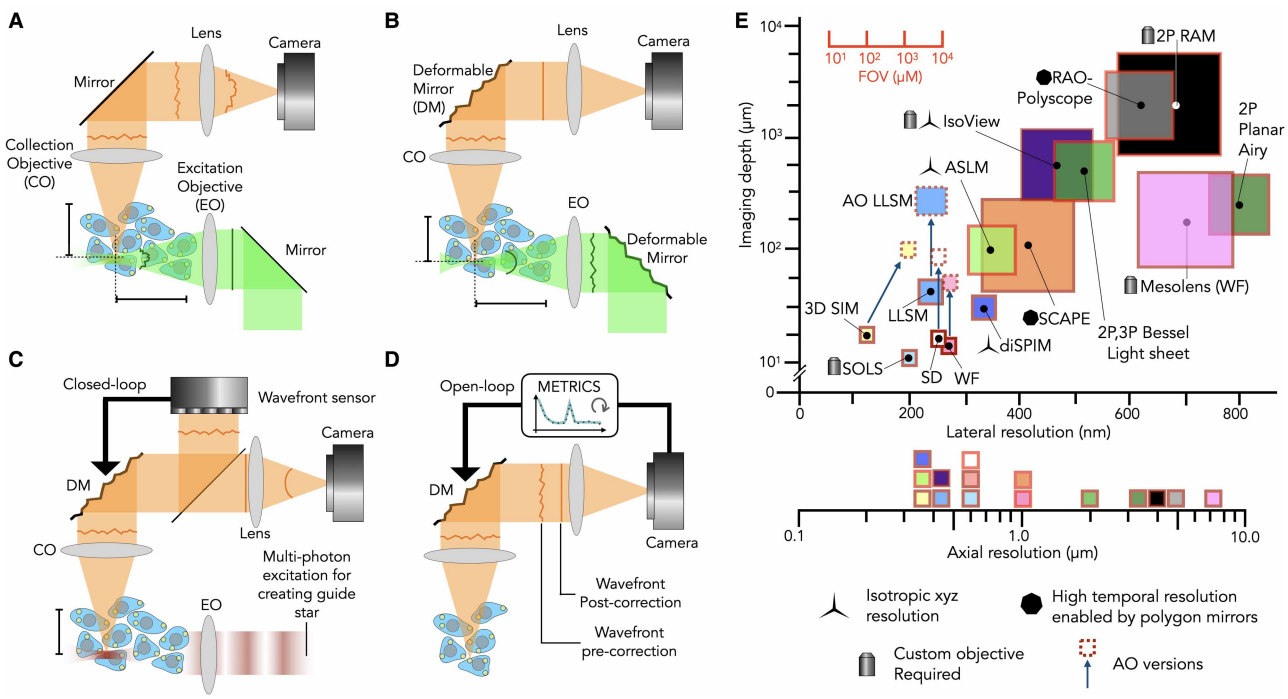

Figure 4. Imaging approaches for studying development across scales.

Imaging approaches (**A**–**D**) Schematic representations of utilisation of Adaptive Optics for gaining depth in imaging. (**A**) Without adaptive optics, deeper imaging quality suffers from both distortions in the excitation as well as the emission wavefronts. (**B**) Ideal situation with deformable motors correcting both excitation profiles and emission wavefronts for best-case scenario. (**C**) Wavefront correction parameters fed into deformable mirrors by inferring the corrections required by sensing wavefront from a 'guide-star' emission created by a multiphoton spot excitation. (**D**) Metrics based parameter iterative optimisation to feed aberration correction to deformable mirrors. (**E**) An overview of different techniques plotted with *x*-axis representing lateral resolution (nm), and *y*-axis the imaging depth (μm). The area of the squares for each technique corresponds to the field of view as scaled to the red length bar (μm). Together with the imaging depth, the 'view-volume' can be approximated. The bottom plot represents the axial resolution of the techniques (μm). Single objective light sheet (SOLS) [110], 3D structured illumination microscopy (3D SIM) [111,112], spinning disk (SD) [113], widefield (WF) [114,115], lattice light sheet microscopy (LLSM) [116,117], dual inverted selective plane illumination microscopy (diSPIM) [118], axially swept light sheet microscopy (ASLM) [119], swept confocally-aligned planar excitation (SCAPE) [120], IsoView [121], 2-photon/3-photon Bessel light sheet [122], raster adaptive optics polyscope (RAO-polyscope) [123], 2-photon random access mesoscope (2P RAM) [124], mesolens-widefield [125], 2-photon planar Airy [126].

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specifically on established and emerging imaging techniques that are most pertinent to studying development, summarised in Figure 4e.

The advent of lattice light sheet microscopy (LLSM) has demonstrably proven to address the requirements of volumetric imaging of subcellular processes [116]. This has been followed by other light sheet modalities operating with higher resolutions (>1.0 NA objectives) employing other ways to implement ultra-thin beams or using axially swept Gaussian beams [119]. An interesting mention, in the context of ease of use is the single objective light sheet by Sapoznik et al. [110] which utilises a standard inverted microscope geometry, making sample preparation and maintaining ambient conditions easier. Most of these techniques are limited to a depth of ∼50 μm or less and need non-standard sample preparation and mounting. Adaptive optics (AO) can be used to mitigate depth-dependent degradation of resolution resulting from aberrations and scattering of light, enabling deep-tissue imaging at subcellular resolutions (Figure 4b). The most sophisticated version of LLSM with AO measures the wavefront distortions directly using a wavefront sensor (Figure 4c) [117]. A 'guide-star' (a focussed multiphoton excitation spot) is used to image and measure the distortions at both, the excitation and emission arms of the microscope. However, this is an effective albeit expensive contraption. Integrating AO with LLSM has resulted in successful observation of exquisite subcellular processes in cells at ∼100 μm deep in live tissues, exemplified by measurement of clathrin coated pits in live zebrafish tissues [117]. A relatively inexpensive way to correct aberrations at the emission arm is to utilise image metrics based AO. This approach utilises iterative processes of optimisation of brightness and sharpness of the observed images, until the appropriate corrective wavefront is obtained (Figure 4d) [128]. This technique has been implemented with various geometries, and more recently with spinning disk, allowing possible modifications of existing set-ups to enable deep-tissue imaging [113].

If we envision going deeper into tissues (>200 μm), with substantial improvement in speed and photo-gentleness based on light sheet geometries, perhaps multiphoton excitation is the most probable option. Non-diffracting beams such as the Bessel or Airy beams which do not have changes in their profile over significant distances have been used in light sheet geometries for deep-tissue imaging with modest success [122,126,129–131]. Exploiting their self-reconstruction properties in combination with multiphoton excitation allows suppression of the concentric ring system in Bessel beams or the lobes in Airy beams [126]. Airy beams also show decreased shadowing artefacts. However, whether these modalities of excitation which, owing to the longer wavelengths have better penetrability, can push the 'imaging at subcellular resolution 'to deeper regimes than AO-LLSM in a developing tissue, in combination with AO is yet to be seen.

A recent trend has been to appreciate the requirements of large field-of-view (FOV) imaging, with higher spatial resolution, resulting in 'mesoscale microscopy'. Swept confocally-aligned planar excitation (SCAPE) approaches large view volumes of $0.6 \times 1 \times 0.55$ mm^3 with resolutions of 0.4–2 μm in XY and 1–3 μm in Z and fills the gap of 'microscopic' resolutions while nearing mesoscopic view volumes [120]. A new custom mesolens has been developed with an NA of 0.47 at 4× magnification, which has significantly improved the lateral as well as axial resolutions available for FOVs as large as 6 mm [125]. A mesoscope combining the advantages of multiphoton excitation and large FOVs is the 2-photon random access mesoscope (2P RAM) [123], with the ability to correct spherical aberrations (including axial chromatic aberrations as a function of depth) effectively using a remote focussing (RF) unit capable of fast axial movements using a fast-moving mirror. This microscope offers a resolution of 0.66 μm in XY and 4.09 μm in Z (at centre) with depth capability up to 1 mm for a FOV of 5 mm. One of the added functionalities of the microscope is fast scans in specific sub-volumes of interest within the entire larger view volume. While the technique has been developed for imaging brain areas in mice, the technique generally may add to 'smart microscopy' that requires context or event dependent adaptation in scanning speeds and resolutions at a specific view volume being tracked and imaged in an ever-changing developing organism.

Similar to imaging deep within a sample, the spatially and temporally variable optical properties of developing tissues can hinder high resolution light sheet based imaging, in large volumetric and developing samples. To combat this, Royer et al. [132] developed an adaptive (different from adaptive optics) 'smart' multi-view light sheet microscopy which employs automated rotation and translation of the excitation and detection objectives to maximise alignment and overlap between the excitation and detection planes [132]. This enables the spatial resolution to be optimised across the imaging volume in real time and continually adjust to any optical changes in the tissue. This was shown to drastically improve resolution and signal strength and has been utilised to image organogenesis at high spatial resolution in a mouse embryo *in toto* [133].

In addition to visualising large volumes during development, it is also beneficial to utilise high resolution imaging to understand the biology at the molecular level. Recently, Hall et al. [134] utilised live cell imaging in

conjunction with 3D-electron microscopy in zebrafish to study endosomal dynamics in skeletal muscle. Through high resolution imaging of endosomal ultrastructure, the authors elucidated mechanisms that lead to T-tubule formation *in vivo*. The combination of the molecular specificity of fluorescence microscopy, provided by targeted fluorophores, together with high resolution ultrastructure revealed by electron tomography is a powerful tool that has been employed to study the ultrastructure of endosomes and plasma membrane tubules [135,136]. Recent work has further expanded on correlative light/electron microscopy (CLEM) by seeking to optimise the method to improve the fluorescence microscopy resolution by enabling single molecule localisation microscopy (SMLM) methods within the same sample [137] and may pave way for such approaches to be used directly on organisms.

In this review, we have only discussed imaging modalities; however, it is critical to note that high content imaging often requires non-trivial post-processing. Briefly, we would like to stress the necessity of good stitching and realignment algorithms required for large volumetric selective plane illumination microscopy (SPIM) time series, especially with long durations of acquisitions. Such imaging also produces large, terabyte-scale data which requires specialised storage infrastructure and analysis pipelines in order to handle the data and extract biologically relevant parameters in an efficient manner. Whilst, there are continual improvements and increases in the accessibility of data storage [138], computational hardware and optimised analysis routines present an ongoing challenge for the widespread adoption of these techniques.

The ability to image long-term, live developmental processes *in vivo* offers huge potential in deciphering the complex biology that underpins developmental processes. This is further compounded by the rise in the development of adjacent molecular tools which have enabled the precise studying of signalling in cellular biology at an unprecedented level. Using rational hybridisation of receptors, adaptors and motor proteins with light sensitive domains has enabled the finessed regulation of protein clustering, localisation and activation in real time [139–142]. These tools can be combined with multiphoton excitation to enable the optical manipulation within a localised region of the sample to complement live imaging studies [143].

Perspectives

- Endosomes have emerged as key players in the regulation of receptor signalling with numerous studies highlighting their involvement across a wide range of receptor families and biological contexts.
- At the same time, the key players and mechanisms which underpin multicellular pattern formation across many organisms and developmental stages are continually being elucidated. However, an understanding of the role that the single cell plays in orchestrating these complex organisations remains elusive.
- Together with the ongoing revolutions in various technologies, including advanced imaging, and fundamental knowledge on single cell biology of intracellular trafficking and signalling, it is an exciting time to aspire towards an integrated ‘molecules-to-tissue’ view of developmental biology.

Competing Interests

The Authors declare no competing interests.

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Abbreviations

2P RAM, 2-photon random access microscopy; AO, adaptive optics; AP-2, adaptor protein-2; ASLM, axially swept localisation microscopy; CLEM, correlative light-electron microscopy; diSPIM, dual inverted selective plane illumination microscopy; Dpp, decapentaplegic; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; Fgf8, fibroblast growth factor 8; FOV, field of view; GPCR, G-protein coupled receptor; LLSM, lattice light-sheet microscopy; NECD, notch extracellular domain; NICD, notch intracellular domain; PNR, perinuclear region; PSM, presomitic mesoderm; RA, retinoic acid; RAO-polyscope, raster adaptive optics polyscope; RF, remote focussing; RTK, receptor tyrosine kinase; SARA, Smad anchor for receptor activation; SCAPE, swept confocally-aligned planar excitation; SD, spinning disk; SIM, structured illumination microscopy; SMLM, single molecule localisation microscopy; SOLS, single objective light sheet; SOP, sensory organ progenitor; SPIM, selective plane illumination microscopy; VEE, very early endosome; WF, widefield.

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