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PERSPECTIVE

In preprints: morphogens in motion

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Morphogen gradients represent one of the most influential ideas in developmental biology. Although the original notion of a morphogen can be traced back more than 80 years, to at least Dalcq and Pasteels' suggestion that gradients of 'morphogenetic substances' guide the differentiation path of cells during embryogenesis (Dalcq and Pasteels, 1937), the concept has evolved over the years (Stapornwongkul and Vincent, 2021). In its current formulation, a morphogen is considered to be a chemical signal that spreads from a localised source to form a concentration gradient in a tissue. Cells within the gradient read the local morphogen concentration to acquire a specific fate. In this view, a morphogen gradient needs to span multiple cell diameters to impart positional information in the developing tissue. Consequently, much attention has focused on how morphogens spread.

A dominant model in the field, the so-called hindered diffusion model, postulates that morphogens diffuse freely in the extracellular space, but that tissue geometry and transient binding interactions affect spreading (Stapornwongkul and Vincent, 2021). Therefore, one can distinguish between free (or local) and effective diffusion rates. Whereas free diffusivity depends on the molecule's size, temperature and viscosity of the environment, effective diffusivity takes into account the effect of morphogen binding and unbinding to receptors on cell surfaces and in the extracellular matrix. Free diffusivity has been successfully measured in a range of model systems using fluorescence correlation spectroscopy (FCS), a method that assays the average time a fluorescently labelled molecule takes to pass through a very small volume (<femtolitre). To infer the effective diffusion coefficient, fluorescence recovery after photobleaching (FRAP) in larger volumes (several cell diameters) is frequently used. However, depending on assumptions about the transport model, FRAP data can result in different values for the effective diffusion coefficient (Zhou et al., 2012). Two recent preprints revisit this issue using new imaging methodologies and technology.

Harish et al. use zebrafish embryos to visualise the Fgf8a morphogen gradient that patterns the developing neural plate (Harish et al., 2022 preprint). Many previous studies have relied on the overexpression of fluorescently tagged morphogens, as visualisation of endogenous morphogen is often complicated by low expression levels (Yu et al., 2009). To rule out artefacts that might derive from such ectopic expression, the authors engineered EGFP into the endogenous *fgf8a* locus. Crucial for imaging this allele was the use of GaAsP hybrid detectors, which are a new type of photodetector particularly suited to sensitive single-photon counting. Imaging the tagged Fgf8a revealed the expected graded distribution along the embryo's animal-vegetal axis. FCS analysis to monitor Fgf8a movement in the extracellular space suggested the presence of two groups of molecules. The majority (92%) of Fgf8a moved with a diffusion coefficient (D) of 56 $\mu m^2\ s^{-1}$, which is similar to that of EGFP in solution, suggesting it is freely diffusing in extracellular space. The remaining Fgf8a was an order of magnitude slower moving, with a D of 4 $\mu m^2\ s^{-1}$.

This suggested that something was hindering Fgf8a movement. Treatment of embryos with heparinase I, an enzyme that cleaves the side chains of heparan sulphate proteoglycans, decreased the amount of ligand in the slow-moving fraction, suggesting that it represented morphogen associated with extracellular matrix constituents. In addition, heparinase I treatment increased the overall levels of Fgf8a-EGFP in the extracellular space. This was likely the result of disassociation of Fgf8a from heparan sulphate proteoglycans, which usually impede Fgf8a movement. Importantly, the authors showed that the longer-range gradient produced by heparinase I treatment resulted in the broadening of Fgf8a target gene expression domains. Finally, the authors demonstrated that versions of Fgf8a attached to transmembrane domains were unable to activate target genes more than one cell diameter away, supporting the conclusion that extracellular diffusion is the predominant mechanism by which Fgf8a mediates its morphogen activity.

These data are consistent with Fgf8a spreading via a hindered diffusion mechanism. However, so far there is no direct evidence that individual molecules switch between free diffusing and bound states. Measuring the rate of exchange of molecules between the slow and fast diffusion fractions will be necessary to test whether hindered diffusion is sufficient to explain the dynamics of Fgf8a gradient formation. This could be accomplished by monitoring individual diffusing morphogen molecules in real time *in vivo*. A second recent preprint, by Kuhn et al., develops methods to do just that (Kuhn et al., 2022 preprint).

Kuhn et al. focus on the TGFβ superfamily ligands Nodal and Lefty, an activator-inhibitor morphogen pair crucial for the formation of mesoderm and endoderm in the zebrafish gastrula (Hill, 2022). Whereas Nodal acts at a short range, Lefty has been shown to inhibit signalling as far as 500 µm from its region of secretion (Müller et al., 2012). Based on FCS and FRAP experiments, both are suggested to spread by hindered diffusion, but differences in transient binding interactions are thought to cause their distinct signalling ranges. In the new work, Kuhn et al. developed proteins fused with HaloTag to observe single molecules of Nodal and Lefty. HaloTag allows the protein of interest to be covalently labelled with synthetic fluorophores that offer superior brightness to fluorescent proteins. By adjusting the concentration of fluorophores, Kuhn et al. were able to label morphogens at sufficiently low density to make single-molecule tracking possible.

To track diffusing morphogens, the extracellular space was divided into regions of close cell-cell contacts and intercellular cavities, which separate the loosely packed cells of the blastoderm. This distinction was important because molecule tracking showed

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differential behaviour in these two regions. In cavities, both Nodal and Lefty molecules exhibited higher mobility than in interfaces. Fitting the distributions of jump distances from single-molecule tracks to a diffusion model revealed three components to ligand movement: a slow component, which probably represented immobile molecules, an intermediate component, and a fast component. Strikingly, in both interfaces and cavities, the diffusion coefficients of intermediate and fast components were similar for Nodal and Lefty molecules. However, there was a higher fraction of immobile Nodal than Lefty; conversely, the fraction of fast-diffusing molecules was larger for Lefty than Nodal. This suggested that the free diffusion coefficients of Nodal and Lefty are comparable, but that the difference in mobility between the ligands arises from increased retention of Nodal in an immobile state. Consistent with this, time-lapse imaging revealed Nodal molecules bound to interfaces with retention times of 10-20 s. Lefty, however, had fewer and shorter duration binding events than Nodal. Importantly, overexpression of the Nodal co-receptor One Eyed Pinhead (also known as Tdgf1) substantially increased the fraction of immobile Nodal molecules, suggesting that the binding of Nodal to cell-surface proteins is at least partly responsible for the lower proportion of molecules with high mobility.

Counterintuitively, despite the higher fraction of immobile and slowly moving molecules observed in interfaces compared with cavities, overall a higher proportion of both Nodal and Lefty was found in extracellular cavities. To explore the reason for this, the authors constructed a computational simulation that modelled blastoderm geometry, molecule movement and receptor binding. This recapitulated the experimental observations. At relatively low receptor density and binding times, molecules tended to localise in cavities. Decreasing the width of the extracellular space in these simulations further increased this tendency, suggesting a role for tissue architecture in morphogen localisation. Conversely, increasing receptor density in the tissue predicted a decrease in molecules in cavities. To test this experimentally, the authors measured the distribution of secreted GFP in embryos injected with increasing amounts of membrane-tethered GFP-binding nanobodies. With low amounts of nanobody, secreted GFP was detected mainly in cavities whereas increasing the amount of nanobody enriched GFP at interfaces.

Similar to the Fgf8a study, this study supports the idea that Nodal and Lefty spread through the blastoderm via hindered diffusion. But is hindered diffusion a universal mechanism of morphogen transport? Other model systems for studying morphogen gradient formation, such as the *Drosophila* wing disc and the vertebrate neural tube, are densely packed pseudostratified epithelia and have fundamentally different tissue architectures compared with the zebrafish blastoderm. Such epithelia typically do not contain extracellular cavities, and the interfaces between cells are estimated to be around 50 times more narrow (~20 nm) than in the zebrafish blastoderm (Stapornwongkul et al., 2020). According to Kuhn et al.'s mathematical model, one would expect that such tissue architecture would slow down diffusion because morphogens are found more frequently in a bound state at the cell surface. Indeed, morphogen spreading in epithelia is believed to operate over shorter distances and on longer time scales compared with the zebrafish blastoderm.

Kuhn et al. also report that they observed only very few endocytosis events over the tracking periods. This is of particular interest because endocytosis followed by recycling and secretion has been proposed to be crucial for spread of the morphogen Dpp in the wing disc (Romanova-Michaelides et al., 2022). Moreover, receptor-mediated endocytosis is commonly assumed to be the dominant mechanism to clear morphogens from the extracellular space. The rate of morphogen degradation modulates morphogen gradient shape, and without a morphogen sink steady-state morphogen gradients cannot form. With single-molecule tracking now considered the new gold standard to assess how morphogens move through complex tissues, it will be exciting to see the adaption of these techniques to other model systems to put current models to the test.

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Competing interests
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