

## Investigating the principles of morphogen gradient formation: from tissues to cells

Anna Kicheva<sup>1</sup>, Tobias Bollenbach<sup>2</sup>, Ortrud Wartlick<sup>3</sup>, Frank Jülicher<sup>4</sup> and Marcos Gonzalez-Gaitan<sup>3</sup>

Morphogen gradients regulate the patterning and growth of many tissues, hence a key question is how they are established and maintained during development. Theoretical descriptions have helped to explain how gradient shape is controlled by the rates of morphogen production, spreading and degradation. These effective rates have been measured using fluorescence recovery after photobleaching (FRAP) and photoactivation. To unravel which molecular events determine the effective rates, such tissue-level assays have been combined with genetic analysis, high-resolution assays, and models that take into account interactions with receptors, extracellular components and trafficking. Nevertheless, because of the natural and experimental data variability, and the underlying assumptions of transport models, it remains challenging to conclusively distinguish between cellular mechanisms.

### Addresses

<sup>1</sup> MRC-National Institute for Medical Research, Developmental Biology, The Ridgeway, Mill Hill, NW7 1AA London, UK

<sup>2</sup> IST Austria, Am Campus 1, A-3400 Klosterneuburg, Austria

<sup>3</sup> Departments of Biochemistry and Molecular Biology, University of Geneva, 30, Quai Ernest-Ansermet, 1211 Geneva 4, Switzerland

<sup>4</sup> Max Planck Institut für Physik komplexer Systeme, Nöthnitzer Str. 38, 01187 Dresden, Germany

Corresponding author: Gonzalez-Gaitan, Marcos  
([Marcos.Gonzalez@unige.ch](mailto:Marcos.Gonzalez@unige.ch))

**Current Opinion in Genetics & Development** 2012, **22**:527–532

This review comes from a themed issue on **Genetics of system biology**

Edited by **James Briscoe** and **James Sharpe**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 6th September 2012

0959-437X/\$ – see front matter, © 2012 Elsevier Ltd. All rights reserved.

<http://dx.doi.org/10.1016/j.gde.2012.08.004>

### Introduction

In recent years it has become clear that the translation of the morphogen gradient into a pattern of gene expression is dynamic and indirect, that morphogen gradients can also regulate growth, and that target cells themselves actively shape gradients (reviewed in [1–3]). Therefore, to study the coordination between morphogen signaling, target gene response and growth which produces patterned tissues requires understanding the mechanisms that cells use to shape gradients.

To understand how the characteristic shape of a morphogen profile is established over a particular developmental time requires a quantitative approach. Most morphogens for which quantitative data exist form approximately exponential concentration profiles (Table 1). Theoretically, exponential gradients are a natural consequence of morphogen secretion from localized sources, spatially uniform degradation and non-directional spreading (reviewed in [4]). This implies that the gradient shape can be characterized by its amplitude and decay length and is determined by three effective kinetic parameters: the morphogen production rate, diffusion coefficient, and degradation rate.

This macroscopic, tissue-level description of morphogen transport does not explicitly consider discrete cells and molecular interactions, such as binding of morphogen molecules to other components and trafficking within and between cells in confined organelles (reviewed in [1]). The kinetic parameters in macroscopic models capture the effects such molecular interactions generate on large scales and thus represent effective tissue-level rates, which capture behaviours on length scales greater than a cell diameter and time scales larger than the time during which the morphogen crosses one cell diameter.

A major challenge has been to develop assays that distinguish between different cellular mechanisms of morphogen transport [1] by clarifying how specific molecular interactions influence the tissue-level effective rates. The combination of biophysical theory, genetics, and *in vivo* imaging techniques has allowed designing a diverse repertoire of experiments. We will review two types of approaches: (i) tissue-level assays, which measure morphogen behaviour on large length scales in different conditions that can help to distinguish between specific cellular mechanisms, and (ii) cellular-level assays where kinetic rates are measured on small scales.

### Unravelling cellular mechanisms with tissue-level assays

Before considering how specific molecular events (e.g. binding and trafficking) control the tissue-level morphogen behaviour and hence gradient shape, it is useful to know what the tissue-level kinetic rates are. In recent years, tissue-level morphogen kinetics has been measured using FRAP, where the observation time scale is similar to the time scale of gradient formation and is usually on the order of hours, rather than seconds or days

**Table 1****Kinetic parameters of some morphogen gradients**

Morphogen and tissue	Decay length ( $\mu\text{m}$ )	Amplitude	Diffusion coefficient $D$ ( $\mu\text{m}^2/\text{s}$ )	Half-life $\tau$ (min)	Time of measurement	Method of measuring $D$ and $\tau$	Reference
<b>Bicoid</b> Drosophila embryo	100	55nM (4750 molec./nucleus)	0.30 (C)		cycle 14	FRAP	[6**,37]
		140 nM	7.4 (N)	40** 36	cycle 14 mid cycle 14	FCS photoswitching	[25,26] [10]
<b>Cyclops</b> Zebrafish embryo	20***		0.7 (TOT)	115	blastula	FRAP PA	[7**]
<b>Dorsal</b> Drosophila embryo	5				cycle 10–14		[20*]
	40*					[34]	
<b>Dpp</b> Drosophila wing disc	20 (7.7 c.d.)	4400 molec./cell	0.1 (TOT)	45	third instar third instar	FRAP	[5**,9**]
			21 (65%); 0.003 (35%) (EX)			FCS	[11*]
<b>Dpp</b> Drosophila haltere	10 (4 c.d.)		0.005 (TOT)	250**	third instar	Production rate reporter assay	[9**]
<b>Fgf8</b> Zebrafish embryo	200 (9 c.d.)		53 (91%); 4 (9%) (EX)	9**	sphere-germ ring	FCS	[24*]
<b>Hh</b> Drosophila wing disc	7 (2.7 c.d.)				third instar		[9**]
<b>Lefty1</b> Zebrafish embryo	80***		11.1 (TOT)	220	blastula	FRAP PA	[7**]
<b>Lefty2</b> Zebrafish embryo	100***		18.9 (TOT)	170	blastula	FRAP PA	[7**]
<b>Shh</b> Chick neural tube	20 (7c.d)				E4		J.B. ****
<b>Squint</b> Zebrafish embryo	40***		3.2 (TOT)	95	blastula	FRAP PA	[7**]
<b>Wg</b> Drosophila wing disc	6 (2.2 c.d.)		0.05 (TOT)	8**	third instar	FRAP	[5**]

All values are reported approximately for rough comparison. For precise values, standard deviations, and further details, see respective reference. Note that many of the reported kinetic parameters apply to morphogen-fluorescent protein fusions, rather than the endogenous proteins, and have been determined in conditions of ectopic or overexpression.

c.d. – cell diameters.

C – cytoplasmic; N – nuclear; TOT – total pool (intra + extracellular), EX – extracellular.

FRAP – Fluorescence Recovery After Photobleaching.

FCS – Fluorescence Correlation Spectroscopy.

PA – photoactivation.

\* The value given is the full width of a Gaussian fit to the gradient profiles at 60% of the maximum.

\*\* The half-life was inferred from the gradient decay length.

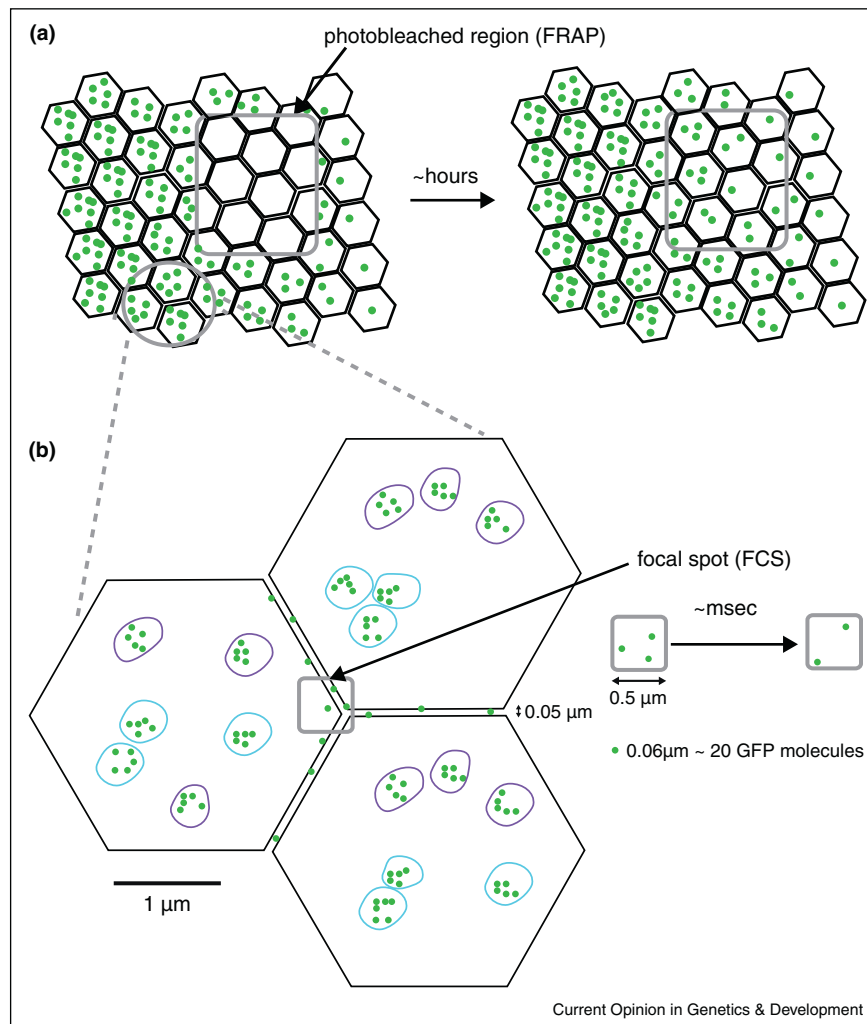
\*\*\* The distance at which the fluorescence decays by 50% from the value at the source boundary.

\*\*\*\* James Briscoe, personal communication.

[5\*\*,6\*\*,7\*\*] (Table 1, Figure 1a). To estimate the effective diffusion coefficient  $D$  and the degradation rate  $k$  from FRAP assays, a solution of the diffusion equation with degradation and production terms for the specific geometry of the bleached region and boundary conditions is fit to the fluorescence recovery curve [5\*\*,8]. The parameters optimized in the fit are  $D$ ,  $k$ , the immobile fraction and the bleaching depth. Depending on the geometry of the bleached region, such a fit might not be sufficiently constrained to determine both  $D$  and  $k$ . In

this case, one of these parameters is determined from the fit to the recovery curve, and the other inferred using the measured gradient decay length  $\lambda$ , which depends on  $D$  and  $k$  as  $\lambda = \sqrt{\frac{D}{k}}$ . Both parameters can also be determined independently without using  $\lambda$  by quantifying and simultaneously fitting the fluorescence recoveries in different subregions of the bleached area, which sufficiently constrains the fit procedure [5\*\*,9\*\*]. Alternatively, the degradation rate can be measured with an independent assay. For example, the half-lives of Nodal

Figure 1



**(a)** Apical view of an epithelium with a morphogen gradient (green). To measure the tissue-level kinetics using FRAP, a region that spans several cell diameters (grey rectangle) is bleached. The time scale of recovery is proportional to the morphogen half-life (Table 1). **(b)** Magnified view of the tissue depicted in A, drawn approximately to scale using measurements from the *Drosophila* wing disc. The relative dimensions correspond to: apical cell area  $5.4 \mu\text{m}^2$ ; extracellular space – 50 nm; each green dot corresponds to the size of  $\sim 20$  GFP molecules (considering width of GFP 3 nm).  $\sim 90\%$  of the morphogen is contained in endosomes (blue – early endosomes, purple – other endosomes) [28], and 10% is extracellular or cortical. Endosome size is not to scale. The FCS focal spot is depicted as a grey square with side  $0.5 \mu\text{m}$ .

and Lefty-Dendra2 in the zebrafish embryo were determined by photoconversion and monitoring the fluorescence decay over time [7<sup>••</sup>]. Similarly, the lifetime of Bicoid-Dronpa in the *Drosophila* embryo was measured using repeated photoswitching [10] (Table 1).

In general, different morphogen transport mechanisms can be consistent with the same effective kinetic rates. To study which specific cellular events underlie the macroscopic morphogen behaviour, tissue-level assays can be modified for instance by photobleaching regions with different geometries. In ‘nested FRAP’, the recovery in the entire bleached area is compared to that in a smaller subregion [5<sup>••</sup>, 7<sup>••</sup>, 11<sup>•</sup>]. If transport occurs by fast

extracellular diffusion, fluorescence recovery is expected almost simultaneously and rapidly in the bleached region because the extracellular pool would recover faster than the observation time-scale (as reported for Dpp-Dendra2 in the wing disc [11<sup>•</sup>]). It would be interesting to test whether such rapid recoveries can be observed far from the source when a very large area is photobleached. If transport is dominated by slower cellular events, like endocytosis, the recovery would start later in the center than at the periphery, thus causing a difference in the shapes of the recovery curves (as for Dpp-GFP in the wing disc and Nodal-GFP in zebrafish embryos) [5<sup>••</sup>, 7<sup>••</sup>]. The observed difference in each individual experiment would be affected by experimental variation. Therefore,

the measurement of experimental variation and the quality of the correspondence between the data and the theoretical curve (as in [5\*\*]) is essential for determining which model the recoveries are consistent with.

Alternatively, tissue-level assays can be applied to mutant conditions with pronounced effects on morphogen kinetics. For instance, FRAP experiments in 'shibire' mutant tissues where endocytosis was partially or fully blocked in a temperature-controlled way, suggested that endocytosis has an effect on the effective diffusion coefficient of Dpp [5\*\*]. That endocytosis is involved in transport through the tissue, rather than purely affecting effective degradation is also supported by observations that the surface receptor and extracellular Dpp levels are not significantly higher in shibire mutant clones than in wildtype tissues [12\*]. Endocytosis could also affect the levels of other surface and secreted molecules involved in morphogen spreading, such as Dally, Dlp and Pentagone [13,14\*\*,15\*\*]. Indeed, Dlp is found to be apically increased in shibire clones, in which Hh and Wg signaling are also affected [16]. To determine how endocytosis affects Dpp spreading, it would be useful to quantitatively measure morphogen kinetics in conditions where these components are perturbed. In addition, developing assays to directly measure receptor occupancy, binding, and trafficking together with the known rates of receptor degradation [9\*\*], would help to distinguish between transcytosis, restricted and free extracellular diffusion as transport mechanisms [5\*\*,11\*,12\*,17].

In addition to FRAP, which creates a non-steady state distribution of fluorescently tagged morphogen, tissue-level kinetics can be assayed using the same principles by photoactivation/photoconversion [11\*]. The fluorescence in the photoconversion area should decrease owing to transport and degradation. However, such experiments are challenging because of the difficulty of establishing non-phototoxic photoactivation conditions (e.g. see [18]) and small numbers of observed molecules compared to FRAP. For instance, no Dpp-Dendra was detected away from the photoconversion region in the wing disc [11\*]. This observation was invoked to suggest that the transported pool is undetectably small, but it remains to be determined whether the process of photoactivation itself does not affect the Dpp trafficking. A complementary tool could be to monitor the fluorescence decrease inside the photoactivated region, similarly to Fluorescence loss in photobleaching (FLIP) experiments, where continuous bleaching of an area leads to observing a halo of bleached molecules in adjacent cells.

FLIP was used to show dynamic nucleocytoplasmic shuttling of Dorsal in the *Drosophila* embryo [19] and a transient compartmentalization of the syncytium during

mitosis, which has potential implications for effective morphogen diffusion [20\*]. This compartmentalization might explain the apparent lack of effect of Bicoid nuclear trapping [21,22] during interphase on the gradient length scale. Thus, FRAP and FLIP approaches where a specific subcellular compartment is bleached, can also be used to address the effects of tissue topology on effective morphogen spreading (see also [7\*\*]).

### Understanding tissue-level behaviour with high resolution assays

A complementary approach to tissue-level assays is to measure the diffusion and trafficking rates on small length scales and investigate how they give rise to effective tissue-level kinetics. Several methods have been developed to measure morphogen dynamics with subcellular resolution. Fluorescence correlation spectroscopy (FCS) quantifies the temporal fluctuations of the fluorescent signal in a volume of  $\sim 0.5 \mu\text{m}^3$  on millisecond timescales and uses an autocorrelation function to estimate the local diffusion coefficient and the concentration of molecules in the focal volume [23]. It is also possible to distinguish whether molecules traversing the spot are free or part of a complex, as well as the relative abundance of different pools. Crucially, diffusion coefficients obtained by FCS characterize diffusion on a subcellular scale and are therefore typically very different from effective diffusion coefficients measured on the tissue scale [1,22] (Table 1, Figure 1).

By performing FCS measurements at different distances to the source of Fgf8-EGFP in injected zebrafish embryos, Yu *et al.* reconstructed the shape of the extracellular Fgf8 gradient and estimated that 91% of Fgf8-GFP diffuse with a diffusion coefficient of  $53 \mu\text{m}^2/\text{s}$  [24\*], which was affected by inhibiting endocytosis. Based on this, it was suggested that the extracellular Fgf8 gradient forms by free diffusion combined with a uniform sink. A slow fraction of extracellular Fgf8, affected by interactions with proteoglycans, was also found. These results demonstrate that FCS is a useful tool for measuring the local kinetics of distinct morphogen fractions. However, understanding how these extracellular fractions and the internalized pool interact on long time scales to produce the long-range tissue-level behaviour, remains a challenge.

Because of the small spatial scale of FCS it might be difficult to reproducibly position the focal spot within large subcellular compartments, such that all morphogen fractions are represented in the data. For instance, the nuclear size in the early *Drosophila* embryo [6\*\*] is  $\sim 100$  times larger than the focal spot, which raises the question of whether the experimental setup allows measuring the kinetics of all fractions of nuclear Bicoid (see [25,26]). Conversely, since FCS is a diffraction-limited technique, it is challenging to apply to subcellular compartments

smaller than  $\sim 200$  nm, such as small vesicles or the extracellular space of very densely packed epithelia like the *Drosophila* wing disc, which could be as narrow as  $\sim 10$  nm (M.G.G. unpublished observation, see also [11<sup>\*</sup>]).

Several modifications of FCS are emerging to overcome this and other technical challenges (reviewed in [23]). For instance, scanning-FCS combined with dual-color cross-correlation has been used to determine the mobility of Fgf receptors along the membrane and their binding affinity for Fgf8 *in vivo* [27]. The use of FCS for studying receptor–ligand interactions in developing tissues could provide key data for distinguishing different transport mechanisms. For instance, the restricted diffusion hypothesis for Dpp in the wing disc [17] predicts high reversibility of morphogen binding, whereas the free diffusion hypothesis requires nearly irreversible binding to receptors [11<sup>\*</sup>]. Besides data on binding kinetics, assays for measuring the relative sizes of morphogen pools located in distinct subcellular compartments are needed. In this regard, further development of techniques based on cross-correlating images of particles containing two different fluorescent labels over time (PICCS) could be useful. PICCS has been used to estimate what fraction of early endosomes contained Dpp [28<sup>\*</sup>].

### Future directions

The combination of theory and quantitative *in vivo* imaging has opened exciting possibilities for studying the cellular mechanisms underlying morphogen gradient formation. However, even for well-studied systems such as Bicoid in the fly embryo and Dpp in the wing disc [1,22], these mechanisms are still not entirely understood. For instance, although it is clear that endocytosis has a key role in Dpp gradient formation, it is unclear how it influences the tissue-level kinetics and whether recycling (transcytosis), restricted or free extracellular diffusion are essential for long-range Dpp transport.

A major challenge in addressing such questions remains to relate cellular-scale models to effective theories on the tissue scale. Numerical simulations are helpful to bridge between scales. Alternatively, the effective tissue-level parameters can be systematically determined from detailed models where different processes occur on separable time-scales [29,30<sup>\*\*</sup>,31<sup>\*</sup>,32<sup>\*</sup>]. The development of better and higher-resolution assays for detecting morphogen interactions will be key for identifying the relevant rate-limiting processes.

Finally, the emergent properties of different trafficking mechanisms could help to distinguish between them. Systems that involve feedback or non-linear diffusion can be more robust to fluctuations in morphogen production [31<sup>\*</sup>,33]. In turn, models where the degradation rate can change over time could account for the scaling of

the gradient amplitude and decay length with tissue size [9<sup>\*\*</sup>]. Indeed, temporally increasing amplitude and in some cases gradient scaling have been observed for several morphogens [6<sup>\*\*</sup>,9<sup>\*\*</sup>,34–36]. Gradient scaling has direct consequences for tissue patterning and the regulation of growth, which emphasizes the fact that growth and gradient formation are intrinsically coupled processes and should be studied in conjunction.

### Acknowledgements

AK is currently supported by an MRC CDF. MGG and OW were supported by the Swiss National Science Foundation, grants from the Swiss SystemsX.ch initiative, LipidX-2008/011, an ERC advanced investigator grant and the Polish-Swiss research program.

### References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Rogers KW, Schier AF: **Morphogen gradients: from generation to interpretation.** *Annu Rev Cell Dev Biol* 2011, **27**:377-407.
  2. Wartlick O, Mumcu P, Jülicher F, González-Gaitán M: **Understanding morphogenetic growth control – lessons from flies.** *Nat Rev Mol Cell Biol* 2011, **12**:594-604.
  3. Kutejova E, Briscoe J, Kicheva A: **Temporal dynamics of patterning by morphogen gradients.** *Curr Opin Gen Dev* 2009, **19**:315-322.
  4. Wartlick O, Kicheva A, González-Gaitán M: **Morphogen gradient formation.** *Cold Spring Harb Perspect Biol* 2009, **1**:a001255.
  5. Kicheva A, Pantazis P, Bollenbach T, Kalaidzidis Y, Bittig T, Jülicher F, González-Gaitán M: **Kinetics of morphogen gradient formation.** *Science* 2007, **315**:521-525.
- In this report, the effective diffusion, degradation and production of Dpp and Wingless are measured in the wing disc by using FRAP. It introduces parallel FRAP, perpendicular FRAP and 'nested' FRAP. The effect on these parameters in a thermosensitive mutant in endocytosis is also studied.
6. Gregor T, Wieschaus E, McGregor A, Bialek W: **Stability and nuclear dynamics of the bicoid morphogen gradient.** *Cell* 2007, **130**:141-152.
- Characterization of the formation of a Bicoid-eGFP gradient and the possible role of nuclear dynamics in this process. Bicoid-eGFP diffusion coefficient measured by FRAP.
7. Müller P, Rogers KW, Jordan BM, Lee JS, Robson D, Ramanathan S, Schier AF: **Differential diffusivity of nodal and lefty underlies a reaction-diffusion patterning system.** *Science* 2012, **336**:721-724.
- Study in zebrafish of the diffusivity of Nodal and Lefty by FRAP and measurements of their clearance by pulse labelling. It shows that differential diffusivity is the major determinant of the differences between Nodal and Lefty ranges.
8. Kicheva A, Holtzer L, Wartlick O, Schmidt T, González-Gaitán M: **Quantitative imaging of morphogen gradients in *Drosophila* imaginal discs.** In *Imaging in Developmental Biology: A Laboratory Manual*. Edited by Sharpe J, Wong R, Yuste R. CSHL Press; 2011:533-550.
  9. Wartlick O, Mumcu P, Kicheva A, Bittig T, Seum C, Jülicher F, González-Gaitán M: **Dynamics of Dpp signaling and proliferation control.** *Science* 2011, **331**:1154-1159.
- A study of the changes in the properties of the Dpp gradient during growth of the imaginal discs: it shows that the gradient scales with tissue size. It also studies the changes of effective diffusion, degradation and production during the growth phase. Measurements include FRAP analysis. It concludes that the gradient scales with the size of the tissue by changing the half-life of Dpp.

10. Drocco J, Grimm O, Tank DW, Wieschaus E: **Measurement and perturbation of morphogen lifetime: effects on gradient shape.** *Biophys J* 2011, **101**:1807-1815.
11. Zhou S, Lo W-cheong, Suhaimi JL, Digman MA, Gratton E, Nie Q, Lander AD: **Free extracellular diffusion creates the Dpp morphogen gradient of the Drosophila wing disc.** *Curr Biol* 2012, **22**:668-675.
- This study analyses the Dpp gradient by 'nested' FRAP, photoconversion and FCS. It proposes a model of gradient formation based on fast extracellular diffusion, quick extracellular clearance by endocytosis and slow lysosomal degradation.
12. Kruse K, Pantazis P, Bollenbach T, Jülicher F, González-gaitán M: **Dpp gradient formation by dynamin-dependent endocytosis: receptor trafficking and the diffusion model.** *Development* 2004, **131**:4843-4856.
- Experimental and theoretical study of the model of Dpp gradient formation by extracellular diffusion. Shows that the levels of extracellular Dpp do not increase in a thermosensitive endocytosis mutant.
13. Yan D, Lin X: **Shaping morphogen gradients by proteoglycans.** *Cold Spring Harb Perspect Biol* 2009, **1**:a002493.
14. Ben-zvi D, Pyrowolakis G, Barkai N: **Expansion-repression mechanism for scaling the Dpp activation gradient in Drosophila wing imaginal discs.** *Curr Biol* 2011, **21**:1391-1396.
- Together with reference [15\*\*], it studies experimentally a model of Dpp gradient scaling based on an expansion-repression model. It proposes that the key molecule (the expander) is Pentagone. In reference [15\*\*] they show that this could act by repressing the degradation of Dpp.
15. Vuilleumier R, Springhorn A, Patterson L, Koidl S, Hammerschmidt M, Affolter M, Pyrowolakis G: **Control of Dpp morphogen signalling by a secreted feedback regulator.** *Nat Cell Biol* 2010, **12**:611-617.
- See reference [14\*\*].
16. Gallet A, Staccini-Lavenant L, Théron PP: **Cellular trafficking of the glypican Dally-like is required for full-strength Hedgehog signaling and wingless transcytosis.** *Dev Cell* 2008, **14**:712-725.
17. Schwank G, Dalessi S, Yang S-F, Yagi R, de Lachapelle AM, Affolter M, Bergmann S, Basler K: **Formation of the long range Dpp morphogen gradient.** *PLoS Biol* 2011, **9**:e1001111.
18. Jacobson K, Rajfur Z, Vitriol E, Hahn K: **Chromophore-assisted laser inactivation in cell biology.** *Trends Cell Biol* 2008, **18**:443-450.
19. Delotto R, Delotto Y, Steward R, Lippincott-schwartz J: **Nucleocytoplasmic shuttling mediates the dynamic maintenance of nuclear Dorsal levels during Drosophila embryogenesis.** *Development* 2007, **137**:4233-4241.
20. Daniels BR, Rikhy R, Renz M, Dobrowsky TM, Lippincott-schwartz J: **Multiscale diffusion in the mitotic Drosophila melanogaster syncytial blastoderm.** *Proc Natl Acad Sci USA* 2012, **109**:8588-8593.
- Analysis of the contribution of instantaneous diffusion coefficients and transient mitotic membrane furrows to effective diffusion rates in the Drosophila syncytial blastoderm on different time and length scales. The importance of this multi-scale diffusion for the interpretation of FRAP experiments is discussed.
21. Grimm O, Wieschaus E: **The Bicoid gradient is shaped independently of nuclei.** *Development* 2010, **137**:2857-2862.
22. Grimm O, Coppey M, Wieschaus E: **Modelling the Bicoid gradient.** *Development* 2010, **137**:2253-2264.
23. Ries J, Schwillie P: **Fluorescence correlation spectroscopy.** *Bioessays* 2012, **34**:361-368.
24. Yu SR, Burkhardt M, Nowak M, Ries J, Petrásek Z, Scholpp S, Schwillie P, Brand M: **Fgf8 morphogen gradient forms by a source-sink mechanism with freely diffusing molecules.** *Nature* 2009, **461**:533-536.
- In this zebrafish study the concentration of extracellular FGF8 and its diffusion are using FCS. It proposes that FGF8 spreads by extracellular diffusion and the gradient is formed by clearance mediated by endocytosis.
25. Porcher A, Abu-arish A, Huat S, Roelens B, Fradin C, Dostatni N: **The time to measure positional information: maternal Hunchback is required for the synchrony of the Bicoid transcriptional response at the onset of zygotic transcription.** *Development* 2010, **137**:2795-2804.
26. Abu-Arish A, Porcher A, Czerwonka A, Dostatni N, Fradin C: **High mobility of bicoid captured by fluorescence correlation spectroscopy: implication for the rapid establishment of its gradient.** *Biophys J* 2010, **99**:L33-L35.
27. Ries J, Yu SR, Burkhardt M, Brand M, Schwillie P: **Modular scanning FCS quantifies receptor-ligand interactions in living multicellular organisms.** *Nat Methods* 2009, **6**:643-645.
28. Semrau S, Holtzer L, González-Gaitán M, Schmidt T: **Quantification of biological interactions with particle image cross-correlation spectroscopy (PICCS).** *Biophys J* 2011, **100**:1810-1818.
- Proposes a quantitative method for the study of internalized ligands (in this case Dpp) while trafficking in different endosomal compartments.
29. Gunawardena J: **A linear framework for time-scale separation in nonlinear biochemical systems.** *PLoS ONE* 2012, **7**:e36321.
30. Sample C, Shvartsman SY: **Multiscale modeling of diffusion in the early Drosophila embryo.** *Proc Natl Acad Sci USA* 2010, **107**:10092-10096.
- In this paper, the authors derive an expression for effective diffusivity as a function of geometry, cytoplasmic diffusivity and nucleocytoplasmic shuttling.
31. Bollenbach T, Kruse K, Pantazis P, González-Gaitán M, Jülicher F: **Robust formation of morphogen gradients.** *Phys Rev Lett* 2005, **94**:018103.
- Together with reference [32\*], these two reports present theoretical studies of the model of spreading of Dpp by intracellular trafficking (planar transcytosis) and considers the high level of robustness of the gradient that this model would deliver.
32. Bollenbach T, Kruse K, Pantazis P, González-Gaitán M, Jülicher F: **Morphogen transport in epithelia.** *Phys Rev E Stat Nonlin Soft Matter Phys* 2007, **75**:011901.
- See reference [31\*].
33. Eldar A, Rosin D, Shilo B-zion, Barkai N: **Self-enhanced ligand degradation underlies robustness of morphogen gradients.** *Dev Cell* 2003, **5**:635-646.
34. Liberman LM, Reeves GT, Stathopoulos A: **Quantitative imaging of the Dorsal nuclear gradient reveals limitations to threshold-dependent patterning in Drosophila.** *Proc Natl Acad Sci USA* 2009, **106**:22317-22322.
35. Kanodia JS, Rikhy R, Kim Y, Lund VK, Delotto R, Lippincott-schwartz J, Shvartsman SY: **Dynamics of the Dorsal morphogen gradient.** *Proc Natl Acad Sci USA* 2009, **106**:21707-21712.
36. Chamberlain CE, Jeong J, Guo C, Allen BL, McMahon AP: **Notochord-derived Shh concentrates in close association with the apically positioned basal body in neural target cells and forms a dynamic gradient during neural patterning.** *Development* 2008, **135**:1097-1106.
37. Gregor T, Tank DW, Wieschaus EF, Bialek W: **Probing the limits to positional information.** *Cell* 2007, **130**:153-164.