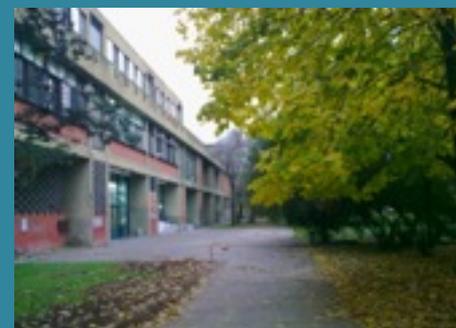


# Fluctuations and transport in cells and the information that can be extracted from optical experiments.

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**Work in collaboration with:** B Pando, L Sigaut, E Perez Ipiña, A Colman-Lerner, J Pearson

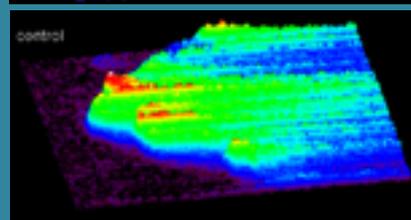
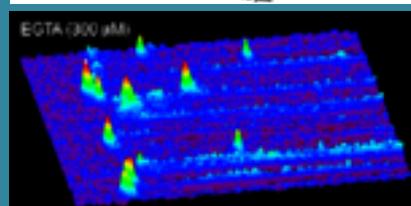
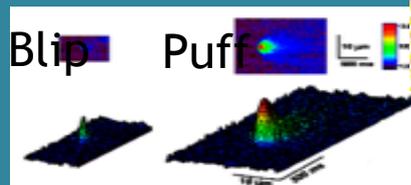
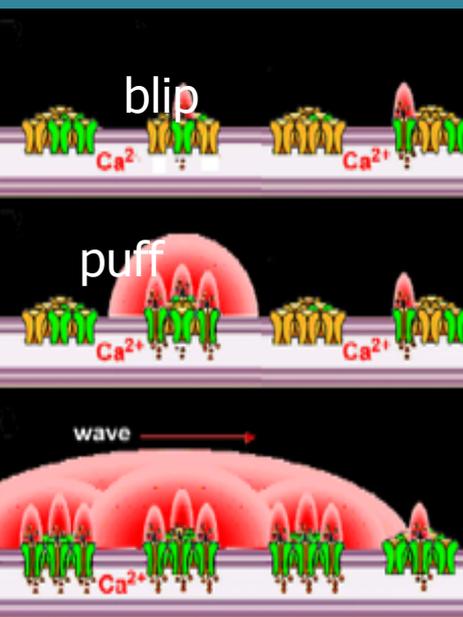
Pando et al, PNAS 2006; Sigaut et al, PRE 2010; Perez Ipiña & SPD, PRE 2013; Sigaut et al, Plos Comp Biol 2014; Perez Ipiña & SPD, BJ 2014, P. Ipiña PhD Thesis, 2015, P. Ipiña & SPD (2016)



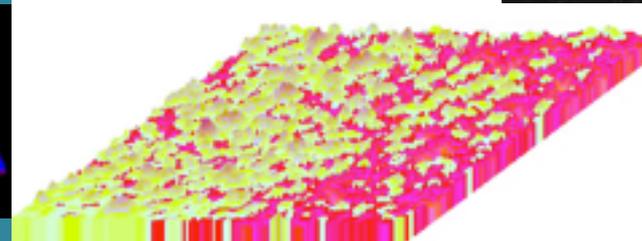
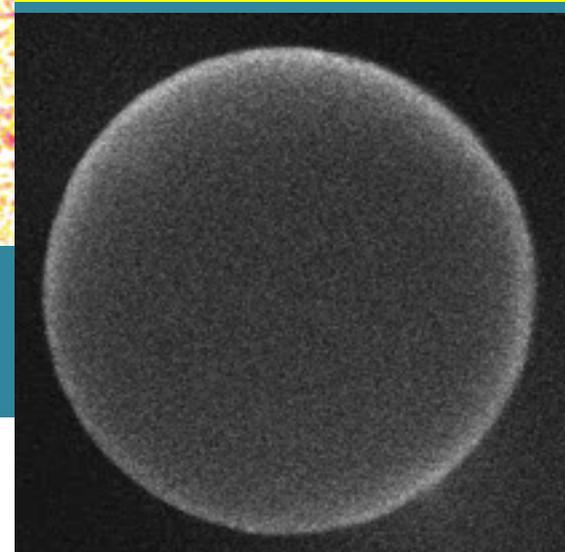
The diffusive transport of substances plays a relevant role in many biological processes.

## Example: $\text{Ca}^{2+}$ signals

In oocytes *Puffs & waves*  
(figs & movies I. Parker)

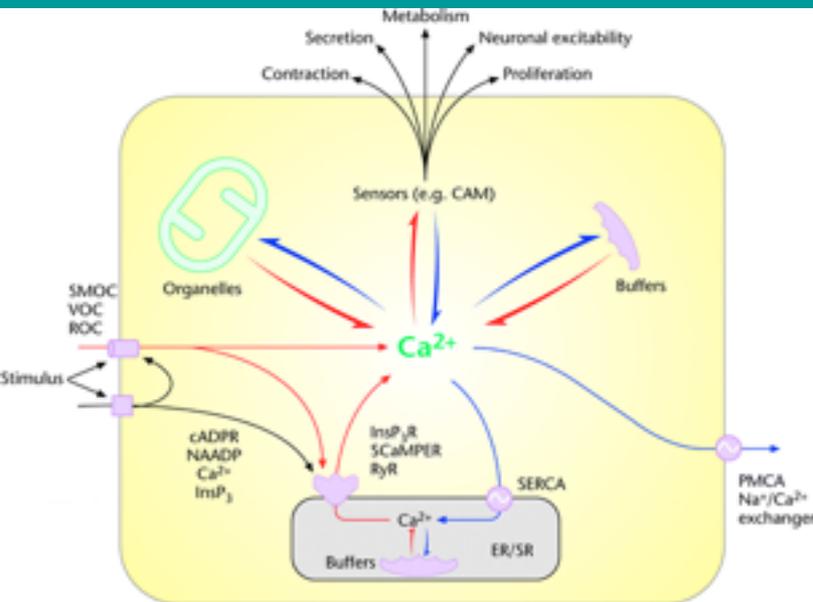


Fertilization wave  
(Fontanilla & Nuccitelli)



The transport rate of the messenger ( $\text{Ca}^{2+}$ ) determines the range of its spatio-temporal distribution and, consequently, the response it elicits.

# But calcium does not diffuse freely inside the cytosol



Scheme of the mechanisms that modulate the distribution of intracellular calcium. From **M D Bootman** and **P Lipp**, **Calcium Signalling and Regulation of Cell Function**

The net transport can be described in terms of an **effective diffusion coefficient** that depends on  $[Ca^{2+}]$ .

Radioactive  $Ca^{2+}$  is added to a cytosolic extract. The radioactivity distribution after a time is fitted with a Gaussian to obtain  $D$ .

The fastest trapping mechanism is buffering. Buffers diffuse more slowly than calcium ions, thus, they slow down the transport of calcium.

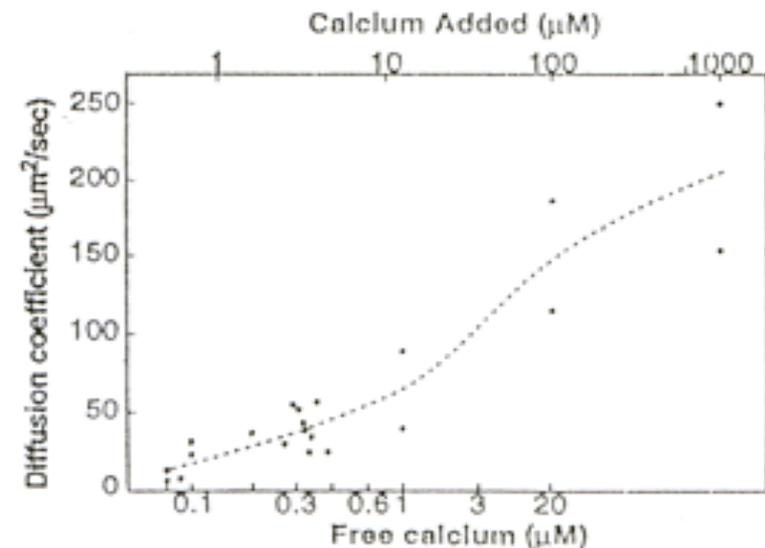


Fig. 3. Dependence of  $D$  for  $Ca^{2+}$  on the concentration of free  $Ca^{2+}$  and the amount of added  $Ca^{2+}$ . The concentration of free  $Ca^{2+}$  was determined as described in the text. The average concentration of added  $Ca^{2+}$  was determined as described (28). The concentration of  $^{45}Ca^{2+}$  was calculated from the amount loaded and the volume over which it diffused. The dashed line was drawn empirically.

Allbritton et al, 1992

What is effective diffusion?

How does it differ from "normal" (free) diffusion?

What is diffusion after all?

# What is diffusion?

It is the macroscopic displacement that results from many randomly directed microscopic steps. In solutions, randomness is the consequence of collisions with “solvent molecules”.

The mean square displacement of a diffusing particle in a simple solvent is proportional to the time elapsed:  $\langle x^2 \rangle = 2Dt$  (in one space dimension)

Explained by Einstein in 1905.

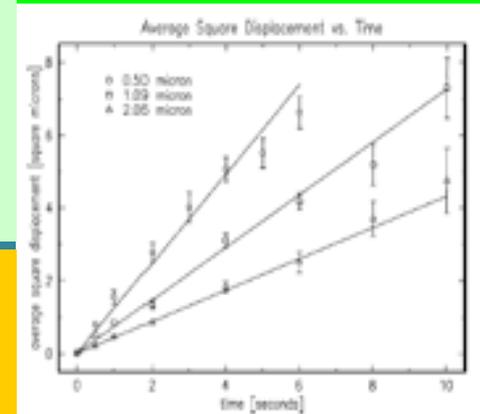
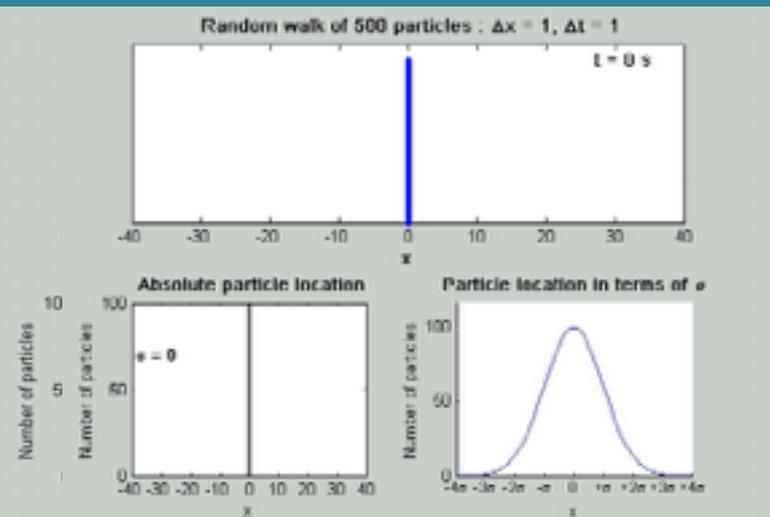


Fig. 2. Mean square displacements as a function of time.



## Einstein, Perrin, and the reality of atoms: 1905 revisited

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Science Center, Harvard University, Cambridge, Massachusetts 02138

478 Am. J. Phys. 74 (5), June 2006

The distribution at time,  $t$ , of a set of particles that diffuse out of  $x=0$  at  $t=0$  is given by a Gaussian of width

$$\Delta x = (2 D t)^{1/2}$$

The diffusion coefficient,  $D$ , characterizes both the mean square displacement of a marked particle as well as the rate at which an initial distribution of particles spreads out with time.

## Diffusion and the central limit theorem

Let  $X_1, X_2, \dots, X_r$  be a set of  $r$  independent random variables with zero mean and finite variance  $\sigma^2$ . Let us define the variable:

$$Z = \frac{X_1 + X_2 + \dots + X_r}{\sqrt{r}}$$

which has a variance  $\sigma^2$ .

In the limit of  $r$  going to infinity the probability density of  $Z$  is:

$$P_Z(z) = [2\pi\sigma^2]^{-1/2} \exp\left[-\frac{z^2}{2\sigma^2}\right].$$

If we consider directly the sum,  $Y = X_1 + X_2 + \dots + X_r$  we conclude that, for large enough  $r$  its probability density is approximately given by:

$$P_Y(y) = [2\pi r\sigma^2]^{-1/2} \exp\left[-\frac{y^2}{2r\sigma^2}\right].$$

We can apply this to the random walker,  $r$  is the number of time steps and  $\sigma^2 = \lambda^2 = 2dD$  with  $D$  the diffusion coefficient and  $d$  the number of dimensions. With  $P_Y(y)$  we can compute  $\langle Y(t) \rangle$  and  $\langle Y^2 \rangle$  and obtain  $\langle Y \rangle = 0$  and  $\langle Y^2 \rangle = 2dDt$

In fact,  $P_Y(y)$  is a solution of the diffusion eq (for certain initial condition).

# (free) Diffusion in a simple solvent

There is a finite time  $\tau$  and the (net) direction of motion during each  $\tau$ -interval is independent of one another.

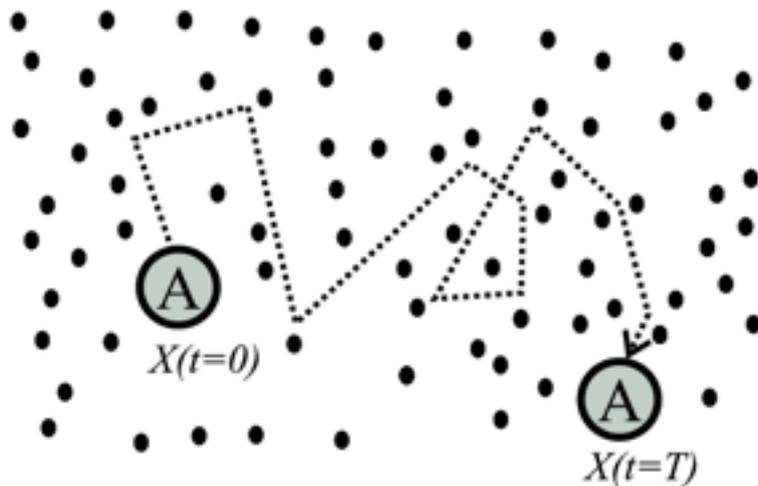
The displacement,  $\mathbf{r}(t)$ , of the random walker after a time  $t \gg \tau$  is the sum of many independent (random) displacements (those performed during the intervals,  $\tau$ ). Due to the central limit theorem the probability that  $\mathbf{r}(t)$  be within a volume of size  $d^3r$  around the value  $\mathbf{r}$  is Gaussian-distributed:

$$P(\mathbf{r}, t)d^3r = \frac{1}{(4\pi Dt)^{d/2}} \exp\left(-\frac{\mathbf{r}^2}{4Dt}\right).$$

The mean square displacement of the random walker is:

$$\langle |\mathbf{r}(t)|^2 \rangle = 2dDt.$$

with  $d$  the number of dimensions and  $D$  the diffusion coefficient ( $[D]=\text{length}^2/\text{time}$ ).



$$L^2 \sim D_A T$$

Figure 1 (a)

If one considers  $N$  particles that perform their random walks independently of one another, then, the Gaussian probability density,  $P$ , also serves to describe the concentration of these random walkers as a function of position and time,  $c(\mathbf{r}, t)$ , for an initial condition in which all the particles are located at the origin (so that the displacement after a time  $t$  of each of them is equal to its position):

$$c(\mathbf{r}, t) = NP(\mathbf{r}, t) = \frac{N}{(4\pi Dt)^{d/2}} \exp\left(-\frac{\mathbf{r}^2}{4Dt}\right),$$

This concentration is a solution of the diffusion equation introduced by Fick:

$$\frac{\partial c}{\partial t} = D\nabla^2 c.$$

The diffusion equation rules the dynamics of the local changes in the mean number of particles (or, equivalently, its concentration) inside a region that is macroscopically small but microscopically large.

The diffusion coefficient,  $D$ , can in principle be estimated from the mean square displacement of a single random walker or from the spread of a population of particles whose concentration evolves as in the solution above.

Due to the linearity of the diffusion equation, if there is an equilibrium situation (i.e., stationary and uniform concentration) and one adds some particles at the origin, then the perturbation to the concentration will change in time according to the diffusion equation and will spread out with the diffusion coefficient  $D$ .

But in the crowded environment of a cell's interior, the transport of molecules hardly ever corresponds to free diffusion in which the dynamics of the molecules is solely determined by their collisions with a simple solvent.

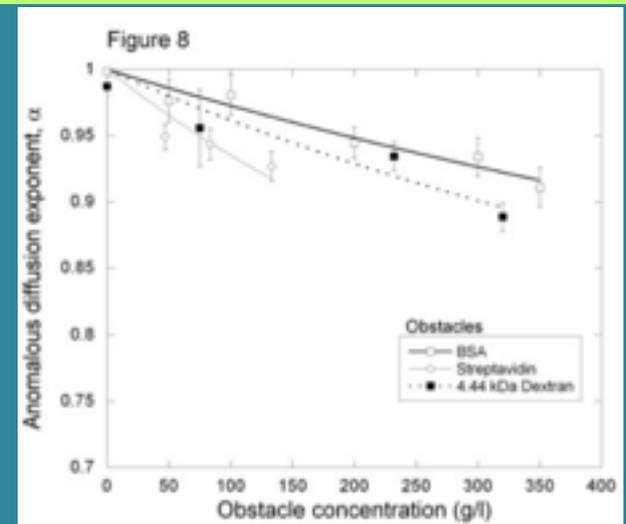
The existence of obstacles or of other interactions can give rise to what is called anomalous diffusion a situation in which the mean square displacement of a particle grows with time as  $\sim t^\gamma$  with  $\gamma$  different from 1.

Subdiffusive transport (i.e. with  $\gamma < 1$ ) has been observed experimentally in porous systems and on cell membranes

Anomalous diffusion exponents associated with the diffusion of streptavidin in solutions crowded with either BSA or non-fluorescent streptavidin for different concentrations of the obstacle proteins, C. Fradin's experiments.

What about the central limit theorem?

Some of the assumptions of the theorem must not hold. In particular, subdiffusion is obtained with random walks in which there is not a well-defined mean time,  $\tau$ , between collisions, but rather those times are taken from a long-tailed distribution

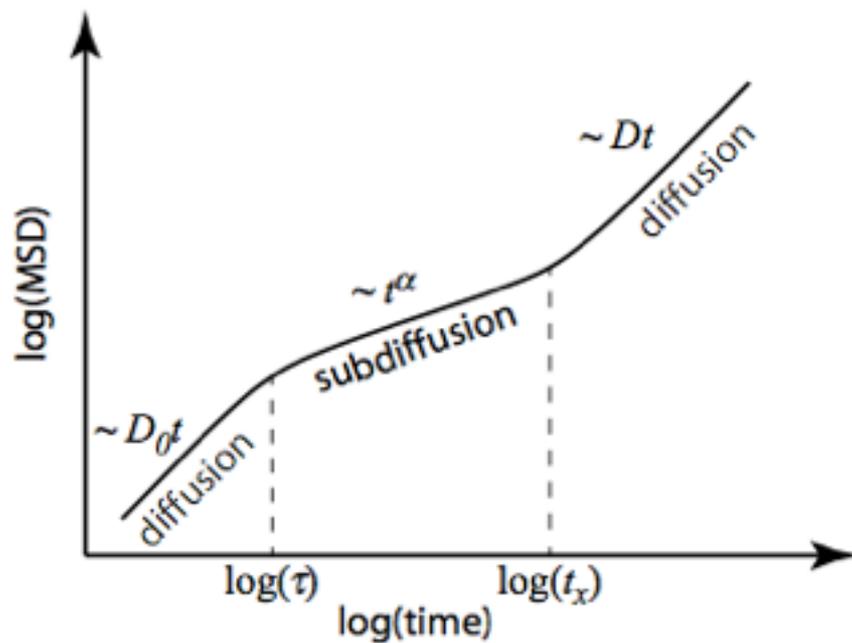


Numerically simulations show that subdiffusion can arise when the random walkers have restrictions to their movement or if they bind to immobile sites.

However, in most of these simulations, the anomalous transport only holds within a certain time window. In those cases, if one computes the mean square displacement of a particle after a long enough time (i.e. for enough averaging over the individual random steps of the particle of interest) the behavior of the linear dependence between the mean-square displacement and the elapsed time predicted by the central-limit theorem is recovered.

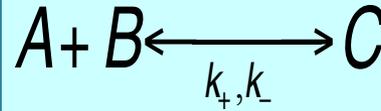
The “diffusion coefficient” in this long time limit is smaller than the one that is obtained in the absence of traps or movement restrictions. It is “effective”.

The truly anomalous transport occurs when the waiting time distribution is long tailed.



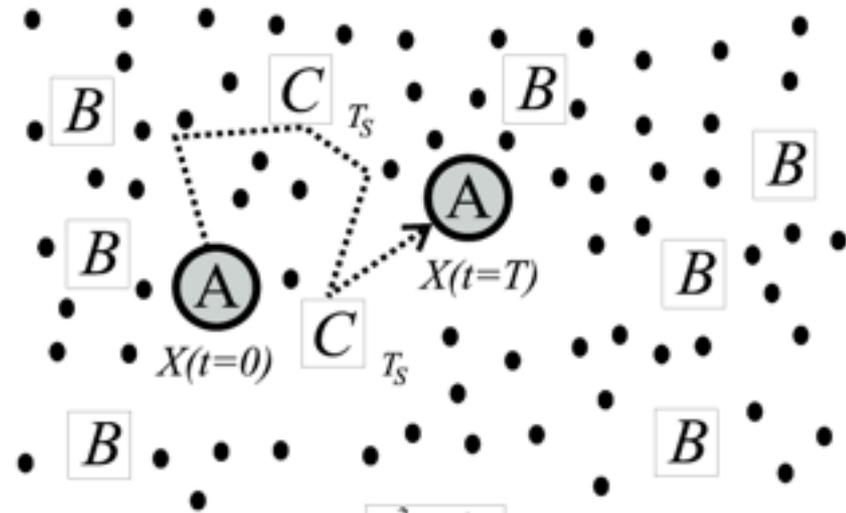
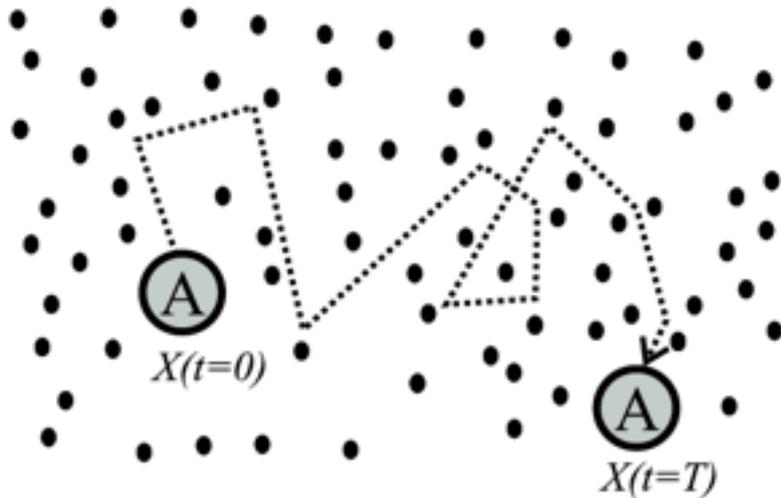
# An intuitive explanation of effective coefficients

Simplest case:



With B and C moving at the same rate ( $m_B \gg m_A$ )

Illustration for B and C immobile:



If the free diffusion of A is  $D_f$ , then:

$$\langle r^2(t) \rangle = 2 d D_f t \text{ when } [B]=0$$

and

$$\langle r^2(t) \rangle = 2 d D_f t_f = 2 d [(D_f t_f)/t] t$$

Thus,  $D_{eff} = D_f \langle t_f \rangle / (\langle t_f \rangle + \langle t_b \rangle)$

If B and C diffuse with coefficient,  $D_S$ , then:

$$D_{eff} = \frac{\langle t_f \rangle D_f + \langle t_b \rangle D_S}{\langle t_f \rangle + \langle t_b \rangle}$$

$\langle t_f \rangle$  and  $\langle t_b \rangle$  are functions of concentrations and reaction rates.

Problem: which ones? Depends on whether we are following a single particle or looking at the behavior of the concentration.

In Pando et al, 2006 we studied the diffusion of particles, P, which diffused with free coefficient,  $D_f$ , and reacted with immobile traps, S, according to:



We assumed an initial equilibrium situation with unmarked particles,  $P_{feq}$ ,

$P_{beq}, S_{eq}$  :  $S_{eq} = \frac{K_D S_T}{K_D + P_{feq}}$  with:  $K_D = k_{off}/k_{on}$

and studied the evolution after:

1. A small amount of marked particles ( $P^t$ ) was added.
2. All particles in a region were initially marked (FRAP).
3. Similar to 1 but with unmarked particles ( $P^u$ ).

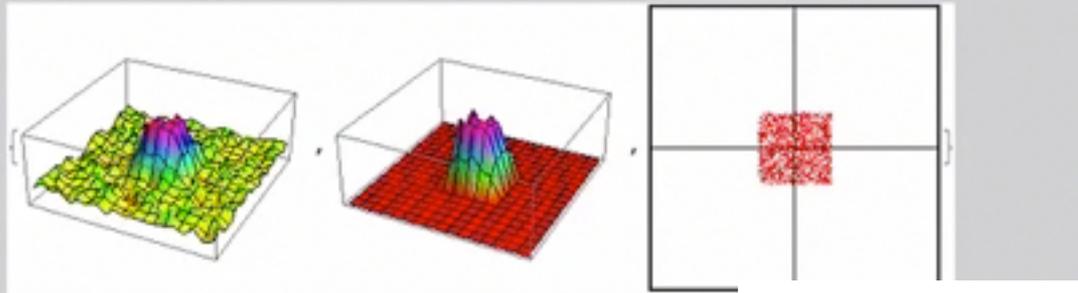
We obtained that, in the long time, the dynamics was approximately diffusive with effective coefficients  $D^t$  &  $D^u$  ( $D^t < D^u$ ) which for  $D_s = 0$  are:

Single molecule:  $D_{sm} = D^t = \frac{D_f}{1 + \frac{S_{eq}}{K_D}}$  Collective:  $D_{coll} = D^u = \frac{D_f}{1 + \frac{S_{eq}}{K_D} \frac{S_{eq}}{S_T}}$

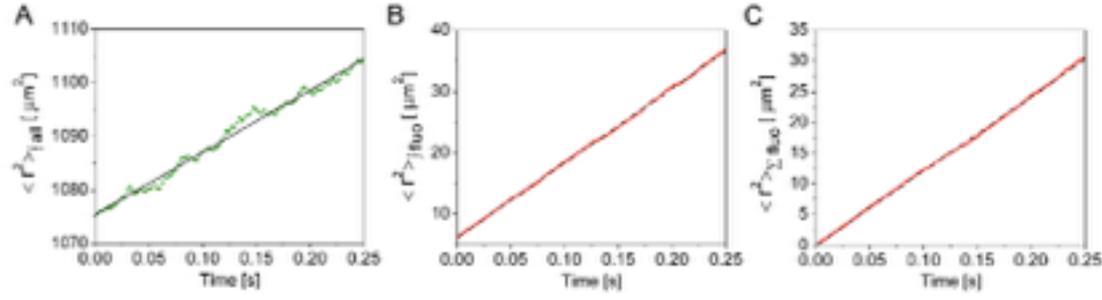
For the mean square displacement of a single marked particle, the spread of a set of marked particles and for FRAP.

For the rate at which a small inhomogeneity (of particles that are undistinguishable from the background) spreads out with time.

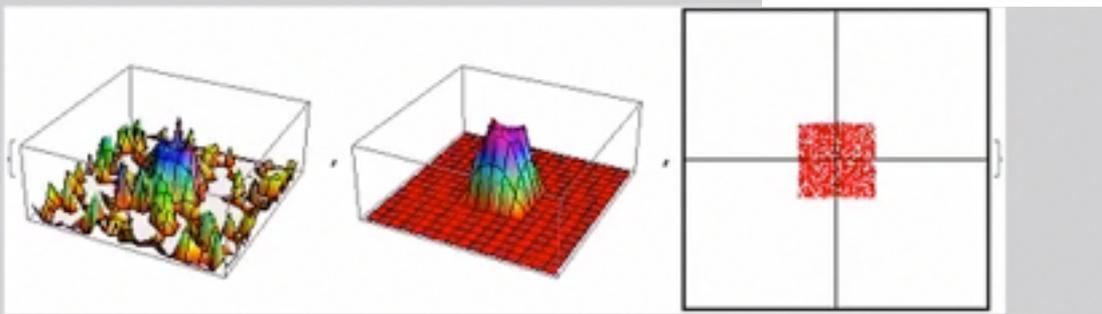
$D^t/D^u$  can be arbitrarily small!



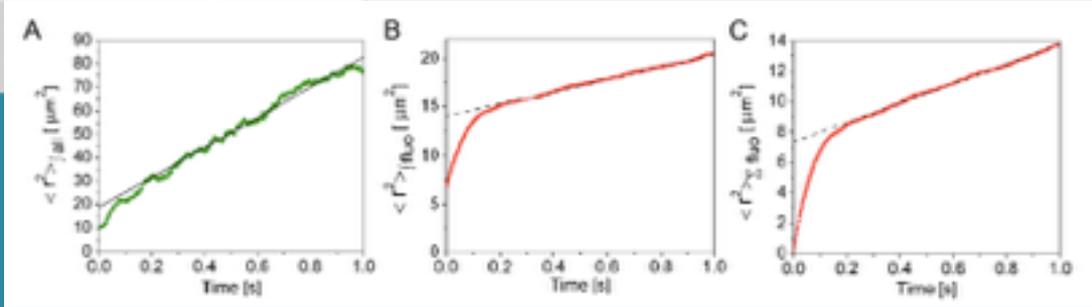
Free Diffusion



D~20 $\mu\text{m}^2/\text{s}$  in all cases



Reaction-diffusion system

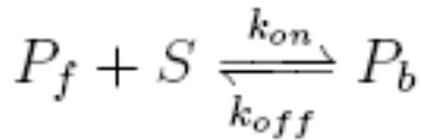


Collective (~10 $\mu\text{m}^2/\text{s}$ )

Single molecule coefficient (~1 $\mu\text{m}^2/\text{s}$ )

The intuitive explanation of effective diffusion is "easy" for  $D_{\dagger}$ :

Given



it is

$$\langle t_f \rangle = \frac{1}{k_{on} S_{eq}}$$

$$\langle t_b \rangle = \frac{1}{k_{off}}$$

and

$$D_{eff} = \frac{\langle t_f \rangle D_f + \langle t_b \rangle D_S}{\langle t_f \rangle + \langle t_b \rangle}$$

gives  $D_{\dagger}$  which is

$$D^{\dagger} = \frac{D_f}{1 + \frac{S_{eq}}{K_D}}$$

for  $D_{S=0}$

It is "easy" when one follows a single particle which does not affect the concentration of the traps S (which remains equal to  $S_{eq}$ ).

If one tries to follow many particles the situation is different: they all compete for the traps, they are not all independent.

# Some intuition for the case with $D_S=0$

Both

$$D^t = \frac{D_f}{1 + \frac{S_{eq}}{K_D}}$$

and

$$D^u = \frac{D_f}{1 + \frac{S_{eq}}{K_D} \frac{S_{eq}}{S_T}}$$

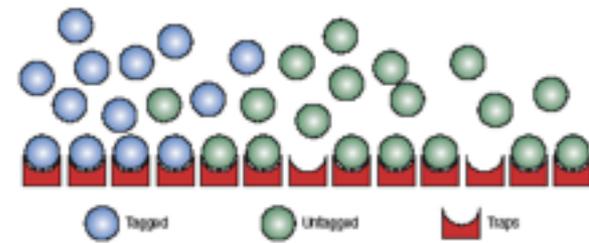
correspond to:

$$D^{ef} = \frac{D_f}{1 + \frac{t_b}{t_f}}$$

But  $D^t$  with:  $t_f = 1/k_{on} S_{eq}$   
 $t_b = 1/k_{off}$

and  $D^u$  with a “dressed”  $t_b$ :  
 $t_b = 1/(S_T k_{off}/S_{eq})$

As the small perturbation spreads out, some particles that were already bound to traps start to be released increasing the rate of spread. This does not happen if the initial perturbation can be distinguished from those previously trapped. The rate of spread is solely determined by the added particles



Let us think of a pipe with its contents in equilibrium and focus at a piece of length  $\lambda$  located at  $x=0$ . At  $t=0$ , we add some colored particles at  $x=-\lambda$ . At  $t=\Delta t$ , the piece at  $x=0$  receives some colored,  $N_c(\Delta t)$  and some uncolored particles,  $N_u(\Delta t)$ . If they are all free,  $N_c(\Delta t) = \alpha N_c(-\lambda, 0)$  and  $N_u(\Delta t) = \alpha N_u(-\lambda, 0)$ . If there are reactions, the added particles will induce a release of bound particles which are initially all uncolored. Some added particles will become bound. Thus,  $N_c(\Delta t) = \alpha N_c(-\lambda, 0)$  and  $N_u(\Delta t) = \alpha' N_u(-\lambda, 0)$  with  $\alpha' > \alpha$  which implies that  $N_c(\Delta t) + N_u(\Delta t) > \alpha (N_c(-\lambda, 0) + N_u(-\lambda, 0))$ . Thus, all the particles diffuse faster than the colored ones.

So, we have one diffusion coefficient,  $D^t$ , ruling the mean square displacement and another one,  $D^u$ , ruling the rate of spread of a perturbation in the concentration (if particles are distinguishable it's  $D^t$  instead!). Something similar happens in non-ideal solutions, i.e., with polymers.

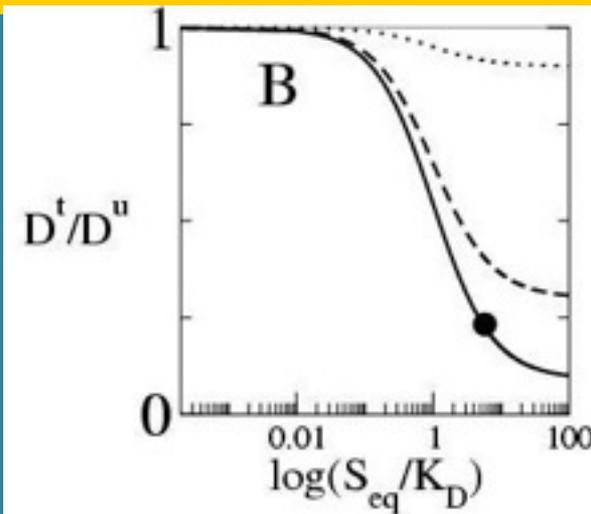
How different between themselves can  $D^t$  and  $D^u$  be?

A lot! Example with  $D_S = 0$

$$D^t = \frac{D_f}{1 + \frac{S_{eq}}{K_D}} = \frac{D_f}{1 + \frac{S_T}{K_D + P_{feq}}} \leq D^u = \frac{D_f}{1 + \frac{S_{eq}}{K_D} \frac{S_{eq}}{S_T}} = \frac{D_f}{1 + \frac{S_T K_D}{(K_D + P_{feq})^2}}$$

They are approximately equal for  $P_{feq} \ll K_D$  or  $P_{feq} \gg K_D$ ,  $S_T$

Their ratio can be arbitrarily small for large  $S_{eq}/K_D$  and small  $S_{eq}/S_T$ .



Solid:  $S_{eq}/K_D = .1$

Dashed:  $S_{eq}/K_D = .3$

Dotted:  $S_{eq}/K_D = 9$

Thus: The messages diffuse faster than the messengers

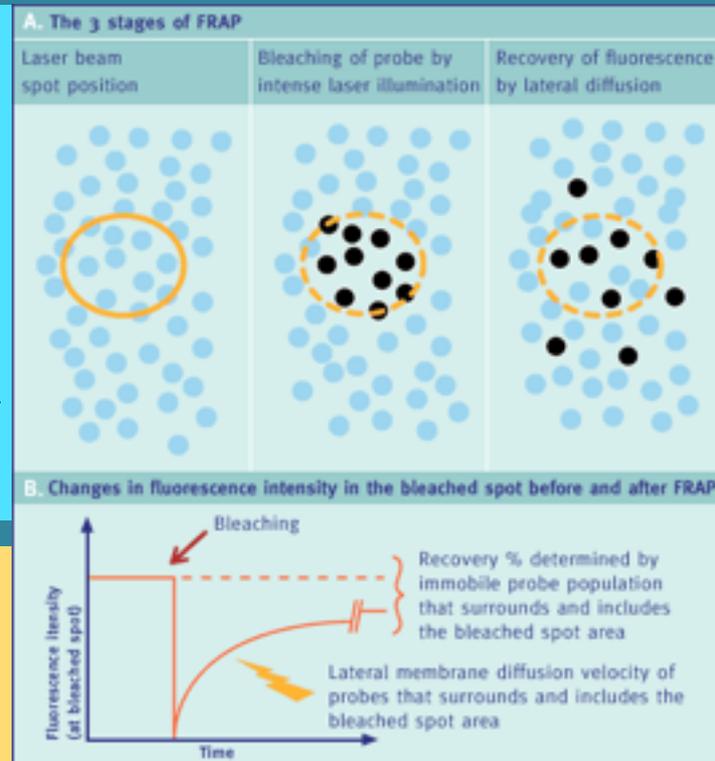
What happens with the optical techniques that are used to estimate diffusion coefficients of biomolecules in intact cells?

## 1. Fluorescence Recovery after Photobleaching (FRAP)

It is used mainly to determine the diffusion of fluorescently labeled proteins.

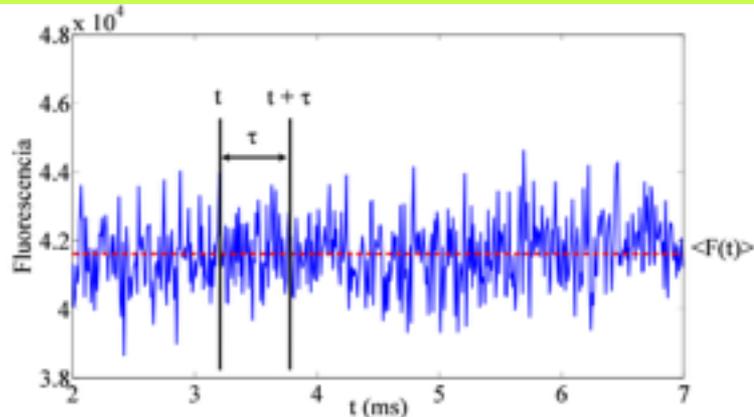
Once the protein is fluorescently labeled, a region is photobleached (fluorescence is turned off). Fluorescence in that region recovers when new fluorescent proteins enter the region. The diffusion coefficient,  $D$ , is determined from the time it takes for the fluorescence to recover.

In the case of RD systems it gives the single molecule coefficient (Sprague et al, 2004).

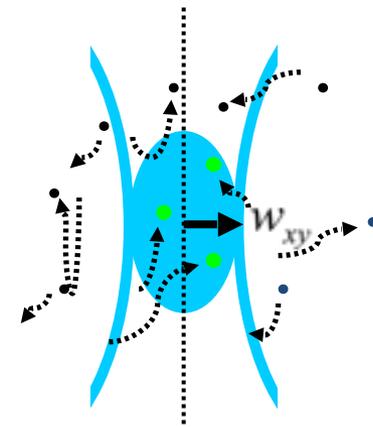


# FCS - Fluorescence Correlation Spectroscopy

FCS measures fluorescence fluctuations in an observation volume of the order of 1 fl.



Example: free diffusion of a single species



The autocorrelation function (ACF) of the fluorescence fluctuations is computed.

$$\delta F(t) = F(t) - \langle F \rangle \quad G(\tau) = \frac{\delta F(t) \delta F(t + \tau)}{\langle F(t) \rangle^2}$$

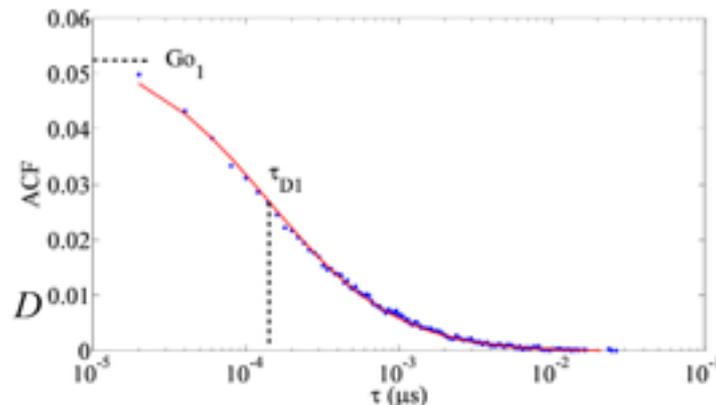
Fitting the ACF the diffusion coefficient can be estimated from the correlation time. For one species:

$$G(\tau) = \frac{G_0}{\left(1 + \frac{\tau}{\tau_D}\right) \left(1 + \frac{\tau}{\omega^2 \tau_D}\right)}$$

with

$$\tau_D = \frac{\omega_r^2}{4D}$$

$$G_0 = \frac{1}{V_{ef} C_{tot}} \propto \frac{1}{N}$$

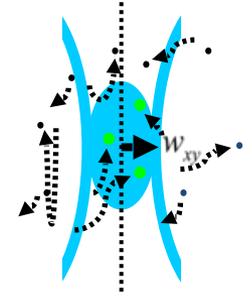


Solution 50 nM TMR-Dex in water.

$V_{ef}$  y  $\omega_r$  are geometric parameters that are determined from a calibration.

# What about RD systems?

$$G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}$$



In Sigaut et al, 2010, we derived analytic expressions for the ACF,  $G(\tau)$ , that correspond to different biophysical models. In the case in which all particles are fluorescent we found, in the limit of fast reactions:

$$G(\tau) = \underbrace{\frac{G_{0S}}{\left(1 + \frac{\tau}{\tau_{DS}}\right) \sqrt{1 + \frac{\tau}{w^2 \tau_{DS}}}}}_{D_S} + \underbrace{\frac{G_{0U}}{\left(1 + \frac{\tau}{\tau_{DU}}\right) \sqrt{1 + \frac{\tau}{w^2 \tau_{DU}}}}}_{D_U}$$

With:

$$\tau_{DS} = w_r^2 / (4D_S)$$

$$\tau_{DU} = w_r^2 / (4D_U)$$

Remarkably, if we assumed that both fluorescent and non-fluorescent particles coexisted in the system we got:

$$G(\tau) = \frac{G_{0S}}{\left(1 + \frac{\tau}{\tau_{DS}}\right) \sqrt{1 + \frac{\tau}{w^2 \tau_{DS}}}} + \frac{G_{0U}}{\left(1 + \frac{\tau}{\tau_{DU}}\right) \sqrt{1 + \frac{\tau}{w^2 \tau_{DU}}}} + \underbrace{\frac{G_{0I}}{\left(1 + \frac{\tau}{\tau_{DI}}\right) \sqrt{1 + \frac{\tau}{w^2 \tau_{DI}}}}}_{D_I}$$

With:

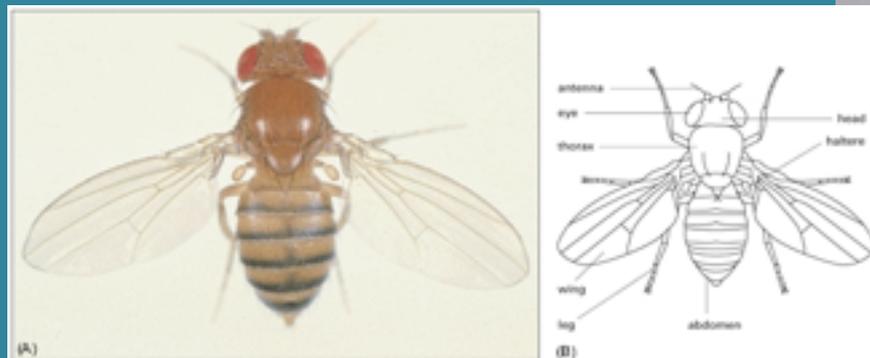
$$\tau_{DI} = \frac{w_r^2}{4D_I}$$

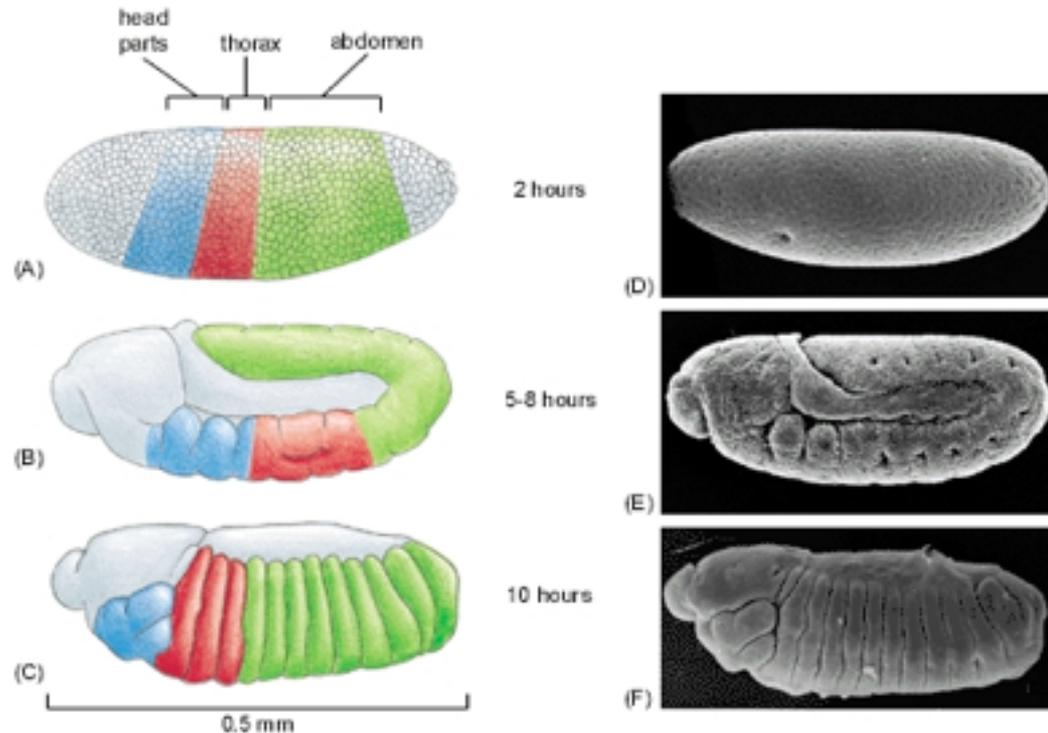
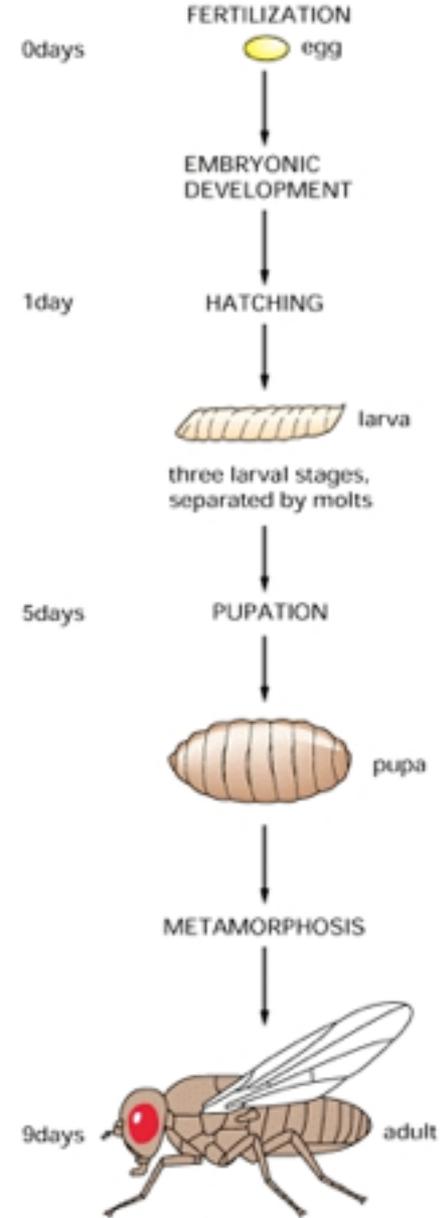
A problem where this difference is relevant.

Diffusion plays a fundamental role for the establishment of spatial structures (patterns) in living organisms.

In embryogenesis there are “morphogens” that determine the fate of otherwise undifferentiated cells depending on their position. Morphogen gradients are key for cellular differentiation.

A case studied in great detail is that of the establishment of the dorso-ventral axis in embryos of *Drosophila* where the protein, Bcd, acts as a transcription factor.





Embryo drawings (A-C) and SEM images (D-F). Two hours after deposition segmentation is not visible, but there is already a “map” that determines the subsequent fate.

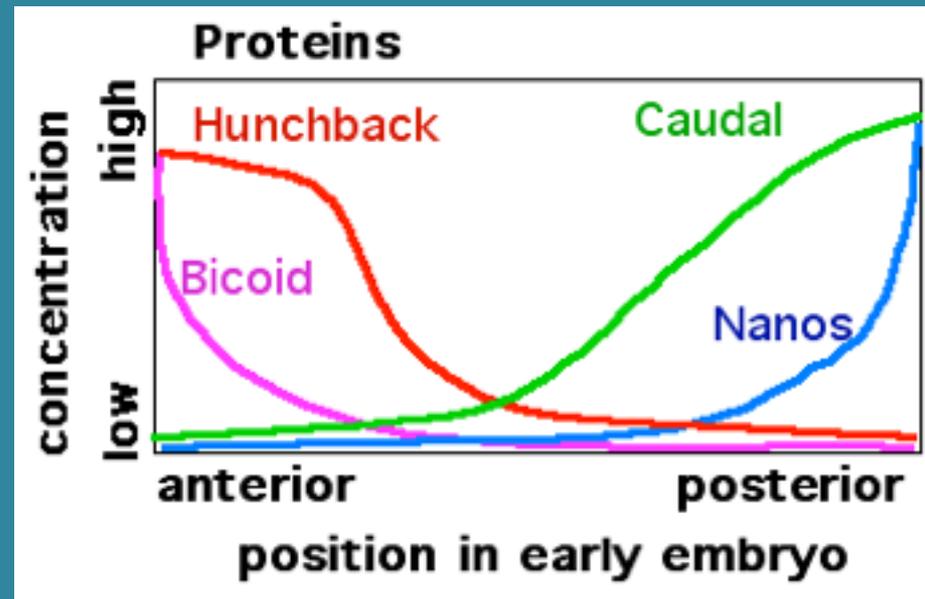
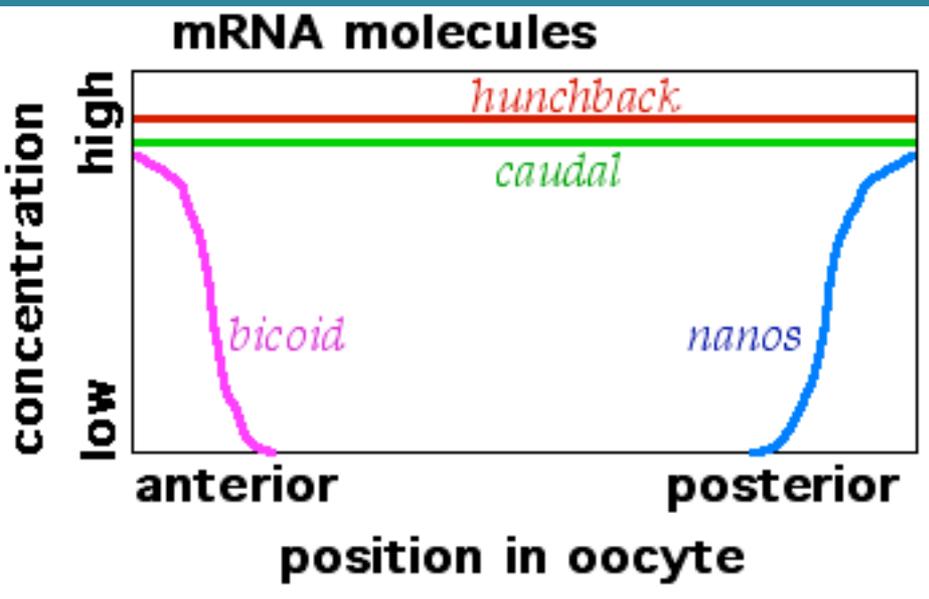
There is an asymmetry in the egg. The positional information is provided by 4 gradients that are established by 4 gene groups.

Synopsis of the development from the egg through the formation of an adult fly.

Molecular Biology of the Cell. 3rd edition.  
Alberts B, Bray D, Lewis J, et al.

During the development of the egg, before it is fertilized, there is a non-uniform distribution of mRNA molecules.

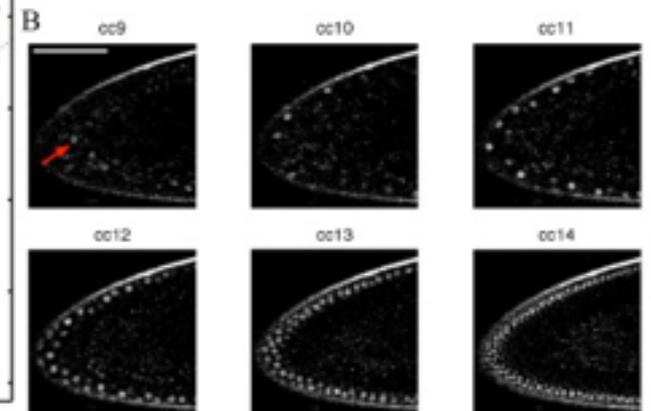
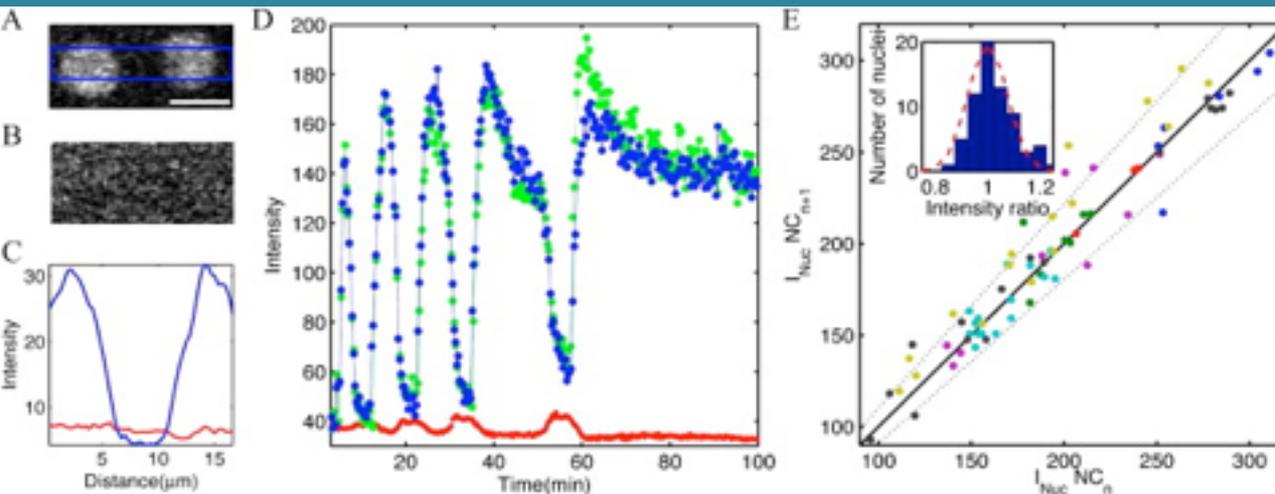
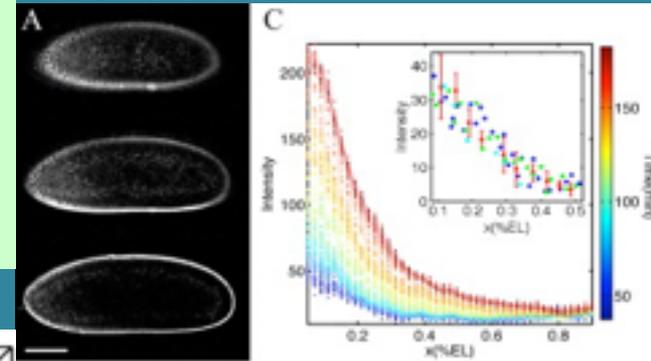
There is a maternal gene, bicoid, which is mainly concentrated in the anterior end of the embryo. The protein encoded by this gene also displays a non-uniform distribution in the embryo.



Bicoid is a transcription factor (a protein that participates of the regulation of DNA transcription). In particular, it promotes the transcription of another maternal gene, hunchback.

Using embryos that express Bcd-GFP Gregor et al determined that the Bicoid gradient is established within 90 minutes after fertilization and remains stable along several nuclear divisions.

Bcd-GFP expressing embryos. (A) Nuclear cycle 12 at 30  $\mu\text{m}$  (top), 60  $\mu\text{m}$  (middle), and 90  $\mu\text{m}$  (bottom) from the surface. (Bar 100  $\mu\text{m}$ ) (B) Each image corresponds to an instant during the interphases 9-14. (C) Images start  $20 \pm 15$  min after egg deposition. (Gregor et al, Cell 2007)



(A) Two nuclei during interphase (B) Same region as in (A) but during mitosis. (C) Intensity profile during interphase. (D) Bcd-GFP in nuclei and in cytoplasm. (E) Intensity peak in a nucleus during cycle  $n$  as a function of the peak during cycle  $n+1$  (Gregor et al, Cell 2007)

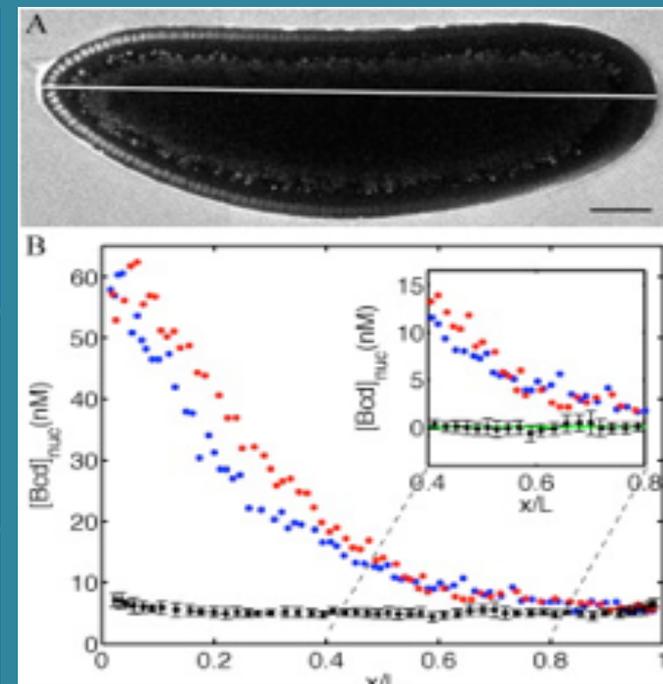
A possible model to explain the gradient formation of the product of bicoid assumes Bcd is produced at the anterior end, it then diffuses and is degraded.

The Bcd diffusion coefficient affects the time and spatial scales of the gradient. Thus, it is important to estimate its value.

FRAP and FCS were used to this end giving estimates of the diffusion coefficient that differ by an order of magnitude. The FRAP diffusion coefficient was too small to explain the formation of the gradient within the observed timescale.

### Our explanation

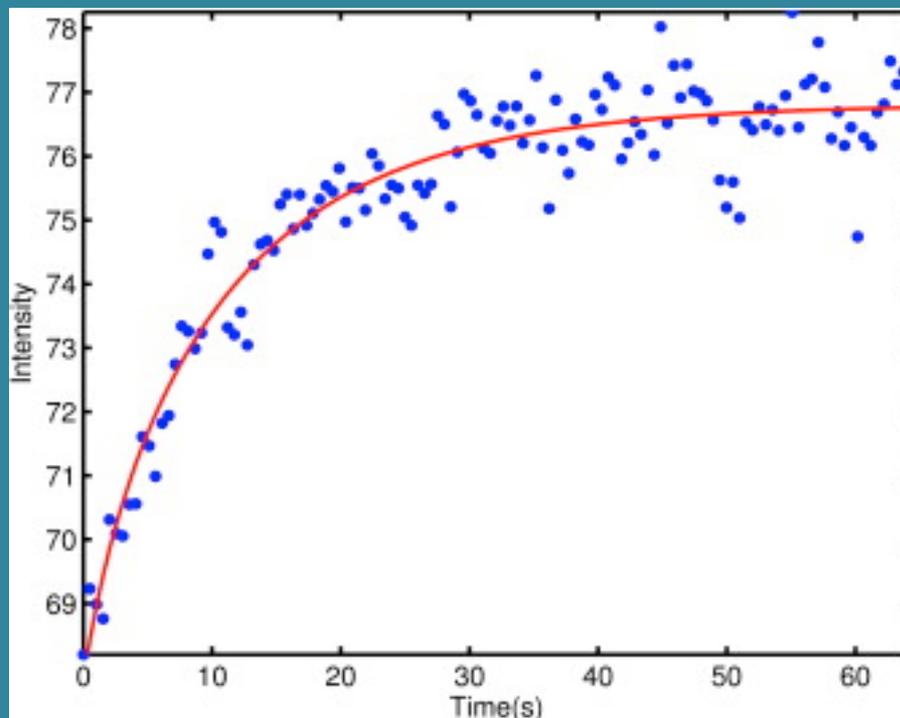
The apparently contradictory results may be explained within a single model by considering that Bcd diffuses and reacts with other substances and that FRAP and FCS provide effective coefficients.



Drosophila embryo expressing Bcd-GFP (Gregor et al., 2007); scale 50 $\mu\text{m}$ . (B) Blue & red: Apparent concentrations in each nucleus.

# The application of FRAP and FCS to the case of Bcd.

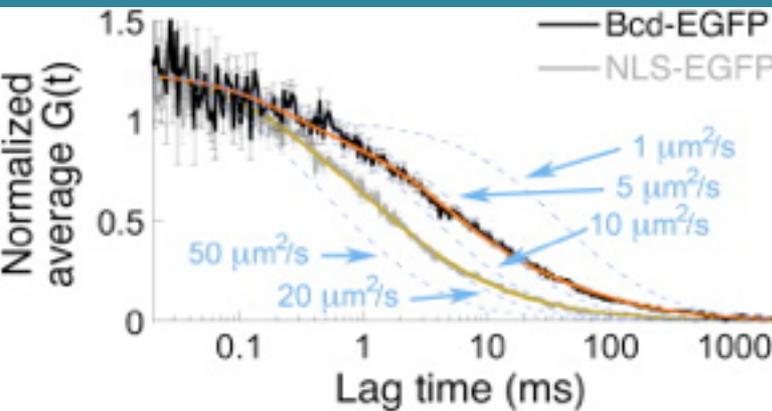
Gregor et al, 2007, did FRAP experiments to determine the diffusion of Bcd.



Recovery curve,  $D = 0.27 \pm 0.07 \mu\text{m}^2/\text{s}$ .

This value is too small to explain the formation of the Bcd gradient within 90 minutes of fertilization.

Abu-Arish et al, 2010 did FCS experiments to determine the diffusion coefficient of Bcd.



Normalized ACF's for Bcd-EGFP and NLS-EGFP in the cortical cytoplasm during the interphase of cycles 12-14. Blue: Expected functions in the case of a single diffusing species for different values of  $D$ .

They tried various fittings to the ACF. The best ones were of the form:

$$G(\tau) = \frac{1}{N} \sum_i \frac{F_i}{\left(1 + \frac{\tau}{\tau_{Di}}\right) \left(1 + \frac{\tau}{\omega^2 \tau_{Di}}\right)} \quad i = 2, 3$$

Doing:

$$D_{Bcd} = \sum_i F_i D_i$$

They obtained:

$$D_{Bcd}^{FCS} \approx [7 - 9] \mu\text{m}^2 / \text{s}$$

They argued there was a technical problem to explain the discrepancy between FRAP and FCS (they did FRAP too and obtained  $D \sim 1 \mu\text{m}^2 / \text{s}$ ).

# FRAP, FCS and Bicoid:

## FRAP

In FRAP the fluorescence is bleached in a small region. Fluorescence recovers because fluorescent particles enter this bleached region.

## FCS

In FCS all particles are fluorescent. Fluorescence fluctuations in a volume,  $V$ , are monitored. Fluctuations occur because particles enter and leave  $V$ .

## The math

Relevant equations for FRAP (all particles in a region are initially marked)

$$\begin{aligned}\frac{\partial P_f^t}{\partial t} &= D_f \nabla^2 P_f^t - k_{on} P_f^t S_{eq} + k_{off} P_b^t \\ \frac{\partial P_b^t}{\partial t} &= k_{on} P_f^t S_{eq} - k_{off} P_b^t\end{aligned}$$

Equations for a small perturbation with respect to equilibrium.

$$\begin{aligned}\frac{\partial P_f}{\partial t} &= D_f \nabla^2 P_f - k_{on} P_f S_{eq} + \frac{S_T}{S_{eq}} k_{off} P_b \\ \frac{\partial P_b}{\partial t} &= k_{on} P_f S_{eq} - \frac{S_T}{S_{eq}} k_{off} P_b.\end{aligned}$$

Effective diffusion is  $D^t$

Effective diffusion is  $D^u$ .

$D^t$  can be inferred if fluorescent and non-fluorescent particles coexist in FCS.

Bicoid

$$D_{FRAP} = 0.3-1 \mu\text{m}^2/\text{s}$$

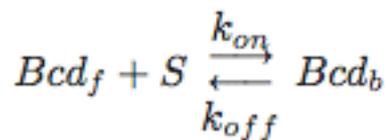
$$D_{FCS} \text{ various diffusing components: } 14, 1.6, 0.095 \mu\text{m}^2/\text{s}$$

# Our explanation (Sigaut et al, PLoS Comp Biol 2014)

Bcd diffuses and reacts with slowly moving or immobile binding sites. Applying our theory on effective diffusion coefficients (and which one is measured by different experimental techniques) we can explain the difference between the estimates of the diffusion coefficient derived using FRAP and FCS.

In fact, we have considered the simplest model with Bcd diffusing and interacting with a single type of traps, S:

Reaction scheme:



Reaction Diffusion eqs:

$$\frac{\partial [Bcd_f^t]}{\partial t} = D_f \nabla^2 [Bcd_f^t] - k_{on} [Bcd_f^t] [S] + k_{off} [Bcd_b^t]$$

$$\frac{\partial [Bcd_f^u]}{\partial t} = D_f \nabla^2 [Bcd_f^u] - k_{on} [Bcd_f^u] [S] + k_{off} [Bcd_b^u]$$

$$\frac{\partial [Bcd_b^t]}{\partial t} = D_S \nabla^2 [Bcd_b^t] + k_{on} [Bcd_f^t] [S] - k_{off} [Bcd_b^t]$$

$$\frac{\partial [Bcd_b^u]}{\partial t} = D_S \nabla^2 [Bcd_b^u] + k_{on} [Bcd_f^u] [S] - k_{off} [Bcd_b^u]$$

$$\frac{\partial [S]}{\partial t} = D_S \nabla^2 [S] - k_{on} [Bcd_f] [S] + k_{off} [Bcd_b],$$

Let us analyze FCS for Bcd assuming diffusion and reactions.

We have:



where  $S$  and bound Bcd diffuse with  $D_S$ , and free Bcd with  $D_f$ .

Then, the ACF is (in the limit of fast reactions):

$$G(\tau) = \underbrace{\frac{Go_s}{\left(1 + \frac{\tau}{\tau_{DS}}\right) \sqrt{1 + \frac{\tau}{\omega^2 \tau_{DS}}}}}_{D_S} + \underbrace{\frac{Go_t}{\left(1 + \frac{\tau}{\tau_{Dt}}\right) \sqrt{1 + \frac{\tau}{\omega^2 \tau_{Dt}}}}}_{D_t} + \underbrace{\frac{Go_u}{\left(1 + \frac{\tau}{\tau_{Du}}\right) \sqrt{1 + \frac{\tau}{\omega^2 \tau_{Du}}}}}_{D_u}$$

which has the same time dependence as the expression used by Abu-Arish et al to fit their experimental ACF:

$$G(\tau) = \frac{1}{N} \sum_{i=2,3} \frac{F_i}{\left(1 + \frac{\tau}{\tau_{Di}}\right) \sqrt{1 + \frac{\tau}{\omega^2 \tau_{Di}}}}$$

We interpret the fitting parameters of Abu-Arish et al in terms of our analytic expression and estimate the biophysical parameters of the problem.

Fitting parameters of the experimental ACF derived from FCS experiments performed in the cytoplasm during interphase at the anterior end of Bcd-EGFP expressing *Drosophila* embryos (Abu Arish et al, 2010)

Our interpretation

3-component fitting parameters	
$D_s$ → <span style="border: 2px solid red; border-radius: 50%; padding: 2px;">D<sub>1</sub></span>	F <sub>1</sub>
$D_t$ → <span style="border: 2px solid green; border-radius: 50%; padding: 2px;">D<sub>2</sub></span>	F <sub>2</sub>
$D_u$ → <span style="border: 2px solid blue; border-radius: 50%; padding: 2px;">D<sub>3</sub></span>	F <sub>3</sub>
2-component fitting parameters	
$D_t$ → <span style="border: 2px solid green; border-radius: 50%; padding: 2px;">D<sub>1</sub></span>	F <sub>1</sub>
$D_u$ → <span style="border: 2px solid blue; border-radius: 50%; padding: 2px;">D<sub>2</sub></span>	F <sub>2</sub>

Abu- Arish et al 2010

$$D_{Bcd}^{FCS} = [7 - 9] \mu m^2 / s$$

Our interpretation

$$D_t^{FCS} = [0.38 - 1.6] \mu m^2 / s$$

Perfectly compatible with the FRAP estimate:

$$D_t^{FRAP} = [0.21 - 1] \mu m^2 / s$$

Detail: FCS during interphase and FRAP during mitosis

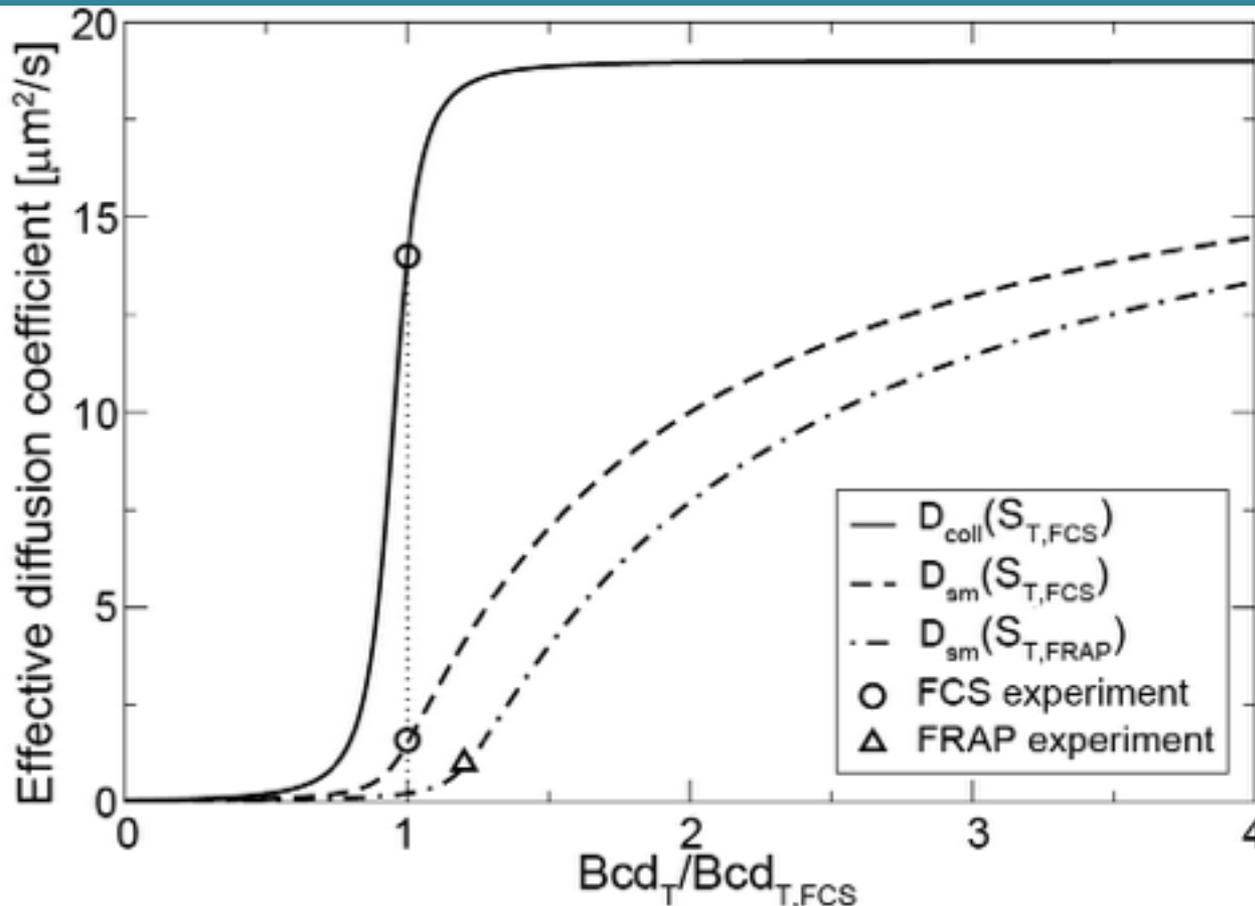
From FCS experiments performed using a freely-diffusing analogue of Bcd (NLS-EGFP) we estimate the free coefficient,  $D_f \sim 20 \text{ um}^2/\text{s}$

Assuming that  $D_f$ ,  $D_S$  ( $\sim 0-0.1 \text{ um}^2/\text{s}$ ) and  $K_D$  have the same values for all the conditions probed in the experiments we can describe the FCS and FRAP results self-consistently. Using our analytic expressions for  $D_u$  and  $D_t$  we derive the ratios of all concentrations and of  $K_D$  for the various conditions probed:

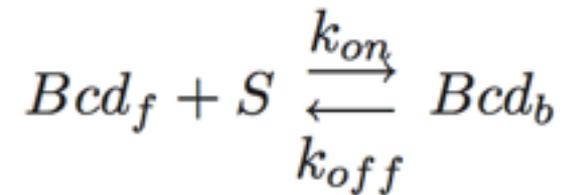
	<b>Cytoplasm, Interphase</b>	<b>Cytoplasm, Mitosis</b>
<i>S/Bcd</i>	0.03	0.05
Bcd	0.92	1.14
<i>Bcd</i>	0.08	0.06
Bcd	1	1.2
<i>S</i>	0.95	1.19
K	0.0026	0.0026

# How to validate our model?

Given these biophysical parameters, by how much could the effective diffusion coefficients vary with  $[Bcd]_T$ ?



A quick reminder:

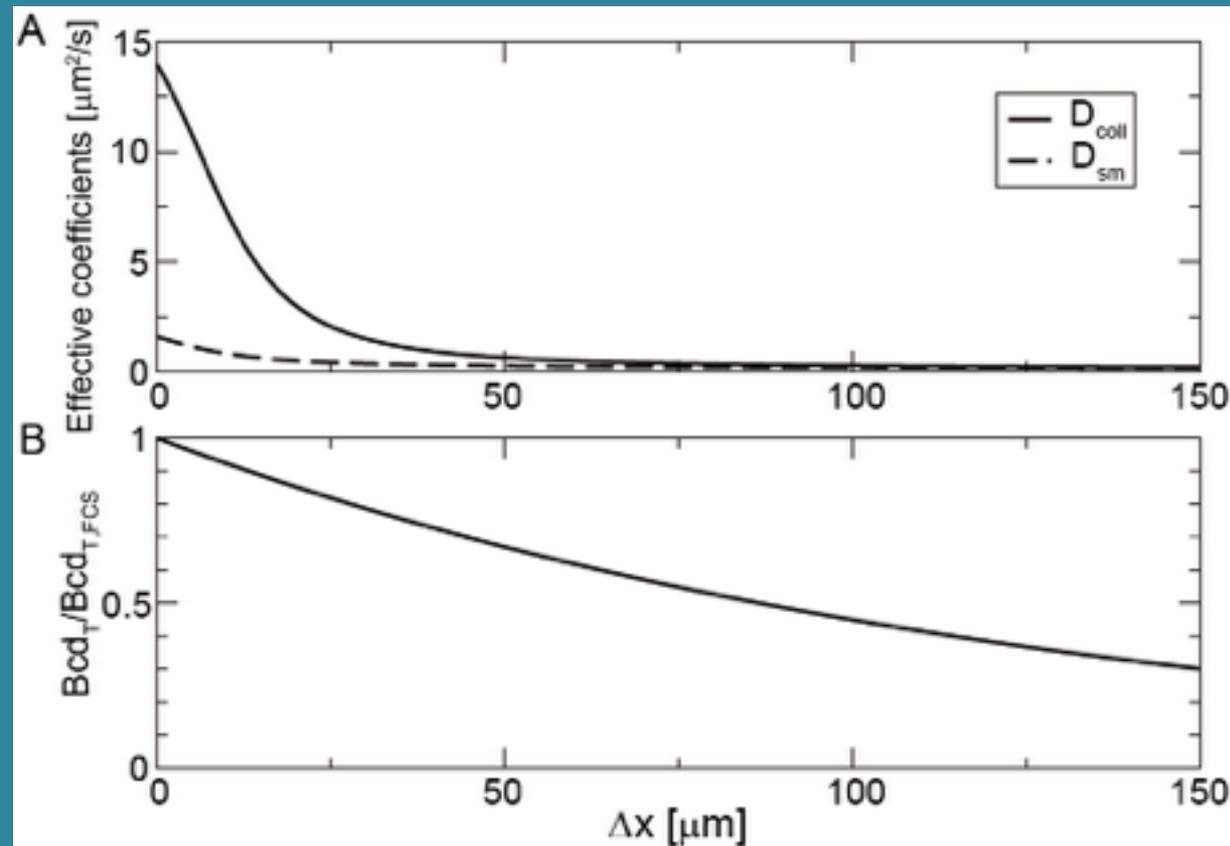


$$D_{sm} = \frac{D_f + \frac{[S]}{K_D} D_S}{1 + \frac{[S]}{K_D}}$$

$$D_{coll} = \frac{D_f + \frac{[S]^2}{K_D [S_T]} D_S}{1 + \frac{[S]^2}{K_D [S_T]}}$$

Effective diffusion coefficients as functions of the total Bcd concentration (normalized to the FCS situation) for 2 uniform trap concentrations:  $S_T = S_{T,FCS}$  (interph) and  $S_T = S_{T,FRAP}$  (mitosis).

Given that  $[Bcd]_T$  depends exponentially on position, how does this translate with positional variation in the embryo?



$$D_{sm} = \frac{D_f + \frac{[S]}{K_D} D_S}{1 + \frac{[S]}{K_D}},$$

$$D_{coll} = \frac{D_f + \frac{[S]^2}{K_D [S_T]} D_S}{1 + \frac{[S]^2}{K_D [S_T]}}.$$

Effective diffusion coefficients and concentration of fluorescent Bcd as functions of the distance to the location of FCS experiments. This gives a hint for future experiments.

So, we can explain the apparently contradictory measurements of the Bcd diffusion coefficient within a single model.

And we have estimates of the free diffusion coefficients of Bcd,  $D_f \sim 20 \text{ } \mu\text{m}^2/\text{s}$ , of its traps,  $D_S$  ( $\sim 0-0.1 \mu\text{m}^2/\text{s}$ ), of the ratio of the various concentrations and the dissociation constant so that we can **estimate** how fast Bcd diffuses everywhere.

How fast Bcd diffuses is key to establish the characteristic timescale of two key processes:

- The time it takes for the gradient of Bcd to be formed.
- The time it takes for the sites on DNA involved in the transcription of certain genes (which are regulated by Bcd) to “read” [Bcd] with a certain precision. In fact, Bcd is a transcription factor and as such it regulates the transcription of different genes, among them, hunchback. The non-uniform distribution of Bcd then results in a non-uniform pattern of expression of hb, i.e., Bcd is a morphogen.

What can we say about these two aspects (that had been previously analyzed using the FRAP estimate of Bcd’s diffusion coefficient?)

Here I will focus on the first of this issue (for the second, let me say that it is the free diffusion coefficient the one that sets the timescale for the read-out precision limit).

About the first point, can our RD model explain the formation of the Bcd gradient during the observed timescale?

Our model includes reactions so it is nonlinear. Thus, it is not so straightforward to tell. In principle, we think it is the collective diffusion coefficient ( $D_{coll} > D_{sm}$ ) the one that determines the timescale of formation (more on this later).

But the collective diffusion coefficient is concentration dependent,

$$D_{coll} = \frac{D_f + \frac{[S]^2}{K_D[S_T]} D_S}{1 + \frac{[S]^2}{K_D[S_T]}}.$$

Therefore, it varies with position: which value should we consider?

We decided to solve the full reaction-diffusion system (an SDID model) and see.

We tried several alternative models, with Bcd degradation only in its free form or in both its free and bound forms and with and without a transformation (during the timescale of the simulation) of Bcd from being non-fluorescent to being fluorescent.

$$\frac{\partial[Bcd]}{\partial t} = D_f \nabla^2[Bcd] - k_{on}[Bcd]([S_T] - [Bcd_b]) + k_{off}[Bcd_b] - \tilde{\alpha}[Bcd] + \theta(z),$$

$$\frac{\partial[Bcd_b]}{\partial t} = D_S \nabla^2[Bcd_b] + k_{on}[Bcd]([S_T] - [Bcd_b]) - k_{off}[Bcd_b],$$

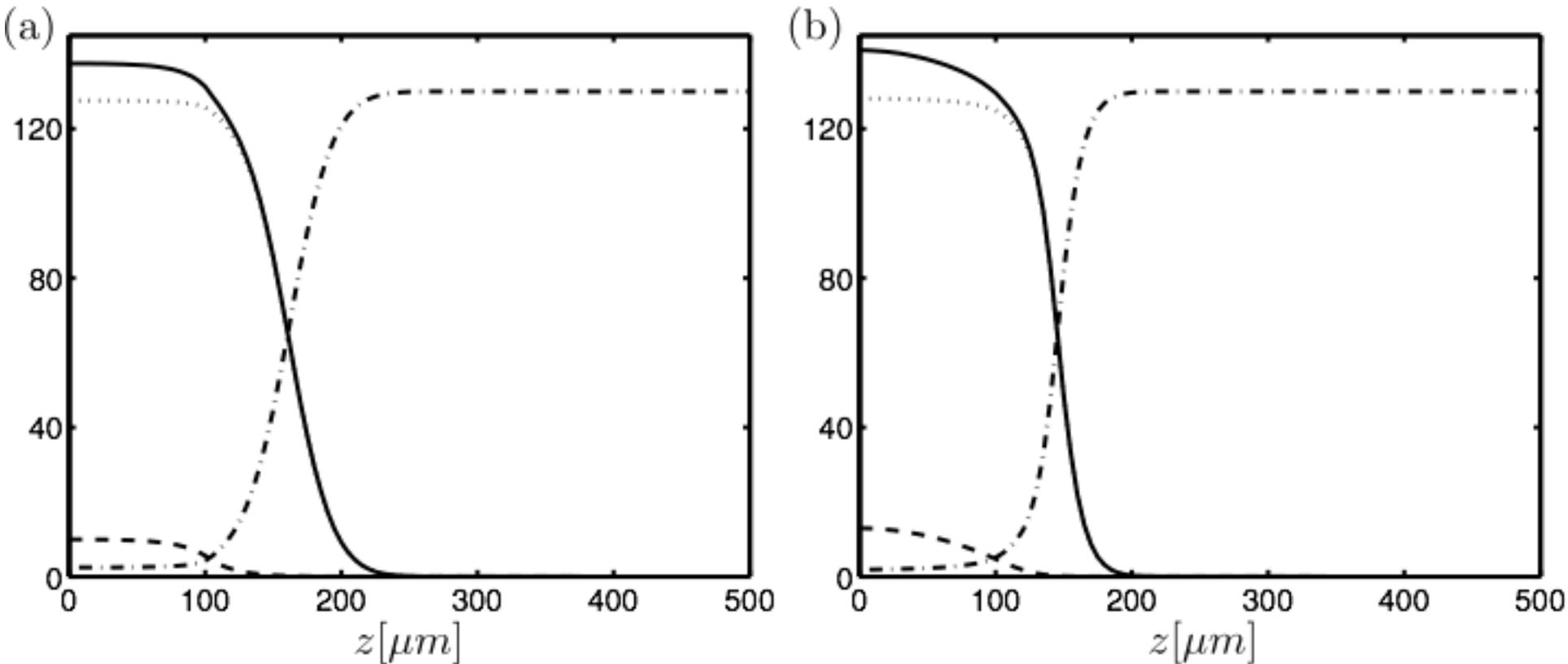
$$\theta(z) = \begin{cases} \frac{\theta_o}{d} & \text{si } z \leq d \equiv 0, 2L \\ 0 & \text{si } z > 0, 2L \end{cases},$$

Solved on a cylindrical domain with no flux boundary conditions, assuming only dependence with  $z$ ,  $0 < z < L$ .

They more or less give similar results (although for different numerical parameters).

Let me mainly focus on the simplest one.

Bcd distributions obtained with the SDID model at the time at which the experiments indicate that the gradient is already established.

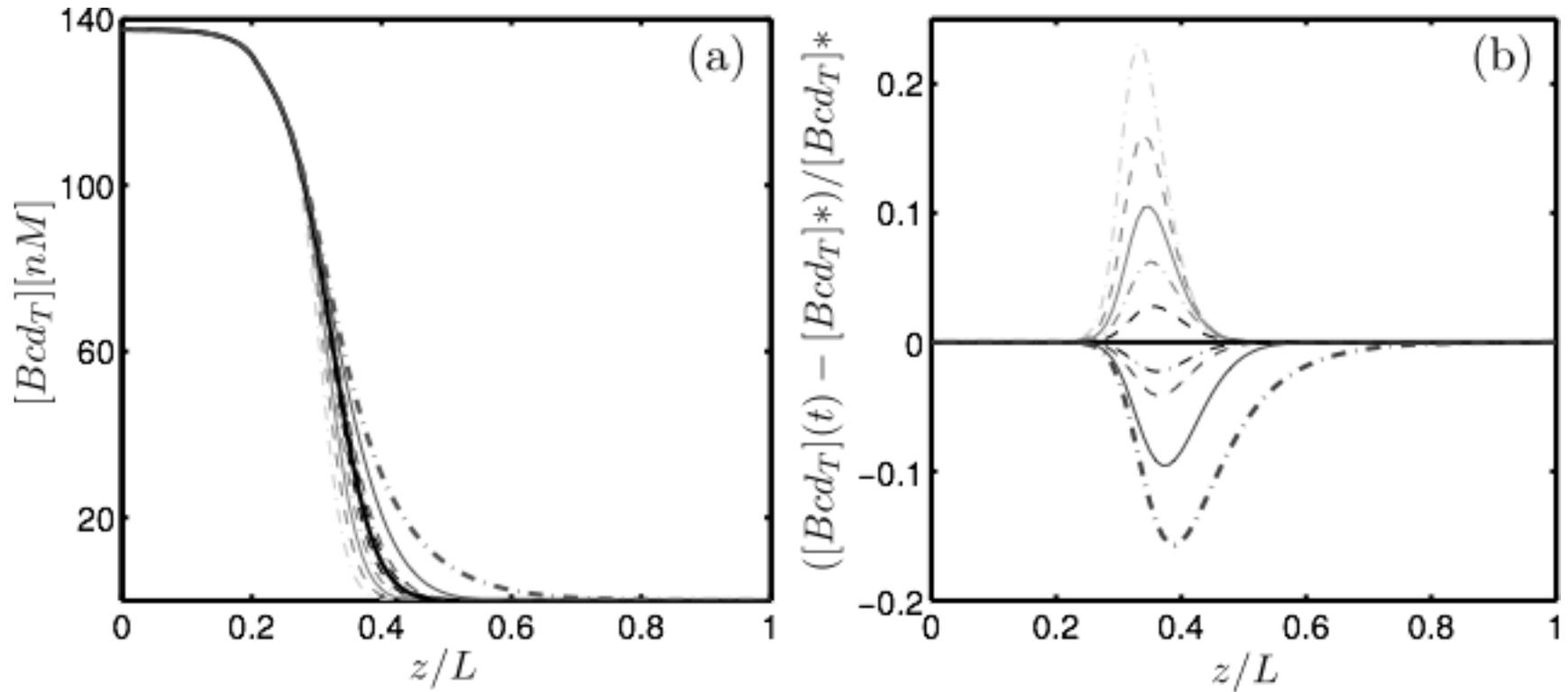


[Bcd<sub>T</sub>] (solid), [Bcd] (dashed), [S] (dashed-dotted), [Bcd<sub>b</sub>] (dotted) vs  $z$  at  $t=100\text{min}$  obtained from simulations of the SDID model with “partial” (a) and “total” (b) degradation.

Parameters:

$\tilde{\alpha} = 0,05s^{-1}$ ,  $\theta_0 = 0,5nMs^{-1}$ , in (a),  $\bar{\alpha} = 0,0005s^{-1}$ ,  $\bar{\theta}_0 = 0,1nMs^{-1}$  in (b).

# SDID model with “partial” degradation: time of convergence of the solution



$[Bcd_T]$  and  $[Bcd]$  vs  $z$  for various times. The differences with respect to the distribution at  $t=100$ min are shown in (b). Most of them are less than 10%

The SDID model with the diffusion coefficients determined in Sigaut et al, 2014 predicts that the gradient is relatively established by 100min for reasonable values of the degradation rate.

Some analytic derivations for the SDID (nonlinear model) assuming  $D_S=0$  and that reactions occur on a fast timescale.

The approximate evolution equation is:

$$\frac{\partial[Bcd]}{\partial t} = D_{coll} \nabla^2[Bcd] - \hat{\alpha}[Bcd] + \hat{\theta}(z).$$

with:

$$\begin{aligned} [Bcd_b] &= [Bcd][S_T]/([Bcd] + K_D), \\ [S] &= K_D[S_T]/([Bcd] + K_D). \end{aligned}$$

This equation for Bcd is similar to that of the linear SDD model:

$$\frac{\partial[Bcd]}{\partial t} = D \nabla^2[Bcd] - \alpha[Bcd] + \theta_o \delta(z),$$

that has a solution that decays in space with:

$$z_o \equiv \sqrt{D/\alpha} \text{ and "travels" with: } v \equiv \sqrt{\alpha D},$$

which time of convergence is:

$$t_{conv}(z) \equiv \frac{z}{v} = \frac{z z_o}{D},$$

Thus, we approximate the length scale of the Bcd gradient in the SDID model by:

$$z_{of} = \sqrt{D_{coll}/\hat{\alpha}} = \sqrt{D_f/\tilde{\alpha}}.$$

which is independent of z and t.

But the observed fluorescence corresponds to  $Bcd_T = Bcd_b + Bcd_f$ , not  $Bcd_f$ . We define:

$$z_{oT} \equiv [Bcd_T]/|\nabla[Bcd_T]|,$$

and obtain:

$$\frac{z_{of}}{z_{oT}} = \frac{D_{sm}}{D_{coll}},$$

For v and  $t_{conv}(z)$  of the Bcd distribution we obtain:

$$v = \sqrt{\tilde{\alpha} \frac{D_{coll}^2}{D_f}}, \quad t_{conv} = \frac{z D_{sm} z_{oT}}{D_{coll}^2},$$

What happens if we use the length-scale,  $l_o$ , and formation time-scale,  $t_o$ , of the observed gradient to infer which value the Bcd diffusion coefficient should take on to explain the observations.

The SDD model:

$$l_o = z_o = \sqrt{D_f/\alpha},$$

$$t_o = t_{conv}(z) = z/\sqrt{\alpha D_f},$$

$$D_f = z \frac{l_o}{t_o}$$

The SDID model:

$$l_o = z_{oT} = \frac{D_{coll}}{D_{sm}} \sqrt{D_f/\tilde{\alpha}},$$

$$t_o = t_{conv}(z) = z \sqrt{\frac{D_f}{\tilde{\alpha} D_{coll}^2}},$$

$$D_{coll} = z \frac{l_o}{t_o} \frac{D_{sm}}{D_{coll}}$$

## Summary

The diffusion of biomolecules plays a relevant role for the transport of information inside cells.

Most often, biomolecules do not diffuse freely inside cells but also react with binding sites (which introduces nonlinearities).

Anyway, the net resulting transport that occurs over long times can be described by effective diffusion coefficients.

In Pando et al 2006 we showed that two different effective diffusion coefficients,  $D_t$  and  $D_u$ , can describe the dynamics depending on the situation.

Collective

$$D_u = \frac{D_f + \frac{S_{eq}^2}{K_D S_T} D_S}{1 + \frac{S_{eq}^2}{K_D S_T}}$$

Single molecule

$$D_t = \frac{D_f + \frac{S_{eq}}{K_D} D_S}{1 + \frac{S_{eq}}{K_D}}$$

They are both weighted averages of the particles,  $D_f$ , and the traps,  $D_S$ , free diffusion coefficients but they can have arbitrarily different numerical values.

Diffusion rates in cells can be estimated experimentally using optical techniques and fluorescently tagged biomolecules. Two widely used techniques are FRAP and FCS.

In Sigaut et al 2010 we compared which effective coefficients can be estimated with them when the biomolecules diffuse and react with non-fluorescent "traps".

In FRAP fluorescent and non-fluorescent versions of the molecules of interest coexist and the technique estimates  $D_+$ .

FCS gives the free trap diffusion coefficient,  $D_S$ , and  $D_u$  if only the fluorescent version of the particles is present and it gives  $D_+$  as well if non-fluorescent particles are present too.

FRAP and FCS experiments were performed in *Drosophila* embryos to estimate the diffusion of the morphogen Bcd obtaining values that differ by an order of magnitude.

The gradient of Bcd is key for the formation of the dorso-ventral axis in flies. The low diffusion coefficient estimated with FRAP could not account for the gradient formation within the observed times

We used a simple biophysical model (with Bcd and traps) to obtain a mechanistic interpretation of the parameters derived with both experimental techniques. We could explain all the results within a comprehensive framework reconciling, as well, the diffusion coefficient estimate and the time it takes for the gradient to be formed.

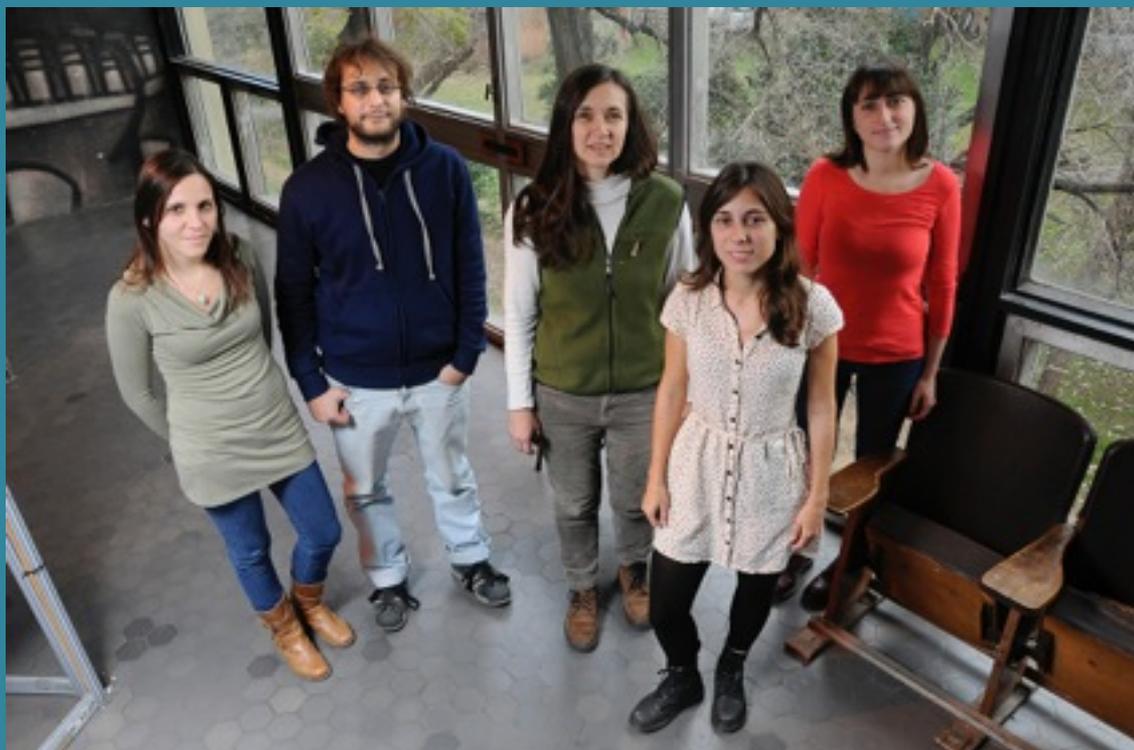
We also estimated other biophysical parameters.

Using the biophysical parameters determined in Sigaut et al we simulated an extended version of the SDD model for the formation of the Bcd gradient (the SDID model) which includes reactions with binding sites.

We probed different variations of the model and obtained distributions that compare relatively well with the experimental observations using reasonable parameters for the Bcd degradation and production.

An important aspect of our SDID model is that the observed fluorescence does not correspond to Bcd, but to Bcd<sub>T</sub>. Inferring parameters assuming that it corresponds to free Bcd can lead to erroneous estimates.

In particular, assuming that the length-scale of the observed gradient is related to the free diffusion coefficient of Bcd may lead to its over-estimation.



Thank you!



# Announcement

## Latin American Workshop on Professional Skills for Young Female Scientists

Buenos Aires, July 12-15, 2016

We expect to have some financial support for participants

Web page: coming soon (stay alert)