

Phase separation of equilibrium polymers of proteins in living cells

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Abstract

A number of proteins polymerise reversibly in living cells. The equilibrium polymers are functional: if mutant proteins are made that cannot polymerise, then these proteins cannot perform their biological functions. Furthermore, these polymers of proteins appear to phase separate inside the cell. The dynamics of one of these polymerising, phase separating, proteins has been studied via Fluorescence Recovery After Photobleaching (FRAP) by Bienz and coworkers. Here, their data is compared with the results of quantitative modelling to gain a better understanding of the dynamics of this protein inside a cell. The protein is called Dishevelled; it is a protein essential to the development of all animals and the name originates in the disruption of hair formation in a mutant version of this protein. It is not known how polymerisation and phase separation enable Dishevelled to perform its biological function but here we propose and discuss two possibilities. The first is that the cell is exploiting the inherently sharp, switch-like, nature of a phase transition to respond in a switch-like way to an external signal. The second is that phase separation dynamically creates a compartment (the more concentrated phase) into which other proteins partition.

1 Introduction

Our bodies are composed of cells. Most of the volume of a cell is taken up by the cytoplasm, the fluid inside of the cell but outside of the nucleus. The cytoplasm of a typical cell is a dynamic, concentrated mixture of about 10^{11} macromolecules, mostly proteins, of around 10^4 different types. Many proteins have quite complex dynamics, they may shuttle into and out of the nucleus, localise to membranes, etc. These dynamics are essential to the biological function of those proteins. Here, we will consider a protein that appears to be both forming equilibrium polymers and phase separating inside cells. Both are apparently essential for its biological function. The protein is called Dishevelled (Dvl). We are motivated to model this protein inside cells by recent work by Bienz and coworkers,¹⁻⁴ and by Dale and coworkers.⁵ They find that Dvl exhibits behaviour that is familiar to physical scientists: phase separation. This is, however, in a context that is unfamiliar to physical scientists: a living cell. Thus, there are opportunities to apply what physical scientists have learnt of phase separation and of equilibrium polymerisation, to understanding how this intriguing protein functions.⁶ Dvl is part of a signalling pathway that is essential for the development of all animals,^{7,8} and it is also implicated in a number of cancers. It is therefore the subject of intense study by biologists. The unusual name of Dishevelled originates in the fact that a mutation in it disrupts polarisation of cells in the wings of fruit flies.⁷ This disruption results in the small hairs on the wings pointing in all directions, making the hairs dishevelled in appearance.

With modern microscopy techniques we can both image the distribution and follow the dynamics of a protein species in a living cell. When this is done it is often found that the protein is not uniformly distributed within the cytoplasm but is concentrated into discrete domains of high concentration, surrounded by the remainder of the cytoplasm where the concentration of the protein is much lower. Examples are the proteins Dvl^{1,5} and TIA-1.^{9,10} TIA-1 stands for T-cell internal antigen-1; the origin of the name is

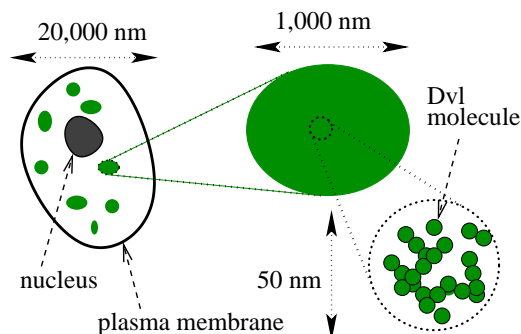


Figure 1: A schematic of a cell containing Dvl puncta. A complete eukaryote cell, with a nucleus at the centre, is illustrated at the left. The cell is approximately $20\mu\text{m}$ across. In the middle is a single punctum, at a larger scale. At the bottom right is a view of a small region inside a punctum, showing the polymers of Dvl.

not relevant here. See Fig. 1 for a schematic of a cell with high concentration domains of Dvl. In Dvl these dynamic aggregates are often called puncta, as they appear as points of light in a microscope. The corresponding domains of TIA-1 are referred to as stress granules as they form in response to stress.^{9,10} Both proteins form highly dynamic aggregates hundreds of nm across in the cytoplasm. As a protein molecule is a few nm across these aggregates are between a few tens and a hundred molecules across. The aggregates are dynamic in the sense that the molecules cycle into and out of them over a timescale of seconds or tens of seconds. This spontaneous formation of domains of high concentration coexisting in dynamic equilibrium with regions of much lower concentration (the remainder of the cytoplasm of the cell) suggests that the proteins Dvl and TIA-1 are phase separating in the cytoplasm of a living cell. This is supported by recent work of Bienz and coworkers.² They showed that Dvl forms equilibrium polymers, and that mutating Dvl so that it cannot polymerise destroys its ability to form the dynamic aggregates. It is well understood that polymerisation can enhance a tendency to phase separate¹¹⁻¹³ and that therefore eliminating polymerisation can shut off phase separation.

Here, we will study a model of a protein that both forms equilibrium polymers and phase separates inside a cell. Our aim is to apply the understanding we have of polymerisation and of phase transitions to better understand how they allow a protein to perform its biological function. The formation of puncta has been shown to correlate strongly with the biological function of Dvl.^{1,2,5}

In the following, we will focus on modelling Dvl and comparing the results to the experiments of Bienz and coworkers,^{1,2} and those of Dale and coworkers.⁵ The literature on the phase separation behaviour of Dvl is reviewed in Ref. 6. The protein Dvl is known to be an essential component of a signalling pathway called the Wnt pathway. This pathway controls a number of processes including cell division and cell polarisation. Despite extensive work, how Dvl functions is not well understood, see for example Kimelman and Xu's recent review.⁸ However, there is a considerable volume of experimental data on the behaviour of Dvl in cells with which models can be compared.

Data on the dynamics of the phase-separated Dvl has been obtained using Fluorescence Recovery After Photobleaching (FRAP).¹ This is a powerful experimental technique used to study the dynamics of proteins inside a cell.¹⁴⁻¹⁸ It can provide quantitative data on the turnover rates of proteins in large dynamic aggregates such as puncta. However, a model is required to interpret this data, for example to disentangle the effects of binding and unbinding of proteins from those of diffusion. Here we develop such a model for the FRAP data.

In the next section, we will define our model for Dvl, and calculate the mean polymer length. In the third section we will determine the phase behaviour. Section four deals with the dynamics of fluorescence recovery. The fifth section is a discussion. There is an appendix devoted to describing our implementation

of the Gillespie¹⁹ algorithm.

2 Equilibrium polymerisation of Dvl

Motivated by the experimental data that shows Dvl polymerising,² we model Dvl by molecules with 2 binding sites: A, and B. Only AB binding occurs, i.e., there are no AA or BB interactions. This binding between two sites is not enough to drive phase separation.^{20–22} To obtain phase separation the polymers must be made sticky, for example by incorporating additional sites in the model. As there is no quantitative data on the coexisting concentrations we do not do this. However, in the next section we will estimate these coexisting concentrations.

In the remainder of this section we will calculate the lengths of the equilibrium polymers formed via AB bonds. If the concentration of Dvl is c then this is also the concentration of A sites. The concentration of free (not bound to B) A sites c_{A0} may be obtained from the mass action equation

$$c = c_{A0} + c_{A0}c_{B0}/K_d^{(AB)}, \quad (1)$$

where c_{B0} is the concentration of free B sites, and $K_d^{(AB)}$ is the dissociation constant for the AB interaction. Note that a dissociation constant K_d is, by definition, one over the corresponding equilibrium constant, K_{eq} : $K_d = 1/K_{eq}$. By symmetry $c_{B0} = c_{A0}$. Defining the fraction of A (or B) sites that are free by $X = c_{A0}/c = c_{B0}/c$, we can rewrite Eq. (1) as

$$1 = X + X^2c/K_d^{(AB)}. \quad (2)$$

This has the solution

$$X = \frac{\sqrt{1 + 4c/K_d^{(AB)}} - 1}{2c/K_d^{(AB)}}. \quad (3)$$

As we have assumed that binding at sites is independent, the value of X allows us to obtain the concentrations of all the species. This has been done before,^{12,23} and comparison with simulation shows that the results obtained are accurate.²³ The concentration of monomers is $c_1 = cX^2$, i.e., it is the concentration of molecules with the A site not bound, cX , times the probability that the B site is not bound, X . The concentration of dimers is $c_2 = cX^2(1 - X)$, i.e., it is the concentration of molecules with site A not bound, cX , times the probability that the molecule's site B is bound, $1 - X$, times the probability that the B site on the second molecule is not bound. Note that we do not need to consider the A site of the second molecule as if the B site on molecule is binding then so must this A site. The general expression for the concentration of n -mers is $c_n = cX^2(1 - X)^{n-1}$.

Now that we have the concentrations of the n -mers, we can calculate the mean polymer length λ , from its definition

$$\lambda = \frac{\sum_{n=1}^{\infty} c_n n}{\sum_{n=1}^{\infty} c_n} = \frac{\sum_{n=1}^{\infty} cX^2(1 - X)^{n-1}n}{\sum_{n=1}^{\infty} cX^2(1 - X)^{n-1}}. \quad (4)$$

The top and bottom sums are c and cX , respectively. Thus the mean length of the equilibrium polymers of Dvl is

$$\lambda = 1/X = \frac{2c/K_d^{(AB)}}{\sqrt{1 + 4c/K_d^{(AB)}} - 1} \quad (5)$$

$$\approx \left(c/K_d^{(AB)}\right)^{1/2} \quad \lambda \gg 1, \quad (6)$$

where the second line holds for strong association. When Dvl forms long equilibrium polymers, their length scales as the square root of the concentration.

We can estimate the dissociation constant for AB binding, $K_d^{(AB)}$, from the data obtained by Schwarz-Romond *et al.*² They used ultracentrifugation to estimate the extent of polymerisation as a function of concentration, see their Fig. 4. They in fact studied not complete Dvl proteins but a domain of this protein called the DIX domain. However, this is the domain known to be responsible for polymerisation.² They estimated that the polymers reached sizes of 6-mers at a concentration of $40\mu\text{M}$. Using Eq. (5) we find that a $K_d = (4/3)\mu\text{M}$ is required to produce a mean length $\lambda = 6$ at $40\mu\text{M}$. This is approximately $K_d^{(AB)} = 10^{-6}\text{nm}^{-3}$, so we will use this value.

3 Phase coexistence

Phase separation results in coexistence between a dilute phase, the cytoplasm, and a more concentrated phase, that in the puncta. We denote the concentrations in the cytoplasm and in the puncta by c_c and c_p , respectively. Because these concentrations are set by the requirement that the chemical potentials be equal in the two phases the concentrations are fixed, i.e., independent of the total amount of Dvl in the cell. Of course, the total volume of the puncta will increase as the total amount of Dvl in the cell increases.

Lee *et al.*²⁴ estimate a total Dvl concentration in *Xenopus* (frog) egg cytoplasm of $100\text{ nM} \simeq 10^{-7}\text{nm}^{-3}$. We take this to be a rough upper bound to the Dvl concentration in the cytoplasm when it coexists with puncta, c_c . At a concentration of 10^{-7}nm^{-3} , Eq. (5) predicts that the Dvl will be almost entirely monomeric. Thus we predict that the Dvl outside the puncta will exist as monomers.

Now, we wish to estimate the concentration of Dvl in the puncta, c_p , and hence the mean length λ of the Dvl polymers there. Typically, phase separation occurs into phases that are highly concentrated, which in this context would imply that the puncta contained a volume fraction of Dvl of order tens of volume %. The total volume fraction of proteins and other macromolecules inside the cytoplasm is estimated to be around 30%.^{25,26} However, we will assume that the volume fraction of Dvl in the puncta is much lower than this, a few % not a few 10%. We do so because Dvl is known to polymerise. Polymers are well known to phase separate at low volume fractions. The volume fraction at the critical point for phase separation scales with the polymer length λ as $\lambda^{-1/2}$.¹¹ See also the work of Bianchi *et al.*²² who studied fluids in which the molecules bound via sticky patches like our A and B sites, and found coexistence between a very dilute phase and a more concentrated phase that itself was only at volume fractions of a few %.

The fact that both Dvl and TIA-1 form equilibrium polymers suggests that there is a specific advantage to polymerising. We speculate that this advantage is that it allows phase coexistence with a higher concentration phase that contains a relatively low, say $< 10\%$, concentration of the protein. This then allows other proteins to rapidly diffuse into and bind the equilibrium polymers in the puncta. Schwarz-Romond *et al.*³ have found that two other proteins, Axin and CKI ϵ (casein kinase ϵ), partition into Dvl puncta. They are present in the puncta at concentrations far above that in the remainder of the cytoplasm and cycle rapidly into and out of the puncta. More experimental work, particularly on measuring c_p , and on further examining the dynamics of Dvl-binding proteins, is needed to test our speculation.

So, if the volume fraction of Dvl is a few % and as Dvl's volume is of order 100 nm^3 , then c_p is of order 10^{-4}nm^{-4} . For $c_p = 10^{-4}\text{nm}^{-4}$, then $\lambda = 10.5$, using Eq. (5) with $K_d^{(AB)} = 10^{-6}\text{nm}^{-3}$. We predict that in the puncta the equilibrium polymers of Dvl are around 10 Dvl molecules long on average.

4 Dynamics: FRAP

Our model of a punctum is a sphere of radius R of the high concentration phase. This is immersed in the cytoplasm, which is taken to be an infinite volume of the low concentration phase. FRAP¹⁴⁻¹⁸ works by bleaching a punctum with a high intensity pulse of laser light, this destroys the ability to fluoresce of all the Dvl in the punctum at the instant that it is hit by the pulse. The fluorescence of the punctum then recovers by the non-fluorescent, bleached, Dvl leaving the punctum and being replaced by unbleached Dvl from elsewhere in the cytoplasm. The fluorescence recovery requires two basic physical processes: i)

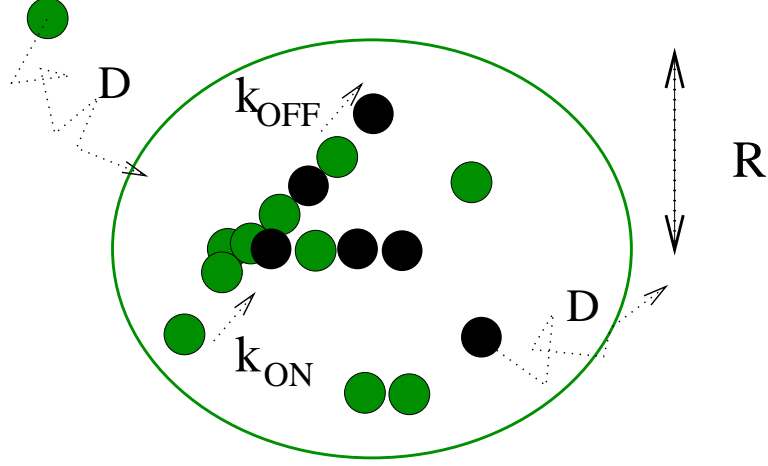


Figure 2: Schematic of a punctum, the green oval, of radius R . Fluorescent Dvl molecules are shown as green discs, bleached molecules as black discs. Note that the puncta observed are much larger in relation to Dvl than shown, and that only a few of the Dvl molecules are shown. The schematic illustrates the processes that FRAP can potentially probe. These are diffusion of Dvl into, out of and across the punctum, and the binding and unbinding reactions.

diffusion into, out of and across the punctum, and ii) reaction: the binding and unbinding of Dvl from the polymers inside the punctum. Both processes are illustrated in Fig. 2. We expect that the rate of recovery of fluorescence will be limited by the slowest process. We will consider both processes, starting with diffusion.

4.1 Diffusion

Here we wish to consider diffusion: a) into and out of a punctum, and b) across a punctum. First, into a punctum. The punctum is assumed to be at steady state in the sense that the total flux of Dvl into it equals the total flux out. Let us consider diffusion into a spherical punctum of radius R . The diffusive flux in is $j_{in} = 4\pi R D c_c$, with D the diffusion constant for Dvl. The diffusion constant of Dvl has not been measured. However, Sprague *et al.*¹⁸ found that the protein GFP (Green Fluorescent Protein) had a diffusion constant in their cells of $D = 15 \mu\text{m}^2\text{s}^{-1}$. Now GFP is considerably smaller than Dvl so we expect Dvl to diffuse more slowly. Therefore, for Dvl we use the estimate $D = 10 \mu\text{m}^2\text{s}^{-1}$.

With this value of D , and a cytoplasmic concentration $c_c = 100 \text{ nM} \simeq 100 \mu\text{m}^{-3}$ (see section 3 and Ref. 24), the flux onto a punctum of radius $R \mu\text{m}$ is $10^4 R \text{ s}^{-1}$. For a concentration in the punctum $c_p = 10^{-4} \text{ nm}^{-3}$, a punctum of radius $R \mu\text{m}$ has approximately $4 \times 10^5 R^3$ Dvl molecules. Thus if FRAP is limited by diffusion into the punctum of Dvl molecules, the FRAP recovery time is given by the ratio of the number of molecules to the flux, i.e.,

$$\text{FRAP time} = \frac{4 \times 10^5 R^3}{10^4 R} = 40 R^2 \text{ s}, \quad (7)$$

with R in μm . Note the fact that the FRAP time scales as R^2 : if fluorescence recovery is diffusion limited then the fluorescence in larger puncta will recover more slowly. For a punctum $1 \mu\text{m}$ across $R = 0.5 \mu\text{m}$ and the diffusion-limited FRAP time is 10 s — close to the experimental value.^{1,2} For puncta 100 nm across the relaxation time is 0.1 s.

As neither D nor c_c have been directly measured, there is considerable uncertainty in our prediction for the rate of fluorescence recovery if it is limited by diffusion. Therefore, we are unable to prove that diffusion is slow enough to be the process that determines the rate at which fluorescence recovers. However, we also cannot rule out that possibility.

Having looked at diffusion into and out of a punctum we turn to diffusion across a punctum. This is very rapid. Assuming that D is the same inside and outside a punctum, the timescale is $R^2/D = 0.025$ s for a punctum of radius $R = 0.5 \mu\text{m}$. This timescale is much smaller than the timescale for diffusion into/out of a punctum and so we neglect it.

Having considered diffusion into the punctum we want to consider reaction. We will consider the timescale for reaction in section 4.2 and compare it to that of diffusion. Section 4.4 will consider reaction and then combined reaction and diffusion in more detail in order to obtain a prediction for the FRAP curve.

4.2 Comparison of rates of diffusion and reaction

Within a punctum, the binding rate for a monomer is $k_{ON}c_pX$, with k_{ON} the rate constant for binding. In dilute solution *in vitro*, rate constants for diffusion-limited protein-protein association are around 10^{-3} to $10^{-2}\mu\text{m}^3\text{s}^{-1}$,²⁷ with the exception of some large rate constants where there is significant electrostatic attraction between the proteins. We take an *in vivo* rate constant that is consistent with these values, $k_{ON} = 10^{-3}\mu\text{m}^3\text{s}^{-1}$. For the concentration inside a punctum, we are assuming $c_p = 10^{-4}\text{nm}^{-3}$. At this concentration the fraction of sites free and available for binding $X \simeq 0.1$. Thus, the rate at which a monomer binds and ceases to be a monomer $k_{ON}c_pX = 10 \text{ s}^{-1}$.

For the purposes of comparison we require the rate of diffusion of monomers out of the punctum. We will neglect the effect of dimers, trimers etc leaving the punctum, and consider only monomers. At steady state, the rate at which Dvl leaves the punctum is equal to that at which Dvl joins the punctum. We estimated that the rate at which Dvl molecules join the punctum is 10^4R s^{-1} , for R in μm . The total number of monomers in the punctum is $4 \times 10^5 R^3 X^2$, where the factor of X^2 is the fraction of the Dvl molecules that are monomers. Now we define r_l as being the rate at which monomers leave the punctum divided by the total number of monomers, i.e., it is rate at which a monomer diffuses out of the punctum. For a punctum of radius $R \mu\text{m}$, $r_l = 10^4R/(4 \times 10^5 R^3 X^2) = 10 \text{ s}^{-1}$ for $R = 0.5 \mu\text{m}$ and $X = 0.1$.

This rate of diffusing out of the punctum is approximately equal to the rate at which a monomer binds to a polymer. Although it should be borne in mind that both rates have large (one or two orders of magnitude) uncertainties, our best guess is that the rates of reaction and diffusion are comparable. Therefore we need to consider this situation. To do this we will start by considering the case where reaction is much slower than diffusion, as that is a simpler case, before extending our analysis to include the situation where both rates are comparable.

4.3 FRAP of binding to fixed sites

Before we consider the timescale of FRAP when it is limited by the rate of binding and unbinding in equilibrium polymers, we will orient ourselves by considering the simpler case of FRAP of a fluorescent protein P that binds to a localised set of static sites S . This has been considered before by Bulinski *et al.*¹⁶ and we follow their treatment of the problem. Here the reaction is simply



with a rate constant k_{ON} for the forward reaction and k_{OFF} for the back reaction; the dissociation constant $K_d = k_{OFF}/k_{ON}$. The forward rate is $k_{ON}c_S c_P$, where c_S is the concentration of free S sites, and c_P is the concentration of free P molecules. The back rate is $k_{OFF}c_{PS}$, where c_{PS} is the concentration of S sites with P bound. Denoting equilibrium concentrations by a superscript (e) we then have that $c_{PS}^{(e)} = c_P^{(e)} c_S^{(e)} / K_d$.

Now, at time $t = 0$, we bleach all the P bound to the S sites. The fluorescence will recover as P diffuses from the rest of the cytoplasm on to the S sites, to replace the bleached P that unbinds from these sites. Now, we assume that the binding/unbinding is the rate limiting step and so the local concentration of free unbleached P is assumed to instantaneously return (due to rapid diffusion) to its equilibrium value,

$c_P^{(e)}$ at time $t = 0$. This is what Sprague *et al.*¹⁸ call the “reaction dominant” limit. They also consider the conditions under which this is a reasonable assumption and the case where diffusion is relatively slow and so must be considered; see Ref. 18 for details. The concentration of free S sites is also unchanged of course, it remains $c_S^{(e)}$. So, if we denote the concentration of *fluorescent* P bound to S by $c_{PS}^{(f)}(t)$, it obeys the differential equation

$$\frac{d\rho_{PS}^{(f)}(t)}{dt} = k_{ON}\rho_P^{(e)}\rho_S^{(e)} - k_{OFF}\rho_{PS}^{(f)}(t). \quad (9)$$

The first term is the binding of fluorescent P from the cytoplasm, and the second term is the unbinding of that portion of the P bound to S that is fluorescent. If we note that at equilibrium we have by definition $k_{ON}c_P^{(e)}c_S^{(e)} = k_{OFF}c_{PS}^{(e)}$ then we can rewrite Eq. (9) as

$$\frac{dc_{PS}^{(f)}(t)}{dt} = k_{OFF} \left[c_{PS}^{(e)} - c_{PS}^{(f)}(t) \right]. \quad (10)$$

Here we introduced the fraction of PS that is fluorescent $f = c_{PS}^{(f)}(t)/c_{PS}^{(e)}$. The boundary condition is then $f(t = 0) = 0$, and the solution is

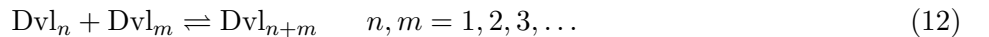
$$f(t) = 1 - \exp(-k_{OFF}t). \quad (11)$$

The fluorescence is predicted to recover exponentially, with a rate constant precisely equal to the rate constant for unbinding.¹⁶

4.4 FRAP of linear polymers

Let us now consider FRAP for a punctum composed of equilibrium linear polymers. In order to consider reaction in isolation, initially we will assume that diffusion into and out of the punctum is instantaneous. Thus once a Dvl molecule has broken its bonds to other Dvl molecules and is monomeric, it leaves the punctum and is instantly replaced with another (unbleached) monomer. We neglect any flux out due to dimers, trimers etc. Towards the end of this section we will relax the assumption that diffusion out of the punctum is instantaneous, in order to consider the situation where diffusion and reaction occur at similar rates and so both contribute to the FRAP time. However, initially we will just calculate the reaction-limited FRAP time.

Now the basic reactions are



with a rate constant k_{ON} for the forward reaction and k_{OFF} for the back reaction. Here Dvl_n is a Dvl n -mer. The binding is mediated by the A and B sites. The dissociation constant for this binding, $K_d^{(AB)}$, is related to the rate constants by $K_d^{(AB)} = k_{OFF}/k_{ON}$. We assume that k_{ON} and k_{OFF} are both independent of n and m . To determine the FRAP time when it is reaction-limited, we require both the rate at which monomers are produced by bond breaking, r_{ub} , and the rate at which monomers bond to chains and other monomers, and cease to be monomers, r_b . We start with r_{ub} .

The rate of bond breaking is just the number of bonds times k_{OFF} . However not all bond breakage results in monomer formation, e.g., if a 10-mer splits in the middle two 5-mers are produced but no monomers. Also, if the bond in a dimer breaks, two monomers are produced; all other bond breaking produces a maximum of one monomer. So, to obtain r_{ub} we sum the contributions from dimers, trimers, etc, starting with dimers. If the Dvl concentration is c , there are $cX^2(1 - X)$ dimers, each with a bond that breaks at a rate k_{OFF} , creating two monomers. Therefore dimers contribute $2cX^2(1 - X)k_{OFF}$ to r_{ub} . Trimers have two bonds, each of which when breaking produces a single monomer. So, as the concentration of trimers is $cX^2(1 - X)^2$, trimers contribute $2cX^2(1 - X)^2k_{OFF}$ to r_{ub} . Only the breaking of one of the

two end bonds of a tetramer produces a monomer, so tetramers contribute $2cX^2(1-X)^3k_{OFF}$, and so on. Summing the contributions of all the n -mers we have

$$r_{ub} = 2ck_{OFF}X^2(1-X) [1 + (1-X) + (1-X)^2 + \dots] = 2ck_{OFF}X(1-X). \quad (13)$$

The rate of binding of monomers, r_b , equals the concentration of monomers, cX^2 , times the concentration of chain ends, $2cX$, times the rate constant k_{ON} , $r_b = 2k_{ON}c^2X^3$.

Having determined the rates of unbinding and binding we now wish to calculate the rate of recovery of fluorescence in Dvl polymers ($n > 1$, all monomers are fluorescent). This equals the rate at which fluorescent monomers bind to polymers, minus the rate at which fluorescent Dvl unbinds from polymers as a monomer. The first rate is just r_b . In order to obtain the second we need to make a further assumption. We will check its validity via comparison with a Gillespie-algorithm simulation. The assumption is that at any time t , the probability that a Dvl molecule is fluorescent is independent both of the length of polymer it belongs to and its position along the polymer. In other words, at any time t , the fraction of Dvl that is bleached is not higher in the middle of a polymer than at its end, and is not higher (or lower) for longer polymers. Then if $f(t)$ is the fraction of Dvl in polymers that is fluorescent, at time t , the rate of unbinding of Dvl from polymers, as monomers, is $r_{ub}f(t)$.

Now that we have both rates, we can obtain a differential equation for the concentration of fluorescent Dvl molecules in polymers (dimers and above). The equation is

$$\frac{d}{dt} [c(1-X^2)f(t)] = r_b - r_{ub}f(t) = 2k_{ON}c^2X^3 - 2k_{OFF}X(1-X)cf(t). \quad (14)$$

Here $c(1-X^2)$ is the concentration of polymers with $n > 1$, i.e., the total concentration minus that of monomers. Now, $2k_{ON}c^2X^3 = 2k_{OFF}X(1-X)c$ because at steady state the two rates must be equal. Indeed, noting that $K_d^{(AB)} = k_{OFF}/k_{ON}$, this follows directly from Eq. (2). Then we have that

$$\frac{df(t)}{dt} = 2k_{OFF} \frac{X(1-X)}{1-X^2} [1 - f(t)]. \quad (15)$$

For bleaching at $t = 0$, the boundary condition is $f(t = 0) = 0$, and then the solution is

$$f(t) = 1 - \exp[-2k_{OFF}(X(1-X)/(1-X^2))t] \quad (16)$$

$$\simeq 1 - \exp[-2k_{OFF}t/\lambda]. \quad (17)$$

The fluorescence is predicted to recover exponentially, with a rate constant equal to the twice the rate constant for the off rate, times $X(1-X)/(1-X^2)$. For long polymers, $X \simeq 1/\lambda \ll 1$ and so this reduces to $2k_{OFF}/\lambda$. So, for these long polymers the recovery time for fluorescence is $\lambda/(2k_{OFF})$. Note that this is radius independent. If the fluorescence recovery is reaction limited then it is independent of the size of the punctum. This offers a way of distinguishing between reaction-limited and diffusion-limited fluorescence recovery, as the time for one increases with punctum radius while the other is constant.

From the ultracentrifugation data of Schwarz-Romond *et al.*,² we estimated a dissociation constant $K_d^{(AB)} = 10^{-6}\text{nm}^{-3}$. As $K_d^{(AB)} = k_{OFF}/k_{ON}$, if we can estimate either one of k_{ON} or k_{OFF} we can determine the other. As earlier, we will take $k_{ON} = 10^{-3}\mu\text{m}^3\text{s}^{-1}$. This is within the range of values for diffusion-limited protein-protein association *in vitro*.²⁷ With this value for k_{ON} , the rate constant for bond breaking $k_{OFF} = 1\text{ s}^{-1}$. For polymers of mean length $\lambda = 10.5$ (see previous section), we then get a predicted FRAP time close to 5s, in good agreement with experiment.¹ A plot of $f(t)$ from Eq. (16) is shown in Fig. 3.

Also shown in Fig. 3 are the results of a Gillespie-algorithm simulation for $f(t)$. This algorithm is described in an Appendix, it simulates explicitly the dynamics of a large number of monomers. It allows us to keep track of each monomer and so we keep track of whether it is bleached or not. The simulation does

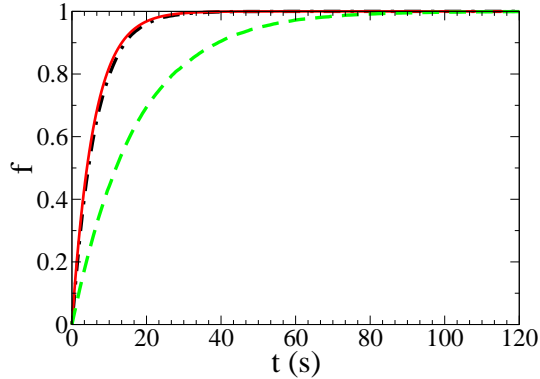


Figure 3: Plot of the fraction of the molecules (excluding monomers) that are fluorescent, as a function of time. This is in a punctum with a concentration $c = 10^{-4} \text{nm}^{-3}$. The on and off rate constants are $k_{ON} = 10^{-3} \mu\text{m}^3 \text{s}^{-1}$ and $k_{OFF} = 1 \text{s}^{-1}$. The red curve is the plot of Eq. (16). The black dot-dashed and green dashed curves are the results of Gillespie-algorithm simulations. The black dot-dashed curve is with diffusion out of the punctum assumed to be instantaneous and the green dashed curve is with diffusion out of the punctum at a rate $r_l = 10 \text{s}^{-1}$.

not assume that the fraction of bleached Dvl is at all times independent of polymer length and location along the polymer. The theory and simulation curves are virtually coincident, so this assumption of the theory introduces almost no error into $f(t)$.

We would like to get a better insight into the assumption of the theory that the fraction of fluorescent Dvl is same all along polymers of all lengths. To do this we have performed a small Gillespie-algorithm simulation. The simulation is of 100 monomers, and at a particular instant part of the way through fluorescence recovery they are formed into 12 polymers, including 3 monomers. In Fig. 4 we have plotted all 12 polymers. In the simulations there is no space dependence so we simply index the polymers 1 to 12 and plot them as columns of monomers. The polymers are part of the way through fluorescence recovery and so some monomers are bleached (black) while some have joined the polymers since bleaching occurred and so are fluorescent (green). We see that polymers of all lengths have relatively random distributions of fluorescent and bleached Dvl along their length. Thus it is reasonable that the theory gives a very accurate FRAP curve, see Fig. 3. The polymers are continually breaking at all points along their length, picking up monomers on the newly created free ends and then binding to other polymers, creating polymers with fluorescent Dvl not only at the ends of the polymer but in the middle.

So far, we have assumed that when a Dvl molecule unbinds from the end of a polymer and becomes a monomer, it instantaneously diffuses out of the punctum and is replaced by a fluorescent molecule. Also that while the Dvl molecule is diffusing out of the punctum there is no opportunity to rebind. Now we will drop this assumption and consider the finite rate of diffusing out of the punctum, r_l , that we considered earlier. We estimated this rate at $r_l = 10 \text{s}^{-1}$ for a punctum of radius $R = 0.5 \mu\text{m}$.

We will incorporate this estimate for r_l , into our Gillespie simulations. Within the simulation monomers diffuse out of the punctum (and are replaced by a fluorescent molecule) at a rate r_l , and this process competes with rebinding to another Dvl molecule in the punctum. Our combined reaction and diffusion rate is now given by Eq. (19) of the appendix. See this appendix for details. Results with $r_l = 10 \text{s}^{-1}$ are given in Fig. 3 as the dashed green curve. We have fitted exponential recovery curves to our Gillespie results both with instantaneous diffusion out of the punctum and with diffusion at rate $r_l = 10 \text{s}^{-1}$. The fits have recovery times of 6.2 and 17.0 s, respectively. Thus, when we allow for the fact that diffusion out of the punctum does not in fact occur much faster than binding and unbinding, the fluorescence recovery time increases by a factor of almost three.

As we would expect, when the rates of unbinding and of diffusion out of the punctum are comparable, they both need to be taken into account when fluorescence recovery is calculated. The relative contribu-

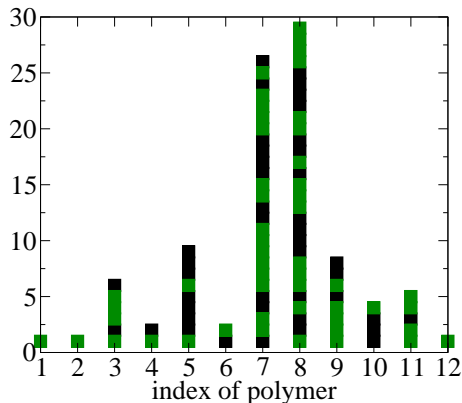


Figure 4: The polymers present at one instant in a small Gillespie simulation of 100 monomers. Twelve polymers are formed and each one is represented by a column of square monomers. Bleached monomers are in black and fluorescent monomers (i.e., those that have joined the punctum since bleaching) are in green. For example, the first two polymers are monomers, the third is a 6-mer of two bleached (second and sixth) and four fluorescent monomers, and so on. The simulations are with $k_{ON} = 10^{-3} \mu\text{m}^3 \text{s}^{-1}$, $k_{OFF} = 1 \text{s}^{-1}$, and $c = 10^{-4} \text{nm}^{-3}$.

tions of reaction and diffusion to the recovery time can be estimated if experiments are done on puncta of different sizes. If say the punctum has a radius $R = 0.1 \mu\text{m}$ then diffusion is much faster and so makes little contribution to the FRAP time, which will then be close to 6.2 s.

5 Conclusion and Future Work

Light microscopy of cells with fluorescent Dvl reveals bright points of light, puncta, indicating domains where the Dvl concentration is much higher than in the remainder of the cytoplasm.^{1,5,6} It appears that Dvl is phase separating inside the cell. Schwarz-Romond *et al.*² have also shown the Dvl polymerises. Here, we focused on modelling FRAP data¹ for the Dvl puncta. Our model is novel, includes polymerisation, and may be useful for other polymerising proteins such as Axin. Using parameters obtained from experiment, together with some physically reasonable estimates, we concluded that it is likely that both diffusion and reaction contribute to the time taken for the fluorescence of a punctum to recover. The model also shows that experiments on puncta of varying size will be able to assess which of reaction and diffusion dominates.

Finally, we will speculate about two possible biological functions of polymerisation and phase separation, and then end by considering some open questions.

5.1 Two possible biological functions of phase separation in a cell

1. Phase separation is an inherently cooperative phenomenon. Traditionally, in biochemistry cooperativity is considered within the context of the Hill model. The Hill model is for binding of molecules, M, to a substrate S, and assumes that the molecules can only bind to S as an n -mer, where n is a model parameter. The probability p that the substrate S is occupied by the n -mer is then given by $p = 1/[1 + (\kappa/c_M)^n]$. Here c_M is the concentration of free M molecules and κ is an effective dissociation constant for binding of the n -mer to the substrate. The probability switches from 0 to 1 more and more sharply as n increases. Phase separation corresponds to an essentially infinite n and so allows a very sharp switch. Cells often need to make decisions in response to external signals, e.g., to divide or not to divide, and so require an internal mechanism that responds in a switch-like manner. We speculate that the phase separation of Dvl may be an internal mechanism that allows cells to respond in a switch-like manner to a signal. Switch-like behaviour due to phase separation

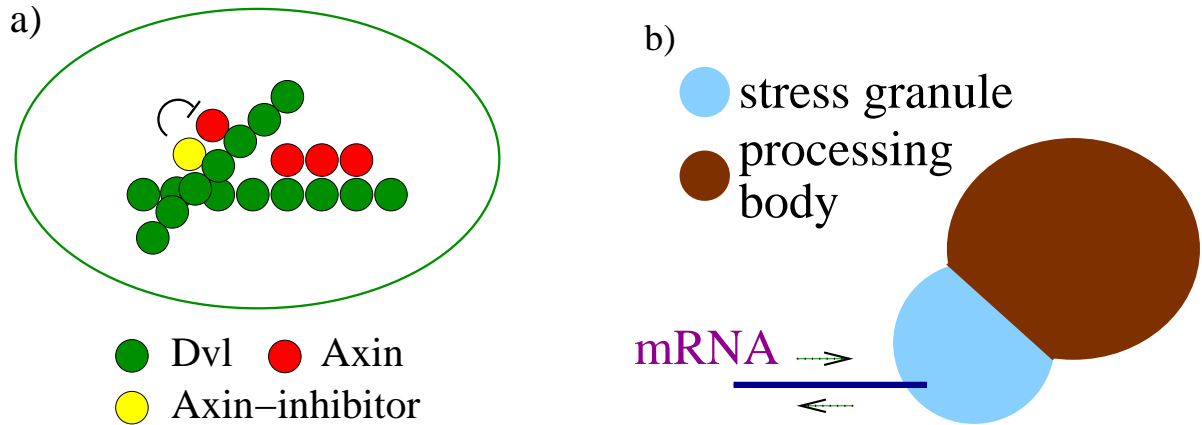


Figure 5: a) and b) are schematics illustrating open questions for puncta and for stress granules, respectively. a) shows both monomeric Axin and a polymer of Axin binding to Dvl. It is an open question whether Axin binds Dvl as a monomer or as a polymer. We also show an Axin-inhibiting protein bound to one Dvl molecule inhibiting an Axin molecule bound to another Dvl molecule. Polymerisation of Dvl may be required to inhibit Axin if a single Dvl molecule cannot simultaneously bind both Axin and an inhibitor. b) shows a touching stress granule and processing body. It illustrates schematically the possibility that mRNA transcripts may be actively transported into or out of stress granules.

at the plasma membrane is modelled in Ref. 6.

- Phase separation also creates two coexisting phases in the cytoplasm, and macromolecules will partition between the two. If the Dvl- or TIA-1-rich phase has a high affinity for a macromolecule then it will tend to partition strongly into the puncta or stress granules, depleting its concentration in the cytoplasm. Stress granules are known to have an affinity for mRNA transcripts,^{9,10} thus they may function by sequestering these transcripts in the granules and so depleting the concentration of mRNA in the bulk of the cytoplasm. In order to allow for this sequestration of what are rather large molecules, it may be helpful for the volume fraction of the TIA-1 polymers in the stress granules to be a few % not 10% or more. This then allows room for the large transcripts. It is well known¹¹ that polymers phase separate to produce a more concentrated phase (the stress granules) that is relatively dilute (see also Ref. 22). Thus we speculate that the function of the polymerisation of TIA-1 and Dvl may be to allow phase separation into a relatively dilute TIA-1- or Dvl-rich phase.

5.2 Future work

Let us consider in more detail how Dvl might function and how future work might enhance our understanding of this function. Dvl is believed to function by disrupting a complex of a number of proteins held together by a protein called Axin. This complex acts to suppress the concentration of a third protein, Armadillo. Thus when Dvl disrupts the Axin-based complex the concentration of Armadillo increases, which switches on a number of genes.

Intriguingly, Axin also polymerises,³ and knocking out Axin’s ability to polymerise speeds up FRAP of Axin in Dvl puncta. FRAP data for a mutant Axin that cannot polymerise³ suggests that monomeric Axin binds to Dvl with a dissociation constant $K_d \simeq 10$ nM. If we compare this with the Dvl concentration measured in *Xenopus* egg extracts of 100 nM,²⁴ we see that we expect significant association between Axin and Dvl even if they both exist as monomers. Future work could model how Axin and Dvl interact and how polymerisation of both proteins effects this interaction. In Fig. 5a) we illustrate schematically a couple of possibilities. Studies of Axin polymerisation could also consider its role in forming the complex

that suppresses the Armadillo concentration. It is known^{28,29} that at least Axin dimerisation is required for it to act to suppress the Armadillo concentration.

Finally, we make two suggestions for future work on stress granules. These granules are believed^{9,10} to sequester mRNA transcripts. Transcripts are large molecules, and it is known that in the cell they move partly via active transport, i.e., they are moved by molecular motors that consume energy in order to move.³⁰ These motors move along tracks called microtubules and actin filaments. Thus our first suggestion for future work is that it could investigate whether transcripts are transported into or out of stress granules. This possibility is illustrated schematically in Fig. 5b).

The second suggestion concerns the fact that puncta and stress granules are not the only domains containing high concentrations of a particular protein. Another example are domains called processing bodies. There are images of cells showing that stress granules and processing bodies are distinct but often touch, see for example Fig. 7B of Ref. 31. Now, interfacial tension tends to cause phase-separated domains to stick together. Future work could consider to what extent do phase-separated domains stick together inside cells and whether this has any biological function.

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Appendix: Gillespie-algorithm simulations

Our Gillespie¹⁹ simulations are straightforward. As usual there is no spatial dependence, only time dependence. We consider N Dvl molecules, each with A and B sites. At any time t , there will be $N_{A0}(t)$ A sites that are not bonded to B, and $N - N_{A0}(t)$ sites that are. The number of B sites that are not bonded $N_{B0}(t) = N_{A0}(t)$, by symmetry. It should be noted that often Gillespie simulations are used to study fluctuations. The puncta contain large numbers of molecules and so N is large and fluctuations relatively small. Thus we are using the Gillespie algorithm not to study fluctuations but because it enables us to follow the individual molecules. This is required in order to follow which Dvl molecules are bleached and which are fluorescent.

Our simulation algorithm starts with the possible reactions at any time t . These are:

1. $N_{A0}(t)N_{B0}(t) = N_{A0}^2(t)$ AB binding reactions, each with a rate k_{ON}/v_P .
2. $(N - N_{A0})$ AB unbinding reactions, each with a rate k_{OFF} .

Here v_P is the total volume of the punctum; $v_P = N/c_P$, for c_P the concentration of Dvl in a punctum. Thus the total reaction rate is

$$\text{rate} = N_{A0}^2 \frac{k_{ON}}{v_P} + (N - N_{A0}) k_{OFF}. \quad (18)$$

The algorithm is then as follows: at time t , one of these reactions is selected at random with a probability equal to the ratio of its reaction rate to the total rate at that time. This reaction is then performed. For example, if it is site A on Dvl molecule 102 binding to site B on molecule 1005, then 1 is subtracted from both N_{A0} and N_{B0} , and it is noted that the site A on molecule 102 is now bound to site B on molecule 1005. The time is then incremented by a random amount selected from an exponential distribution of mean the inverse of the total rate. Then another reaction is selected, and the process is repeated as often as required.

To study bleaching we first run the simulation to equilibrate the polymerisation reaction. Then we set $t = 0$ and mark all N molecules as bleached. The simulation is then run on and each time an unbinding reaction is performed we check to see whether any monomers are produced. If they are then they are assumed to instantaneously diffuse out of the punctum and be replaced by a molecule that is fluorescent.

Thus all monomers are fluorescent and so when a monomer binds to a polymer, the fraction of fluorescent Dvl in the polymers is increased. The simulation is run until all the bleached Dvl have been replaced by fluorescent Dvl.

When the rates of diffusion and reaction are comparable we need to consider both. To do this we need to consider the rate at which Dvl molecules leave a punctum and add this rate to Eq. (18). If the number of monomers at time t is $N_0(t)$ and the rate at which a single monomer leaves is r_l , then this rate is $N_0 r_l$. With this term the total reaction rate is

$$\text{rate} = N_{A0}^2 \frac{k_{ON}}{v_P} + (N - N_{A0}) k_{OFF} + N_0 r_l \quad (19)$$

As before, at time t one reaction is selected at random with a probability equal to the ratio of its reaction rate to the total rate. The difference is that if a monomer is created it is no longer assumed to be replaced instantaneously by a fluorescent monomer; it is simply created. Only if a leaving “reaction” is selected is the monomer assumed to diffuse from the punctum and be replaced by a fluorescent monomer.

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