

Tuning Protein Diffusivity with Membrane Tethers

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S Supporting Information

ABSTRACT: Diffusion is essential for biochemical processes because it dominates molecular movement on small scales. Enzymatic reactions, for example, require fast exchange of substrate and product molecules in the local environment of the enzyme to ensure efficient turnover. On larger spatial scales, diffusion of secreted signaling proteins is thought to limit the spatial extent of tissue differentiation during embryonic development. While it is possible to measure diffusion *in vivo*, specifically interfering with diffusion processes and testing diffusion models directly remains challenging. The development of genetically encoded nanobodies that bind specific proteins has provided the opportunity to alter protein localization and reduce protein mobility. Here, we extend the nanobody toolbox with a membrane-tethered low-affinity diffusion regulator that can be used to tune the effective diffusivity of extracellular molecules over an order of magnitude in living embryos. This opens new avenues for future applications to functionally interfere with diffusion-dependent processes.

Diffusion is fast over short distances but slow over longer spatial scales. It can therefore theoretically limit the dispersal and action range of signaling proteins within tissues, for example during early development.^{1,2} Most multicellular organisms develop from an embryo that initially consists of equivalent stem cell-like “naive” cells. A long-standing concept in developmental biology is that a subset of cells, the source, secretes signals that diffuse into the surrounding tissue and instruct naive cells to form embryonic organs. The idea that extracellular signaling molecules spread by diffusion appears to be straightforward due to the passive nature of diffusion,^{3,4} but the relevance of extracellular diffusion for the dispersal of signaling molecules from source to target tissues is still largely unclear. Despite evidence for free diffusion of the *Drosophila melanogaster* bone morphogenetic protein (BMP) homolog Decapentaplegic (Dpp),⁵ models of active Dpp transport have also been proposed.⁶ Similarly, the extent to which extracellular diffusion of the vertebrate signaling molecules BMP^{7–9} and Nodal^{4,10–14} is required for their endogenous function is unclear. Thus, classical models in which diffusion determines signal dispersal and tissue patterning are still being debated.^{1,15,16}

To examine the mechanisms underlying signal dispersal and to probe diffusion models, the Affolter lab has recently pioneered the morphotrap approach, in which a high-affinity anti-GFP nanobody¹⁷ (reviewed in ref 18) is targeted to the cell surface with a transmembrane domain tagged with

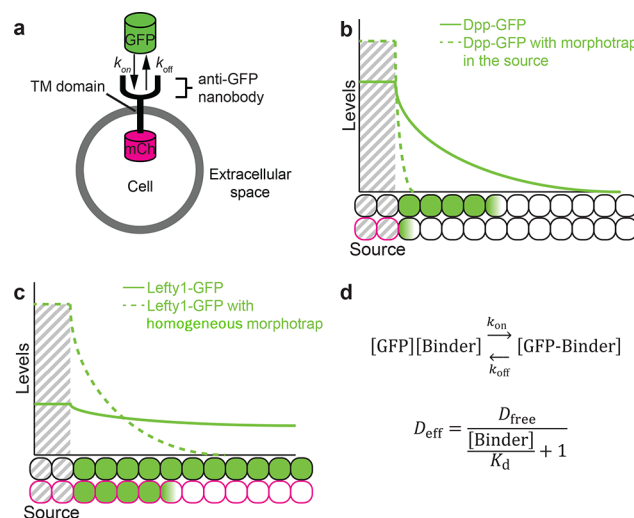


Figure 1. Using membrane-tethered nanobodies to immobilize extracellular GFP fusion proteins. (a) The morphotrap consists of a high-affinity anti-GFP nanobody, a transmembrane (TM) domain, and an intracellular mCherry (mCh) tag. Binding of extracellular GFP or GFP fusion proteins holds them on the plasma membrane.¹⁹ (b) Illustration of the Dpp-GFP distribution in the *D. melanogaster* wing disc upon expression of the morphotrap from a localized source.¹⁹ Normally, Dpp-GFP spreads from the source through the surrounding tissue (cells illustrated below the *x*-axis), forming a concentration gradient (green line). Cells that receive sufficiently high signal levels respond and induce downstream signaling (green cells). When the morphotrap is co-expressed in the source tissue (magenta cell outlines), Dpp-GFP is retained in the source and the formation of a relevant signaling gradient is abolished (green dashed line). Only cells in the immediate vicinity of the source receive signaling, whereas cells at a distance do not. (c) Illustration of the Lefty1-GFP distribution expressed from a localized source in zebrafish embryos.²⁴ Lefty1-GFP forms a long-range gradient from the source tissue (green solid line). When the morphotrap is homogeneously expressed in zebrafish embryos, Lefty1-GFP mobility is reduced, resulting in a steep gradient ~ 2 h after the onset of Lefty1-GFP production (green dashed line). The range of Lefty1-GFP is illustrated by the green cells below the *x*-axis. (d) If GFP reversibly interacts with a binder such as an anti-GFP nanobody, GFP’s effective diffusion coefficient D_{eff} is predicted to be modulated by the concentration of the binder as well as its GFP binding affinity,^{21,22} K_d .

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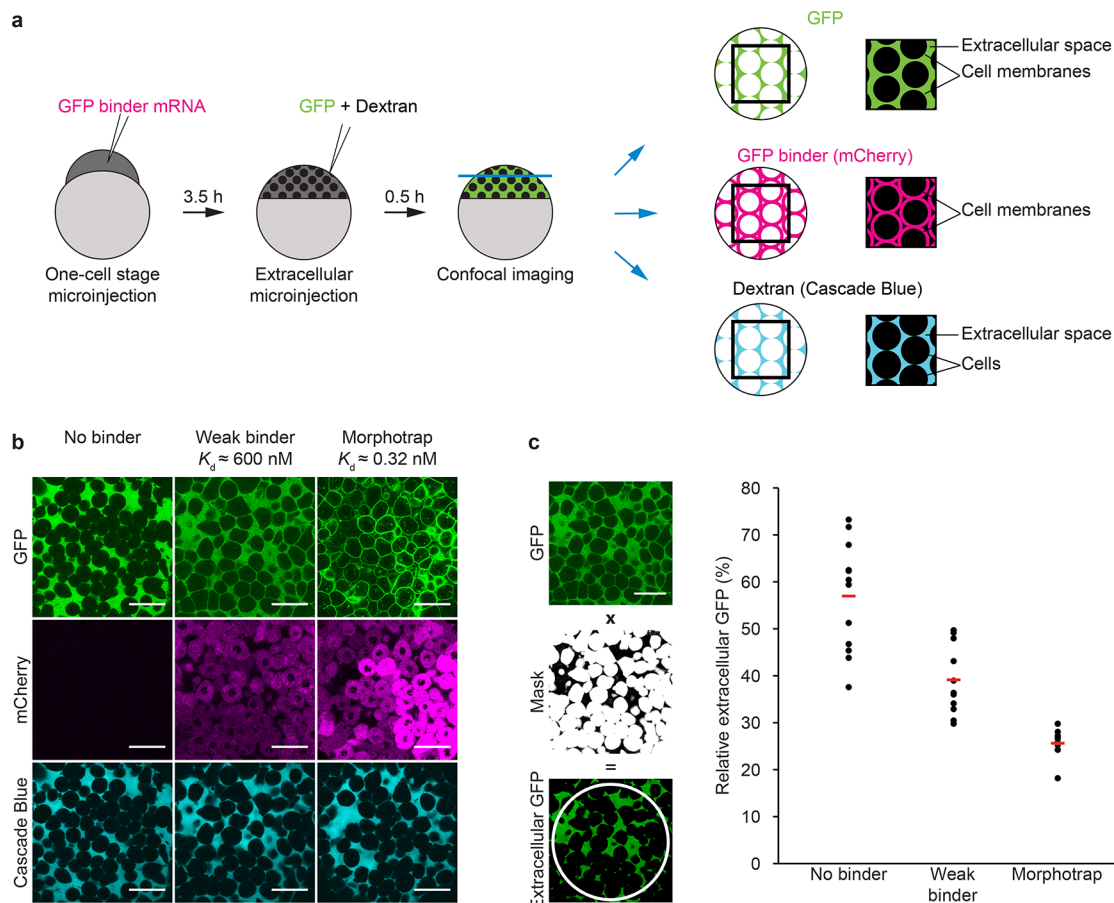


Figure 2. A low-affinity GFP binder partially tethers extracellular GFP to cell membranes in zebrafish embryos. (a) Schematic of the localization assay. GFP binders were expressed in zebrafish embryos by microinjecting 100 pg of the corresponding mRNAs at the one-cell stage. After 3.5 h of embryonic development, GFP and a fluorescent dextran were injected extracellularly followed by confocal microscopy to determine the localization of GFP, the GFP binder (mCherry), and dextran (Cascade Blue). The panel on the right illustrates the localization of the three fluorescent signals shown in panel b. (b) Without GFP binders, GFP is distributed homogeneously in the extracellular space. In embryos expressing the weak GFP binder, GFP can be detected both on cell membranes and in the extracellular space. In the presence of the morphotrap, the majority of GFP localizes to cell membranes. Scale bars correspond to 50 μ m. (c) A mask was created from the extracellular dextran signal and used to extract the GFP signal in cell-free areas within a circular region of interest (ROI, white). The graph shows measurements of extracellular GFP normalized to total GFP in the ROI from single embryos (black dots). Red lines indicate mean values. The scale bar corresponds to 50 μ m.

mCherry (Figure 1a, refs 19 and 20). The morphotrap binds extracellular GFP-tagged signaling molecules and thus tethers the signal to the cell membrane. In a tissue expressing the morphotrap, this presumably transient GFP–nanobody interaction slows the long-distance transport of the extracellular GFP-tagged population, resulting in a reduced “effective” diffusivity.^{4,21,22} In the context of development, where signaling typically occurs in a defined time frame, decreasing the signal mobility results in a reduced signaling range, shortening the spatial domain in which signaling is active. Thus, the morphotrap provides a means to directly test the effect of decreasing the spatial range of GFP-tagged signaling molecules on the development of living systems.

Three examples have shown how the morphotrap can be used with GFP-tagged signals to test the importance of their mobility for biological functions. First, in the developing fly wing, Dpp-GFP normally exhibits a graded distribution away from producing cells, but co-expression of the morphotrap in these source cells abolished Dpp-GFP spreading¹⁹ (Figure 1b) and resulted in a loss of Dpp-dependent signaling outside of the source. Second, a morphotrap was used in

Caenorhabditis elegans to tether a fluorescently labeled Wnt homolog to membranes and prevent its extracellular spreading, resulting in a loss of cell migration.²³ Third, we have recently used the morphotrap in living zebrafish embryos to drastically slow the spreading of the normally highly mobile Nodal antagonist Lefty1-GFP, which leads to defective body size scaling after experimental shortening²⁴ (Figure 1c). The strong effect on the mobility of GFP fusion proteins can be explained by the high-affinity anti-GFP nanobody used in the morphotrap (*in vitro* dissociation constant K_d of approximately 0.32 nM¹⁷). However, to understand the extent to which signal diffusion determines the range over which the signal acts, it is crucial to decrease signal mobility in a gradual, fine-tuned manner.

To enable fine-tuned regulation of signaling molecule diffusivity, we have generated an alternative GFP binder by swapping the morphotrap nanobody¹⁹ with an anti-GFP nanobody that has a dissociation constant of 600 nM *in vitro*.²⁵ The lower affinity of this GFP binder should result in a reduced degree of GFP membrane tethering compared to the morphotrap and thus a weaker effect on overall GFP

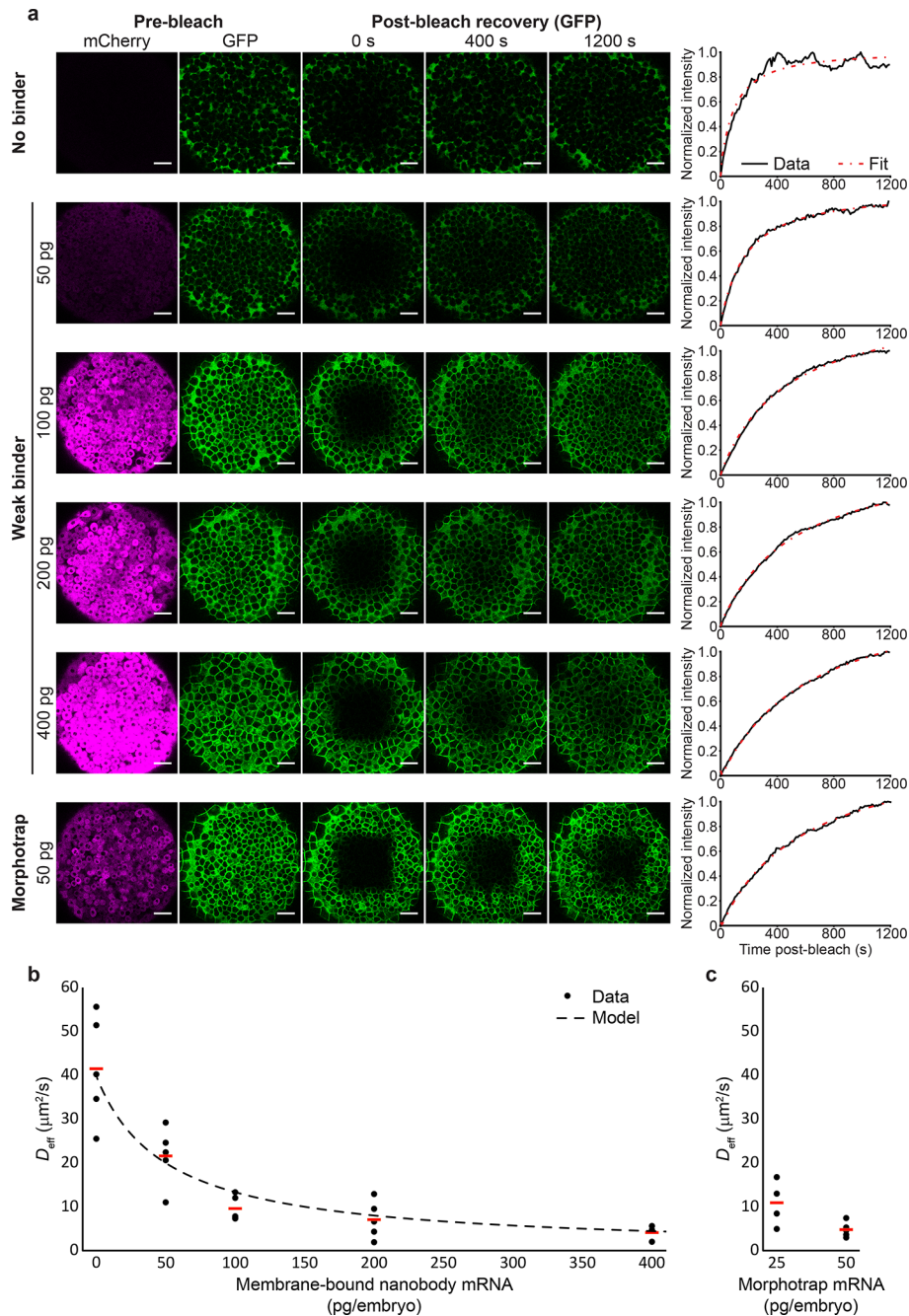


Figure 3. Titration of a low-affinity GFP binder modulates the mobility of extracellularly injected GFP in zebrafish embryos. (a) Different amounts of mRNA encoding the weak binder ($K_d = 600 \text{ nM}$ *in vitro*) were injected into zebrafish embryos at the one-cell stage (50, 100, 200, or 400 pg); negative controls were left uninjected (0 pg of mRNA), and positive controls were injected with 50 pg of mRNA encoding the morphotrap ($K_d = 0.32 \text{ nM}$ *in vitro*). Before the embryos were mounted for FRAP experiments at blastula stages, they were injected extracellularly with approximately 100 pg of recombinant GFP. FRAP experiments were performed as previously described^{8,11} and analyzed using PyFRAP.²⁷ Scale bars correspond to 50 μm . (b and c) The effective diffusion coefficients (D_{eff}) of independent experiments executed as described for panel a are shown as black dots, and red lines indicate mean values. The dashed line in panel b shows an overlay with the effective diffusion model calculated from the equation in Figure 1d (see the Supporting Information for details).

mobility. Binding of GFP by membrane-tethered binders can be described by a second-order chemical reaction (Figure 1d). Because the binder and the GFP–binder complex are immobilized on the cell surface, formation of the GFP–binder complex decreases the amount of free GFP diffusing with the molecular diffusion coefficient D_{free} . If k_{on} and k_{off} (Figure 1d) of the GFP binding reaction are fast, the effective diffusion coefficient D_{eff} which describes GFP mobility over tissue-wide scales, is decreased in the presence of the binder.

D_{eff} depends on the concentration of the binder and the dissociation constant $K_d = k_{\text{off}}/k_{\text{on}}$ of the binding reaction^{4,21,22,26} (Figure 1d).

The nanobody LaG-42 is a well-characterized GFP binder with an *in vitro* K_d of approximately 600 nM.²⁵ To test the effect this low-affinity GFP binder has on the localization of extracellular GFP, we expressed the weak GFP binder or the original morphotrap in zebrafish embryos and subsequently injected recombinant GFP into the extracellular space (Figure

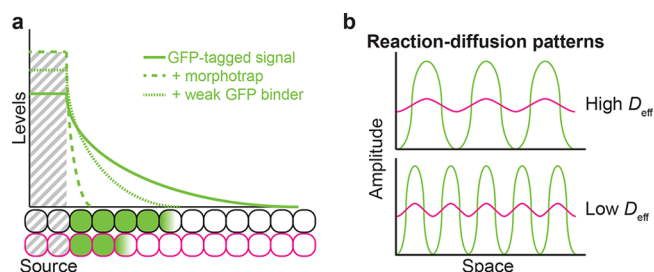


Figure 4. Potential future applications of weak GFP binders. (a) Homogeneous expression of weak GFP binders is predicted to moderately shorten gradients of GFP-tagged signaling molecules. (b) Potential use of weak GFP binders to change the characteristic wavelength of reaction–diffusion patterning systems.

2a). Assuming that the total levels of GFP and its binders are similar between experiments, a higher concentration of free GFP is expected for the weak binder compared to the morphotrap. Indeed, extracellular GFP levels were higher for the binder with a higher K_d in measurements in which independent masks for the extracellular space were used (see Figure 2b,c and the Supporting Information).

The relationship between D_{eff} , D_{free} , and binder levels in the equation of Figure 1d predicts that weak GFP binders could be used to fine-tune the effective diffusivity of extracellular GFP by using different binder concentrations. To test this prediction, we injected different amounts of mRNA encoding the low-affinity GFP binder into zebrafish embryos at the one-cell stage. Following extracellular injections of GFP at blastula stages, we performed FRAP experiments^{8,11} and determined the resulting effective diffusion coefficients.^{27,28} Our results show that the mobility of extracellular GFP can be fine-tuned by expressing different levels of the weak GFP binder (Figure 3). In good agreement with previous measurements in zebrafish embryos,^{11,27} we found a mean effective diffusion coefficient of $42 \mu\text{m}^2/\text{s}$ for extracellular GFP in the absence of a GFP binder. Strikingly, this effective diffusivity was reduced stepwise after microinjection of 50, 100, 200, and 400 pg of mRNA encoding the weak binder to 22, 10, 7, and $4 \mu\text{m}^2/\text{s}$, respectively (Figure 3b). In contrast, just 50 pg of morphotrap-encoding mRNA reduced the effective GFP diffusivity to $5 \mu\text{m}^2/\text{s}$ (Figure 3c). Although a reduced level of morphotrap expression resulted in a higher GFP mobility (Figure 3c), fine-tuning GFP mobility using even lower morphotrap expression levels may be difficult. GFP binders are expected to saturate more easily at low levels, and free diffusion may then dominate fluorescence recovery.²² Interestingly, the difference in GFP mobilities with 50 pg of morphotrap mRNA [$D_{eff} \approx 5 \mu\text{m}^2/\text{s}$ (Figure 3c)] and 50 pg of weak binder mRNA [$D_{eff} \approx 22 \mu\text{m}^2/\text{s}$ (Figure 3b)] was weaker than expected on the basis of the *in vitro* dissociation constants, which differ by a factor of 2000. It is therefore possible that the *in vivo* dissociation constants of the nanobodies in zebrafish embryos are different from the values measured *in vitro*,^{17,25} and fluorescence correlation spectroscopy (FCS) measurements might be able to dissect these potential differences in future experiments.^{14,29,30}

Controlled expression of a GFP binder allowed us to tune the effective diffusivity of extracellular GFP over an order of magnitude, whereas alternative approaches that change the molecular weight of the attached fluorophore would be

expected to have a much smaller effect size. Since the free diffusion of spherical molecules is proportional to their radii, even tripling the number of attached GFP molecules would at most lead to a 30% reduction in diffusivity,^{3,11} for instance. To gradually reduce the mobility of a GFP-tagged signaling molecule, it is theoretically also possible to use distinct GFP binders with different affinities. In our experience, however, there are two caveats when comparing different GFP binders in combination with a GFP fusion protein. First, GFP binders can reduce the biological activity of a GFP fusion protein, depending on the nanobody used. Second, nanobody binding can increase or decrease GFP fluorescence.³¹ We therefore chose to titrate a single GFP binder to obtain a gradual reduction of GFP mobility (Figure 3).

Long-range diffusion of ligands is responsible for the propagation of signaling in classical models of tissue patterning.^{32,33} However, the requirement of signal mobility for patterning has so far only in a few cases been directly tested by tethering extracellular signaling molecules to cell membranes with a strong GFP binder.^{19,20,23,24} Our proof-of-principle experiments demonstrate that a weak GFP binder can be used to reduce the effective diffusivity of extracellular GFP in a tunable manner between 2- and 10-fold. We expect that this control over signal mobility will allow the range of GFP-tagged signals to be shortened in future experiments, allowing a functional assessment of the extent to which diffusion controls signaling range during development (Figure 4a). Furthermore, this tool could be used to probe previously postulated self-organizing reaction–diffusion patterning systems,^{11,34} whose characteristic wavelength should change with reduced effective diffusivities of the involved signaling molecules^{35–37} (Figure 4b). For biological processes that are controlled by intracellular reaction kinetics, low-affinity anti-GFP nanobodies could be incorporated into the intracellular morphotrap²⁰ to generate weak binders that modulate the mobility of cytoplasmic proteins. For example, the kinetics of Pom1-GFP gradient formation in *Schizosaccharomyces pombe* could be perturbed to test the importance of Pom1-GFP distribution for symmetric cytokinesis.³⁸ Finally, purification of weak GFP binders might find useful applications in *in vitro* reaction–diffusion networks, such as the Min system,^{39–41} to modulate pattern formation processes.

To date, nanobodies have been generated against various proteins and together with other types of small protein binders (recently reviewed in ref 42) could be used to alter the mobility of several signaling molecules. Future experiments combining these tools have the potential to revolutionize *in vivo* studies by testing the importance of signal diffusion in various biological settings.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b01150.

Experimental procedures (PDF)

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Notes

The authors declare no competing financial interest.

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