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### ► To cite this version:

Mathias Heltberg, Judith Miné-Hattab, Angela Taddei, Aleksandra Walczak, Thierry Mora. Physical observables to determine the nature of membrane-less cellular sub-compartments. *eLife*, 2021, 10, <10.7554/eLife.69181>. <hal-03815448>

**HAL Id: hal-03815448**

**<https://hal.science/hal-03815448v1>**

Submitted on 18 Oct 2022

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# Physical observables to determine the nature of membrane-less cellular sub-compartments

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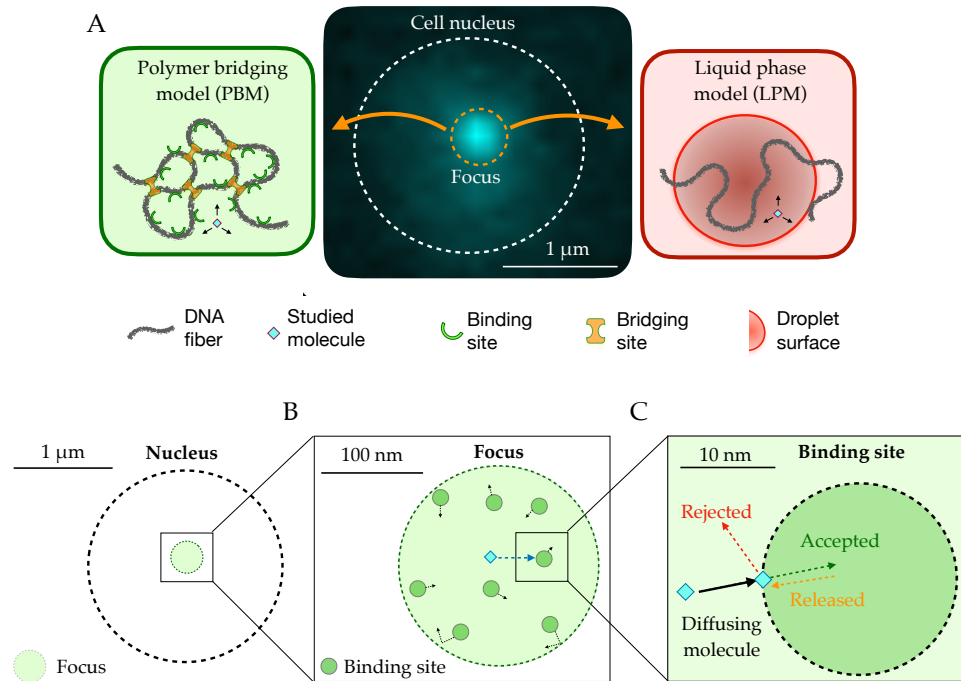
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**Abstract** The spatial organization of complex biochemical reactions is essential for the regulation of cellular processes. Membrane-less structures called foci containing high concentrations of specific proteins have been reported in a variety of contexts, but the mechanism of their formation is not fully understood. Several competing mechanisms exist that are difficult to distinguish empirically, including liquid-liquid phase separation, and the trapping of molecules by multiple binding sites. Here we propose a theoretical framework and outline observables to differentiate between these scenarios from single molecule tracking experiments. In the binding site model, we derive relations between the distribution of proteins, their diffusion properties, and their radial displacement. We predict that protein search times can be reduced for targets inside a liquid droplet, but not in an aggregate of slowly moving binding sites. We use our results to reject the multiple binding site model for Rad52 foci, and find a picture consistent with a liquid-liquid phase separation. These results are applicable to future experiments and suggest different biological roles for liquid droplet and binding site foci.

## Introduction

The cell nucleus of eukaryotic cells is not an isotropic and homogeneous environment. In particular, it contains membrane-less sub-compartments, called foci or condensates, where the protein concentration is enhanced for certain proteins. Even though foci in the nucleus have been observed for a long time, the mechanisms of their formation, conservation and dissolution are still debated (Strom *et al.*, 2017; Altmeyer *et al.*, 2015; Larson *et al.*, 2017; Patel *et al.*, 2015; Boehning *et al.*, 2018; Pessina *et al.*, 2019; McSwiggen *et al.*, 2019a,b; Oshidari *et al.*, 2020; Gitler *et al.*, 2020; Erdel *et al.*, 2020). An important aspect of these sub-compartments is their ability to both form at the correct time and place, and also to dissolve after a certain time. One example of foci are the structures formed at the site of a DNA double strand break (DSB) in order to localize vital proteins for the repair process at the site of a DNA break (Lisby *et al.*, 2001). Condensates have also been reported to be involved in gene regulation (Hnisz *et al.*, 2017; Bing *et al.*, 2020) and in the grouping of telomeres in yeast cells (Meister and Taddei, 2013; Ruault *et al.*, 2021). More generally, a vast number of membrane-less cellular sub-compartments that have been reported in the literature with different names. Here, we consider a focus to be a spherical condensate of size smaller than a few hundreds nanometers.

Different hypotheses have been put forward to explain focus formation in the context of chromatin, among which two main ones (discussed in the particular context of DSB foci in (Miné-Hattab and Taddei, 2019)): the Polymer Bridging Model (PBM) and the Liquid Phase Model (LPM). The Polymer Bridging Model is based on the idea that specific proteins form bridges between different chromatin loci by creating loops or by stabilizing interactions between distant loci on the DNA (Fig. 1A, left). These interactions can be driven by specific or multivalent weak interactions between chromatin binding proteins and chromatin components. In this case, the existence of sub-compartments relies on both the binding and bridging properties of these proteins. By contrast, the LPM posits that membrane-less sub-compartments arise from a liquid-liquid phase separation. In this picture, first proposed for P granules involved in germ cell formation (Brangwynne *et al.*,



**Figure 1. Schematic setup of the models.** **A.** In the middle, the observed signal from a fluorescently tagged Rad52 protein inside the nucleus following a double strand break. Left: Schematic figure showing the Polymer Bridging Model (PBM). Proteins binding specifically to the chromatin stabilize it, effectively trapping the motion of other molecules. Right: Schematic figure showing the Liquid Phase Model (LPM). Liquid-liquid phase separation results in the formation of a droplet foci with a different potential and different effective diffusion properties than outside the droplet. **B.** Details of the PBM model. Particles diffuse freely with diffusivity  $D_n$  until they hit one of the  $N$  spherical binding sites, themselves diffusing with diffusivity  $D_b$ . The focus is formed due a high concentration of binding sites. The binding sites are only partially absorbing, so that not all collision events result in a binding event. Once bound, the particle stays attached to the binding site, and then unbinds with rate  $k_-$ .

2009), proteins self-organize into liquid-like spherical droplets that grow around the chromatin fiber, allowing certain molecules to become concentrated while excluding others (Fig. 1A, right).

Although some biochemical and wide field microscopy data support the LPM hypothesis for DSB foci (Altmeyer et al., 2015; Larson et al., 2017; Strom et al., 2017; McSwiggen et al., 2019a), these observations are at the optical resolution limit, and a more direct detection of these structures is still missing. Coarse-grained theoretical models of the LPM exist (Statt et al., 2020; Grmela and Öttinger, 1997), but predictions of microscale behaviour that can be combined with a statistical analysis of high resolution microscopy data to discriminate between the hypotheses has not yet been formulated. Previously, we analyzed in detail single-particle tracking data in the context of yeast DSB foci (Miné-Hattab et al., 2021). We found that the behaviour of Rad52 foci was consistent with a liquid droplet, based on several observations, including the diffusion coefficient of proteins inside the focus relative to that of the whole focus, the size of the focus following 2 double-strand breaks, and its dissolution upon adding aliphatic alcohol hexanediol.

Here, we build a general physical framework for understanding and predicting the behaviour of each model under different regimes. The framework is general and applicable to many different types of foci, although we chose to focus on the regime of parameters relevant to yeast DSB foci, for which we can directly related our results to experimental measurements. While the LPM and PBM models have often been presented in the literature as opposing views, here we show under what conditions the PBM may be reduced to an effective description that is mathematically equivalent to the LPM, but with specific constraints linking its properties. We discuss the observables of the LPM and PBM and derive features that can be used to discriminate these two scenarios.

## 71 Results

### 72 Two models of foci

73 To describe the situation measured in single particle tracking experiments, we consider the diffusive  
74 motion of a single molecule within the nucleus of a cell in the overdamped limit, described by the  
75 Langevin equation in 3 dimensions (using the Itô convention, as we will for the rest of this work):

$$d\mathbf{r} = dt \left[ \nabla D(\mathbf{r}) - \frac{D(\mathbf{r})}{k_B T} \nabla U(\mathbf{r}) \right] + \sqrt{2D(\mathbf{r})} d\mathbf{W}, \quad (1)$$

76 where  $\mathbf{W}$  is a 3-dimensional Wiener process,  $U(\mathbf{r})$  is the potential exerted on the particle, and  $D(\mathbf{r})$   
77 is a position-dependent diffusion coefficient. The  $\nabla U$  term corresponds to a force divided by the  
78 drag coefficient  $k_B T / D(\mathbf{r})$ , which is given in terms of  $D$  and temperature according to Einstein's  
79 relation. The  $\nabla D$  term comes from working within the Itô convention. The steady state distribution  
80 of particles is given by the Boltzmann distribution:

$$p(\mathbf{r}) = \frac{1}{Z} \exp \left[ -\frac{U(\mathbf{r})}{k_B T} \right], \quad (2)$$

81 where  $Z$  is a normalization constant.

In the LPM, we associate the focus with a liquid droplet characterized by a sudden change in  
the energy landscape. We model the droplet as a change in the potential  $U(r)$ , and a change in  
the diffusion coefficient  $D(r)$  inside the droplet focus compared to the diffusion coefficient in the  
rest of the nucleus  $D_n$ . We assume both the diffusion coefficient and the potential are spherically  
symmetric around the center of the focus, and have sigmoidal forms:

$$D(r) = D_0 + \frac{D_n - D_0}{1 + e^{-b(r-r_f)}}, \quad (3)$$

$$U(r) = \frac{A}{1 + e^{-b(r-r_f)}}, \quad (4)$$

82 where  $D_0$  is the diffusion coefficient inside the focus,  $r_f$  is the radial distance to the center of  
83 the focus, and the coefficients are defined in Table 1. Different relations between the diffusion  
84 coefficient and the surface potential are possible.

In the PBM we describe the dynamics of particles using a model where the focus has  $N$  binding  
sites, each of which is a partially reflecting sphere (Bryan, 1891; Duffy, 2015; Carslaw and Jaeger,  
1992) with radius  $r_b$  (Fig. 1B and C). Binding sites can themselves diffuse with diffusion coefficient  
 $D_b$ , and are confined within the focus by a potential  $U_b(\mathbf{r})$ , so that their density is  $\rho(\mathbf{r}) \propto e^{-U_b(\mathbf{r})}$   
according to the Boltzmann distribution. While not bound, particles diffuse freely with diffusion  
constant  $D_n$ , even when inside the focus. However, the movement of the particle is affected by  
direct interactions with the binding sites. Binding is modeled as follows. As the particle crosses  
the spherical boundary of a binding site during an infinitesimal time step  $dt$ , it gets absorbed with  
probability  $p_b = \kappa \sqrt{\pi dt / D_n}$  (Fig. 1C), where  $\kappa$  is an absorption parameter consistent with the Robin  
boundary condition at the surface of the spheres,  $D\mathbf{n} \cdot \nabla p(\mathbf{x}) = \kappa p(\mathbf{x})$  (Erban and Chapman, 2007;  
Singer et al., 2008), where  $\mathbf{x}$  is a point on the surface of the sphere, and  $\mathbf{n}$  is the unit vector normal  
to it.

87 While bound, particles follow the motion of their binding site, described by:

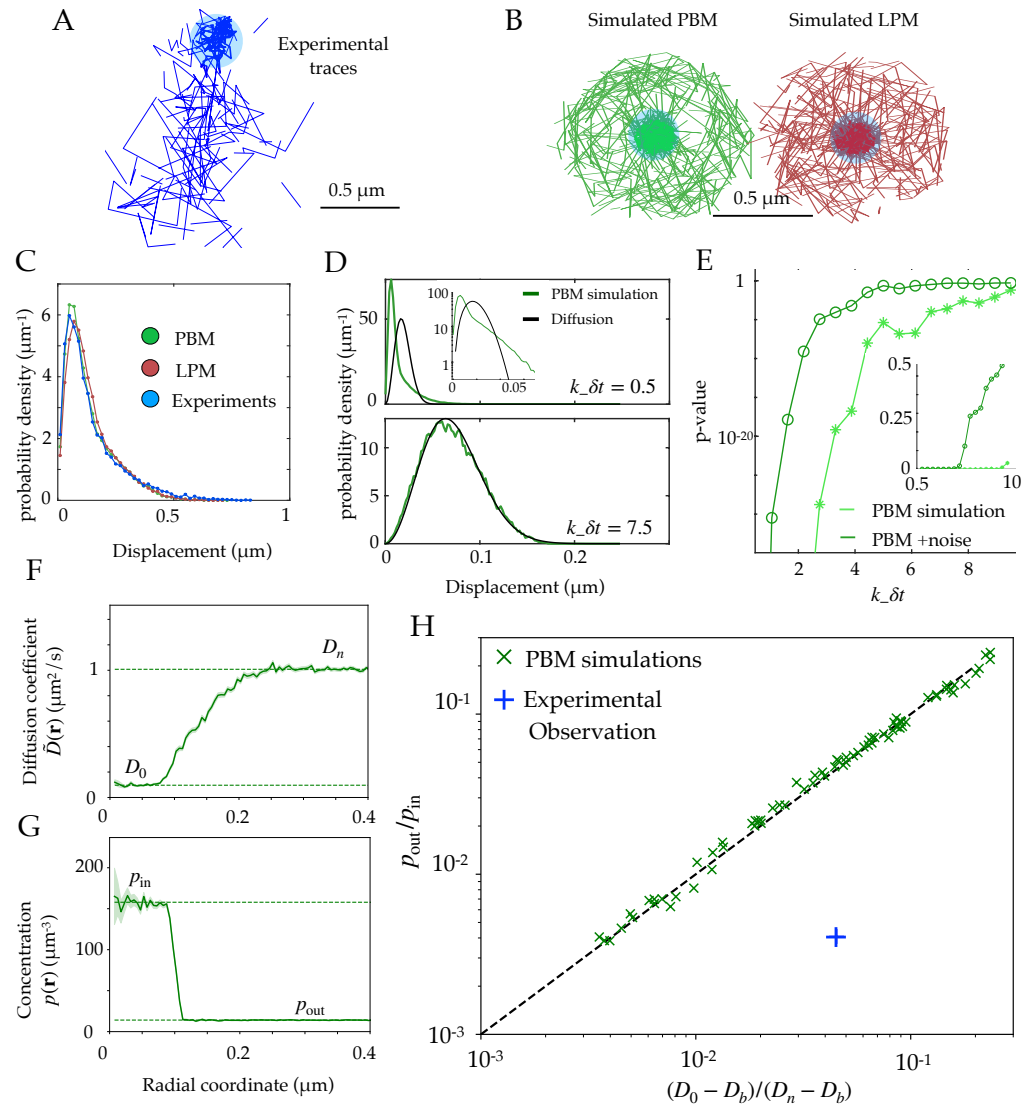
$$d\mathbf{r} = -dt \frac{D_b}{k_B T} \nabla U_b(\mathbf{r}) + \sqrt{2D_b} d\mathbf{W}, \quad (5)$$

88 where  $\mathbf{W}$  is a 3-dimensional Wiener process. A bound particle is released with a constant rate  $k_-$ .  
89 Since the potential  $U_b$  is constant within the bulk and its only function is to keep binding sites within  
90 the focus, the PBM can be described by 5 parameters:  $N$ ,  $r_b$ ,  $D_b$ ,  $\kappa$  and  $k_-$ . Their typical values can  
91 be found in Table 1.

### 102 Comparison between simulated and experimental traces

103 In recent experimental work (Miné-Hattab et al., 2021), we used single particle tracking to follow  
104 the movement of Rad52 molecules, following a double-strand break in *S. cerevisiae* yeast cells, which  
105 causes the formation of a focus. These experiments show that temporal traces of Rad52 molecules  
106 concentrate inside the focus, as shown for a representative cell in Fig. 2A.

107 Using both the PBM and LPM models described above, we can construct traces that look  
108 similar to the data (Fig. 2B). To mimic the data, we only record and show traces in two dimensions  
109 and added detection noise corresponding to the level reported in the experiments (Miné-Hattab



**Figure 2. Diffusion properties and effective free energy.** **A.** Example of experimental tracking of Rad52 molecules visiting a double-strand break (DSB) focus. Different connected traces correspond to distinct Rad52 molecules. **B.** Example trajectory of a particle visiting the focus from simulations in the PBM (left) and LPM (right). The simulated trajectories are visually similar to the data in B. **C.** Displacement histogram (jump sizes) for the PBM, LPM and experiments, for an interval  $\delta = 20$  ms. **D.** Displacement histogram for the PBM for small values of  $k_- \delta t$  (top) and high values (bottom). Here we varied the interval from  $\delta t = 1$  ms (top) to  $\delta t = 15$  ms (bottom). **E.** Hypothesis testing using a two sided KS-test, comparing the displacement histogram of a free diffusion process (black line in D) and the displacement histogram of diffusion inside the focus (green line in D). Parameters are the same as in D.  $\delta t$  was varied from 1 to 25 ms. **F.** Effective diffusion coefficient as a function of distance to the focus center  $r$ , estimated from simulations of the PBM calculating  $\bar{D} = \langle \delta \mathbf{r}^2 \rangle / (2d \delta t)$  in each radial segment. **G.** Particle density  $p(r)$  as a function of  $r$ , estimated from simulations of the PBM. Error bars are standard errors on the mean. **H.** Relation between the ratio  $(D_0 - D_b)/(D_n - D_b)$  versus the ratio of densities inside and outside the focus (From Eq. 11), both estimated from simulations of the PBM (green crosses), compared to the identity prediction (Eq. 12, black line). Blue cross shows the experimental observation for Rad52 in DSB loci (see text related to Fig. 7 and Table S1 in *Miné-Hattab et al. (2021)*). Parameter values as in Table 1 except:  $r_n = 1 \mu\text{m}$  for B;  $A = 2.5 k_B T$  for B-C;  $r_n = 0.3 \mu\text{m}$ ,  $r_f = 0.15 \mu\text{m}$  and  $D_n = 0.5 \mu\text{m}^2/\text{s}$  for D-E,  $\kappa = 300 \mu\text{m}/\text{s}$  for D,  $r_n = 0.75 \mu\text{m}$  for F-H. In H we varied  $\kappa = 1-400 \mu\text{m}/\text{s}$ ,  $k_- = 1-1,500 \text{s}^{-1}$ , and  $\rho = 2.4-4.8 \cdot 10^4 \mu\text{m}$ .

**Figure 2-Figure supplement 1. Effect of crowding.**

Variable	Model	Description	Value	Range	Exp. value	Units
$r_f$	both	radius of focus	100	50-200		nm
$r_n$	both	radius of nucleus	500	300-1000		nm
$D_n$	both	Diffusion coefficient in nucleus	1.0	0.5-2.0	1.08	$\mu\text{m}^2/\text{s}$
$\sigma$	both	Experimental noise level	30	30	30	nm
$D_0$	LPM	Diffusion coefficient inside droplet	0.05	0.01-0.5	0.032	$\mu\text{m}^2/\text{s}$
$A$	LPM	Surface potential	5.0	0-10	5.5	$k_B T$
$b$	LPM	Steepness in potential	1000	500-10000		$\mu\text{m}^{-1}$
$\rho$	PBM	Density of binding sites inside focus	$4.8 \cdot 10^4$	$1 \cdot 10^3$ - $8.4 \cdot 10^4$		$\mu\text{m}^{-3}$
$D_b$	PBM	Diffusion coefficient of binding sites	0.005	0-0.1	0.005	$\mu\text{m}^2 \text{s}^{-1}$
$r_b$	PBM	Radius of binding sites	10	5-20		nm
$k_-$	PBM	Unbinding rate	500	10-10000		$\text{s}^{-1}$
$\kappa$	PBM	Absorption parameter	100	0-1000		$\mu\text{m}/\text{s}$

**Table 1.** Parameters used in this study with their typical values, and the ranges we have considered. Experimental values are from (Miné-Hattab *et al.*, 2021) (see Materials and Methods for details on estimating diffusion coefficients and free energy differences).  $D_0$  and  $A$  are model parameters in the LPM, but also effective observables in the PBM. The diffusivity of binding sites is taken to be that of Rfa1 molecules in the focus, which bind to single-stranded DNA in repair foci, and are thus believed to follow the diffusion of the chromatin (Miné-Hattab *et al.*, 2021). The number  $N$  of binding sites is related to their density  $\rho$  inside the focus through  $N = (4/3)\pi\rho r_f^3$ .

110 *et al.*, 2021). Based on these simulations, we gather the statistics of the particle motion to create  
 111 a displacement histogram representing the probability distribution of the observed step sizes  
 112 between two successive measurements. For this choice of parameters (see Fig. 2 caption), both of  
 113 the models and the experimental data look very similar (Fig. 2C).

114 In principle, we could have expected the displacement histogram of particles inside the bulk  
 115 of the focus (where traces are not close to the boundary) to look markedly different between the  
 116 PBM and the LPM. While the LPM should follow the prediction from classical diffusion (given by  
 117 a Gaussian radial distribution,  $p(|\delta\mathbf{r}|) \propto |\delta\mathbf{r}|^2 e^{-|\delta\mathbf{r}|^2/(4D\delta t)}$  for a small interval  $\delta t$  in the bulk), the PBM  
 118 prediction is expected to be in general non-Gaussian because of intervals during which the particle  
 119 is bound and almost immobile (as the chromatin or single-stranded DNA carrying the binding sites  
 120 moves very slowly), creating a peak of very small displacements. Simulations show that departure  
 121 from Gaussian displacements is most pronounced when the binding and unbinding rates are slow  
 122 compared to the interval  $\delta t$  (Fig. 2D, top), but is almost undetectable when they are fast (Fig. 2D,  
 123 bottom). With our parameters, the binding rate is  $k_+ \rho \approx 3,000 \text{ s}^{-1}$ , and  $k_-$  ranges from 10 to 10,000  
 124  $\text{s}^{-1}$ , with  $\delta t = 20 \text{ ms}$ . For comparison, assuming weak binding to DNA,  $K_d = k_+/k_- \approx 1 \mu\text{M}$  would  
 125 give  $k_- \sim 40 \text{ s}^{-1}$ , and assuming strong specific binding,  $K_d \sim 1 \text{ nM}$ , implies  $k_- \sim 0.04 \text{ s}^{-1}$ . We stress  
 126 that there is a lot of uncertainty in the values for experimentally measured rate constants, and  
 127 a recent study (Saotome *et al.*, 2018) found the dissociation constant  $k_d$  for Rad52 in yeast to  
 128 vary between two observed sites from 5.6 nM to 101 nM. Fig. 2E shows how the detectability of  
 129 non-Gaussian displacements gets worse as  $k_- \delta t$  increases, and is further degraded by the presence  
 130 of measurement noise.

131 The experimental findings of single Rad52 molecules in yeast repair foci (Miné-Hattab *et al.*,  
 132 2021) suggest that the movement inside the focus are consistent with normal diffusion and its  
 133 Gaussian distribution of displacements (Fig. 2C). This observation excludes a wide range of slow  
 134 binding and unbinding rates in the PBM, as this would lead to non-Gaussian statistics (Fig. 2D, top).  
 135 However, it does not rule out the PBM itself, which is undistinguishable from classical diffusion for  
 136 fast binding and unbinding rates (Fig. 2D, bottom). In addition, separating displacements inside the  
 137 focus from boundary-crossing ones can be very difficult in practice, and errors in that classification  
 138 may result in spurious non-Gaussian displacement distributions that would confound this test.  
 139 Therefore, it is important to find observables that can distinguish the two underlying models.

### 140 Effective description of the Polymer Bridging Model

141 Motivated by experimental observations, we want to find a coarse-grained description of the PBM  
 142 that can be reduced to a classical diffusion process under an effective potential and with an effective  
 143 position-dependent diffusivity, and relate its parameters to the properties of the binding sites. To  
 144 do so we analyze the PBM in a mean-field approximation, which is valid in the limit where binding  
 145 and unbinding events are fast relative to the traveling time of the particles. In this regime, a particle  
 146 rapidly finds binding sites with rate  $k_+ \rho(\mathbf{r})$  (where  $\rho(\mathbf{r})$  is the density of binding sites) and unbinds  
 147 from them with rate  $k_-$ . While in principle rebinding events complicate this picture, it has been

148 showed that the period where rebindings to the same binding site occurs can be included in the time  
 149 they are bound, and thus can be renormalized into a lower effective unbinding rate (*Kaizu et al.,*  
 150 **2014**). Assuming that interactions between binding sites do not affect their binding to the particle  
 151 of interest, the binding rate can be approximated in the presence of partially reflecting binding sites  
 152 by the Smoluchowski rate (*Nadler and Stein, 1996; Berezkhovskii et al., 2019*) (Appendix 1):

$$k_+ = \frac{4\pi D_n r_b}{1 + \frac{D_n}{r_b \kappa}}. \quad (6)$$

153 If the processes of diffusion, binding, and unbinding are in equilibrium, the steady state distribution  
 154 of a particle can be derived using the Boltzmann distribution. The equilibrium assumption is  
 155 justified by the fact that our time of observation is much smaller than the time scales of focus  
 156 formation, and that the focus is of constant size during our observations. It is possible that active  
 157 fluctuations are present inside the focus, but the Rad52 molecules that we are observing are not  
 158 actively involved in the chemical reactions that take place over the measurement timescale. In  
 159 this sense Rad52 can be considered a passive agent, and this description is therefore an effective  
 160 description of its motion inside the focus. This is supported by the fact that the Rad52 diffusion  
 161 properties look constant across our observation period.

162 At each position  $\mathbf{r}$ , the unbound state is assigned weight 1, and the bound state weight  $\rho(\mathbf{r})/K_d$ ,  
 163 where  $K_d = k_-/k_+$  is the dissociation constant. Then the probability distribution of the particle's  
 164 position is given by:

$$p(\mathbf{r}) \propto \left(1 + \frac{\rho(\mathbf{r})}{K_d}\right) = \frac{1}{p_u(\mathbf{r})}, \quad (7)$$

165 where

$$p_u(\mathbf{r}) = \frac{k_-}{k_- + k_+ \rho(\mathbf{r})} \quad (8)$$

166 is the probability of being unbound conditioned on being at position  $\mathbf{r}$ .

Here we assume that binding and unbinding is fast compared to variations of  $\rho(\mathbf{r})$  experienced  
 by the tracked particles in the measured time intervals. This assumption holds if the density of  
 binding sites is large, which is a fundamental assumption of the PBM. In this limit, the dynamics of  
 particles are governed by an effective diffusion coefficient, which is a weighted average between  
 the free diffusion of tracked molecules, and the diffusion coefficient of the binding sites:

$$\tilde{D}(\mathbf{r}) = p_u(\mathbf{r})D_n + (1 - p_u(\mathbf{r}))D_b = \frac{D_n k_- + D_b k_+ \rho(\mathbf{r})}{k_- + k_+ \rho(\mathbf{r})}. \quad (9)$$

167 Likewise, particles are pushed by an effective confinement force: when they are bound to binding  
 168 sites, they follow their motion which is confined inside of the focus. The resulting drift is given by  
 169 that of the binding sites, but weighted by the probability of being bound to them:

$$\begin{aligned} \langle d\mathbf{r} \rangle &= -dt(1 - p_u(\mathbf{r})) \frac{D_b}{k_B T} \nabla U_b(\mathbf{r}) \\ &= dt \left[ -\frac{\tilde{D}(\mathbf{r})}{k_B T} \nabla \tilde{U}(\mathbf{r}) + \nabla \tilde{D}(\mathbf{r}) \right], \end{aligned} \quad (10)$$

170 where in the second line we have rewritten the dynamics in terms of an effective potential  $\tilde{U}(\mathbf{r}) =$   
 171  $k_B T \ln(1 + k_+ \rho(\mathbf{r})/k_-)$ , using  $\rho(\mathbf{r}) \propto e^{-U_b(\mathbf{r})/k_B T}$ . Thus the effective dynamics may be described by the  
 172 Langevin equation of the same form as the LPM (1) but with the relation between  $\tilde{U}(\mathbf{r})$  and  $\tilde{D}(\mathbf{r})$   
 173 constrained by their dependence on  $\rho(\mathbf{r})$ :

$$\tilde{U}(\mathbf{r}) = k_B T \ln \left[ \frac{\tilde{D}(\mathbf{r}) - D_b}{D_n - D_b} \right], \quad (11)$$

174 with the convention that  $\tilde{U} = 0$  far away from the focus where  $\rho = 0$ . As a consistency check, one can  
 175 verify that the equilibrium distribution  $p \propto e^{-\tilde{U}/k_B T}$  gives back Eq. 7. Eq. 11 reveals a fundamental  
 176 relation about the dynamics of molecules inside the PBM, and is therefore an important fingerprint  
 177 to test the nature of foci.

## Scaling relation between concentration and diffusivity in the PBM

Experiments or simulations give us access to the *effective diffusivity* through the maximum likelihood estimator  $\tilde{D} = \langle \delta \mathbf{r}^2 \rangle / (2d \delta t)$ , where  $\delta t$  is the time between successive measurements,  $\delta \mathbf{r}$  is the measured displacement between two measurements, and  $d$  the dimension in which motion is observed. Within the PBM, Eq. 11 allows us to establish a general relation between the particle concentration  $p(r)$ , which can also be measured, and the effective diffusivity  $\tilde{D}$ , through:

$$p(\mathbf{r}) \propto \frac{1}{\tilde{D}(\mathbf{r}) - D_b}. \quad (12)$$

Typically in experiments we have  $D_b \ll \tilde{D} \ll D_n$ , in which case this relation may be approximated by  $p(\mathbf{r})\tilde{D}(\mathbf{r}) = \text{const.}$

We validated Eq. 12 in simulations of the PBM. We divided the radial coordinate  $r$  into small windows of  $10^{-3} \mu\text{m}$  and plotted the measured effective diffusion coefficient  $\tilde{D}(r)$ , as a function of  $r$  (Fig. 2F), as well as the density of tracked particles  $p(r)$  (Fig. 2G).  $\tilde{D}(r)$  takes an approximately constant value inside the focus, defined as  $D_0$  by analogy with the LPM, and is equal to  $D_n$  well outside the focus where diffusion is free. Likewise the density  $p(r)$  decreases from  $p_{\text{in}}$  inside to  $p_{\text{out}}$  outside the focus. Note that  $D_0$  in the PBM is not a free parameter, but rather emerges from the mean-field description and depends on the properties of binding site. We extracted those values numerically from the simulations. Fig. 2H shows that Eq. 12 predicts well the relationship between these 4 numbers, for a wide range of parameter choices of the PBM (varying  $\kappa$  from 1 to 400  $\mu\text{m}/s$ ,  $k_-$  from 5 to 1500  $s^{-1}$  and  $\rho$  from 23873 – 47746  $\mu\text{m}^{-3}$ , while keeping  $D_b = 5 \cdot 10^{-3} \mu\text{m}^2/s$  and the other parameters to values given by Table 1). While this relation was derived in the limit of fast binding and unbinding, it still holds for the slower rates explored in our parameter range (see Fig. 2H). However, it breaks down in the limit of strong binding, when we expect to see two populations (bound and unbound), making the effective diffusion coefficient an irrelevant quantity (see Fig. 2D).

We can compare this prediction to estimates from the experimental tracking of single Rad52 molecules in yeast repair foci (*Miné-Hattab et al., 2021*) (see Materials and Methods for details), assuming that the diffusivity of the binding sites is well approximated by that of the single-stranded DNA-bound molecule Rfa1, measured to be  $D_b = 5 \cdot 10^{-3} \mu\text{m}^2/s$ . This experimental point, shown as a blue cross in Fig. 2H, substantially deviates from the PBM prediction: Rad52 particles spend much more time inside the focus than would be predicted from their diffusion coefficient based on the PBM. To agree with the data, the diffusion coefficient of binding sites would have to be increased to  $D_b = 0.0314 \mu\text{m}^2/s$ , which is almost an order of magnitude larger than what was found in experiments. The existence of multiple binding sites could in principle lead to an enhanced level of molecular crowding. This would in fact decrease the effective diffusion coefficient inside the focus, moving points of the PBM simulations in Fig. 2H to the left, further away from the experimental observation. However we checked numerically that this effect was small, by adding inert spheres of the same size as the binding sites to generate crowding (Fig. 2-Figure supplement 1).

## Diffusion coefficient and concentration predict boundary movement in the PBM

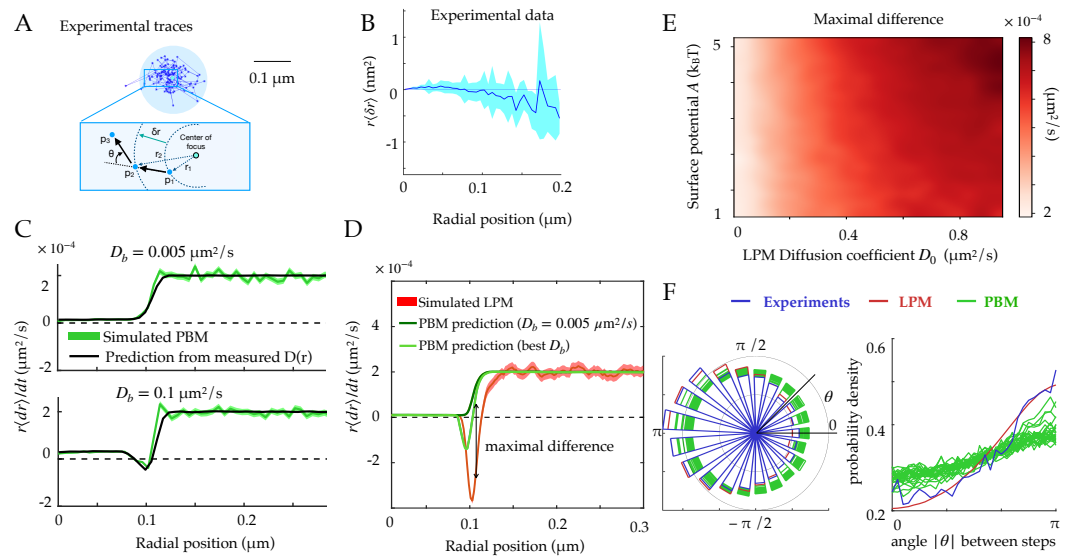
Another observable that is accessible through simulations and experiments is the radial displacement near the focus boundary. In practice, we gather experimental traces around the focus, and estimate the radius of the focus as shown in Fig. 3A. Using many traces, we can find the average radial displacement  $\langle \delta r \rangle$  during  $\delta t$ , as a function of the initial radial position of the particle  $r$  (Fig. 3B). Under the assumption of spherical symmetry, within the PBM this displacement is given by:

$$\begin{aligned} \langle \delta r \rangle &\simeq \delta t \left( -(1 - p_u(r)) \frac{D_b}{k_B T} \partial_r U_b(r) + \frac{\tilde{D}(r)}{r} \right) \\ &= \delta t \left( -\frac{\tilde{D}(r)}{k_B T} \partial_r \tilde{U}(r) + \partial_r \tilde{D}(r) + \frac{\tilde{D}(r)}{r} \right), \end{aligned} \quad (13)$$

where the term  $\tilde{D}/r$  comes from the change to spherical coordinates.

The first line of Eq. 13 shows that the average change in radial position of single particles  $\langle \delta r \rangle$  cannot be negative in the PBM for steady binding sites ( $D_b = 0$ ). This result does not hold for moving binding sites ( $D_b > 0$ ), as we will see below. This is reproduced in simulations, for different absorption probabilities, as shown in Fig. 3C.

By contrast, in the LPM there is no constraint on the sign of the displacement  $\langle \delta r \rangle$  since the relation between the diffusion coefficient and the surface potential is not constrained like in the PBM. Even when binding sites can move, this prediction can be used to falsify the PBM. Eq. 13 makes a prediction for the average radial displacement of the tracked molecule in the PBM, solely



**Figure 3. Radial and angular dynamics.** **A.** Experimental trace of a single Rad52 in a DSB focus (Miné-Hattab *et al.*, 2021). The inset shows the definition of the radial movement  $\delta r$ . Here concentric circles are shown to define the radius relative to the focus center, where a particle moves a specific distance away from the center of the focus, as well as the angle  $\theta$  between consecutive displacements. **B.** Data extracted from experiments (Miné-Hattab *et al.*, 2021) to estimate the average radial displacement of the tracked particle multiplied by the radius. Here and throughout the x-axis represents the radial position at the beginning of the timestep. Error bars are standard errors on the mean. **C.** Simulations showing the radial displacements in the PBM with slowly (top) and rapidly (bottom) moving binding sites. Black lines are predictions based on the measurement of  $\bar{D}$  (Eq. 12). Error bars are standard deviations on the mean. **D.** Radial displacement from simulations of the LPM. Light-green line shows the (wrong) prediction made while assuming the PBM, using the measurement of the effective diffusion coefficient  $\bar{D}(r)$  (Eq. 13). We call the discrepancy between data and the PBM prediction the “Maximal positive difference.” Error bars are standard errors on the mean. **E.** Heatmap showing the maximal positive difference in LPM simulations as a function of  $D_0$  and  $A$ . **F.** Distribution of angles (represented radially on the left, and linearly on the right) between the displacements of consecutive steps of length  $\delta r$ , from experiments and simulations. Multiple curves for the PBM correspond to different parameter choices corresponding to the points of Fig. 2H. Parameter values as in Table 1, except  $r_n = 1 \mu\text{m}$  for F. For F parameters are varied with  $\kappa = 100\text{--}300 \mu\text{m/s}$ ,  $k_- = 500\text{--}1,500 \text{s}^{-1}$ ,  $r_f = 0.1\text{--}0.14 \mu\text{m}$ .

**Figure 3-Figure supplement 1.** Asymmetry coefficient for an infinite PBM focus.

228 as a function of the diffusivity and concentration profiles  $\tilde{D}(r)$  and  $p(r)$ , using  $\tilde{U}(r) = k_B T \ln p(r)$ .  
 229 Accordingly, this prediction agrees well with simulations of the PBM (Fig. 3C).

230 Using Eq. 13 that is derived for the PBM, along with the definition of  $\tilde{U}$  as a function of  $\tilde{D}$  in  
 231 Eq. 11, to analyze a simulation of the LPM leads to large disagreement between the inferred and  
 232 true parameters. This PBM-based analysis underestimates the depth of the potential (Fig. 3D, green  
 233 lines compared to the red). It predicts a negative displacement  $\langle dr \rangle$  when  $D_b$  is inferred using the  
 234 PBM formula  $p_{in}/p_{out} = (D_n - D_b)/(D_0 - D_b)$ , although its magnitude is underestimated. But when  
 235 taking the experimental value of  $D_b = 0.005 \mu\text{m}^2/\text{s}$ ,  $\langle dr \rangle$  is always positive even at the boundary.  
 236 This spurious entropic “reflection” is an artifact of using the wrong model, since the distinct relation  
 237 between the observed diffusion coefficient and the equilibrium distribution for the PBM leads to a  
 238 specific shape around the boundary which is not the same for the LPM. The inference using the  
 239 PBM of such a positive displacement at the surface of the focus can therefore be used to reject the  
 240 PBM. Fig. 3E represents the magnitude of that discrepancy as a function of two LPM parameters  
 241 — diffusivity inside the droplet and surface potential — showing that the PBM is easier to reject  
 242 when diffusivity inside the focus is high.

243 In summary, the average radial diffusion coefficient can predict the radial displacement of  
 244 tracked molecules within the PBM, and deviations from that prediction can be used as a means to  
 245 reject the PBM using single-particle tracking experiments.

### 246 **Distribution of angles between consecutive time steps**

247 To go beyond the average radial displacement, we considered a commonly used observable to  
 248 study diffusive motion in complex environment: the distribution of angles between two consecutive  
 249 displacements in 2 dimensions. While this distribution is uniform for a homogenous environment  
 250 (*Liao et al., 2012*), it is expected to be asymmetric in presence of confinement and obstacles (*Izeddin*  
 251 *et al., 2014*).

252 We computed this distribution from simulations of the PBM and LPM, and compared them  
 253 to experiments in yeast repair foci (Fig. 3F), calculating the angle between the vector relating the  
 254 first two points and the vector relating the last two points. These distributions are all asymmetric,  
 255 with an enrichment of motion reversals (180 degree angles). Since the LPM assumes standard  
 256 diffusion within a potential, the asymmetry in that model can be entirely explained by the effect  
 257 of confinement, which tends to push back particles at the focus boundary. With the parameters  
 258 of Table 1, the LPM agrees best with the data, while the PBM shows a more moderate asymmetry  
 259 across a wide range of parameters. Therefore, both the LPM and the PBM are expected to show  
 260 asymmetric diffusion around the boundary of the focus, but one could expect that the PBM (and  
 261 not the LPM) revealed an additional asymmetry inside the bulk of the focus, due to the interactions  
 262 of the tracked molecules with the binding sites, which causes reflections and hinders motion. To  
 263 isolate this effect from boundary effects, we simulated the PBM in an infinite focus with a constant  
 264 density of binding sites (Fig. 3-Figure supplement 1) and found that this expectation is confirmed.  
 265 However, this asymmetry is seen only when the measurement time step is small or comparable to  
 266 the binding time. For finite foci, it must also be corrected for boundary effects. These difficulties  
 267 make the asymmetry criterion unfit to discriminate between the two models in the context of yeast  
 268 repair foci.

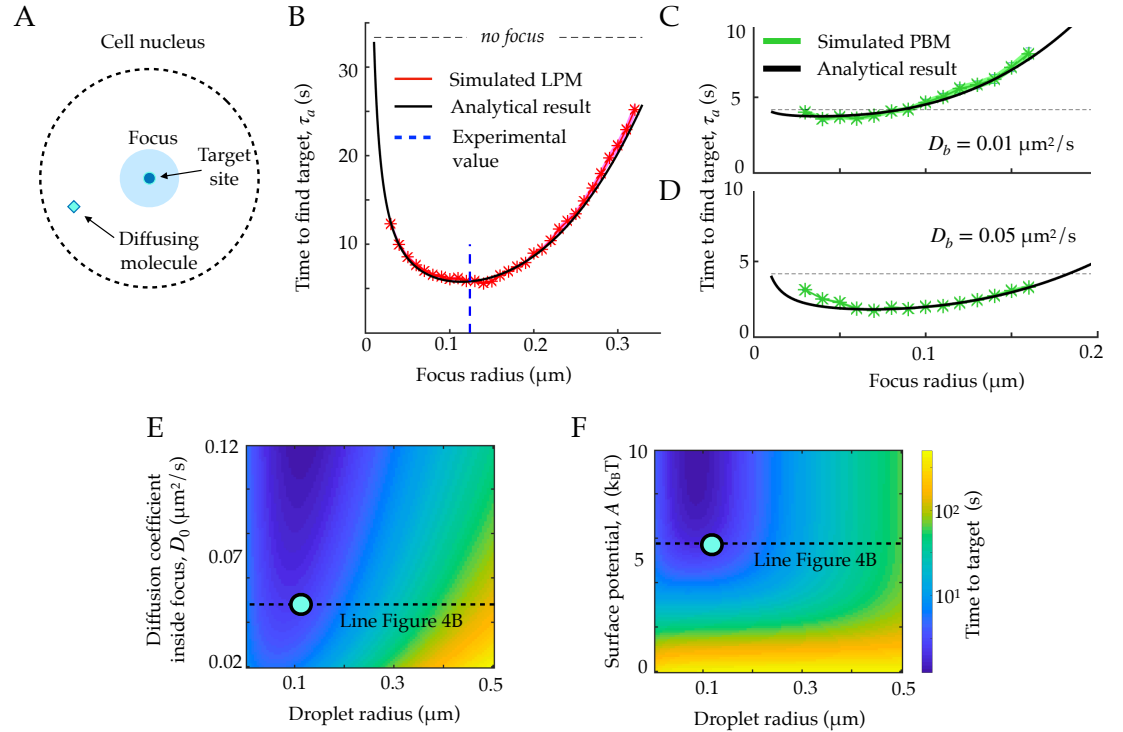
### 269 **Foci accelerate the time to find a target, but only moderately in the PBM**

270 Foci keep a higher concentration of molecules of interest within them through an effective potential.  
 271 We wondered if this enhanced concentration of molecules could act as a “funnel” allowing molecules  
 272 to find their target (promoter for a transcription factor, repair site, etc) faster.

273 To address this question, we consider an idealized setting with spherical symmetry, in which  
 274 the target is a small sphere of radius  $r_0$  located at the center of the focus, of radius  $r_f$  (Fig. 4A). We  
 275 further assume that the nucleus is a larger sphere of radius  $r_n$ , centered at the same position. We  
 276 start from a general Langevin equation of the form in Eq. 1, and assume that the target is perfectly  
 277 absorbing, creating a probability flux  $J = \tau_a^{-1}$ , equal to the rate of finding the target for a single  
 278 particle. The corresponding Fokker-Planck equation can be solved at steady state, giving (Appendix  
 279 2):

$$\tau_a = \int_{r_0}^{r_n} dr r^2 e^{-U(r)/k_B T} \int_{r_0}^r \frac{dr'}{D(r')r'^2} e^{U(r')/k_B T}. \quad (14)$$

280 Taking the particular form of Eqs. 3 and 4, with a sharp boundary  $br_f \gg 1$ , the integral can be



**Figure 4. First passage times to a target site inside the focus.** **A.** Schematic figure showing the setup of the tracked molecule and the effective target. **B.** Time to reach the specific target in simulations for the LPM. Black curve shows the predicted result from the analytical derivation (Eq. 15). Here we use parameters:  $A = -5.5k_B T$ ,  $D_0 = 0.05 \mu\text{m}^2/\text{s}$ ,  $r_n = 1.0 \mu\text{m}$ ,  $D_n = 0.8 \mu\text{m}^2/\text{s}$ . **C.** Same as B, but for the PBM. Same parameters as in B, but with  $D_0 = D_n \cdot e^{U(r)/k_B T}$ . **D.** Heatmap showing the expected search time as a function of the droplet size (x-axis) and the focus diffusion coefficient (y-axis) (Eq. 15). Green point corresponds to experimental observations. **E.** Heatmap showing the expected search time as a function of the droplet size (x-axis) and the height of the surface potential (y-axis) (Eq. 15). Green point corresponds to experimental observations. Parameter values as in Table 1, except  $b = 2,000 \mu\text{m}^{-1}$ ,  $D_0 = 0.04 \mu\text{m}^2/\text{s}$ ,  $A = 5.5k_B T$  for B, E and F,  $\kappa = 50 \mu\text{m}^2/\text{s}$ ,  $k_- = 100 \text{s}^{-1}$ ,  $\rho = 2.4 \cdot 10^4 \mu\text{m}^{-3}$  for C-D.

281 computed explicitly:

$$\begin{aligned} \tau_a = & \frac{r_f^3 - r_0^3}{3D_0r_0} + \frac{r_0^2 - r_f^2}{2D_0} + e^{-\frac{A}{k_B T}} \left( \frac{r_n^3 - r_f^3}{3D_0r_0} + \frac{r_f^3 - r_n^3}{3D_0r_f} \right) \\ & + \frac{r_n^3 - r_f^3}{3D_n r_f} + \frac{r_f^2 - r_n^2}{2D_n}. \end{aligned} \quad (15)$$

282 In the limit  $r_0 \ll r_f \ll r_n$  and of a strong potential  $A \gg k_B T$ , Eq. 15 simplifies to:

$$\tau_a \approx \frac{r_f^3}{3D_0r_0} + \frac{r_n^3}{3D_n r_f}, \quad (16)$$

283 which is exactly the sum of the time it takes to find the focus from the edge of the nucleus, and the  
284 time it takes to find the target from the focus boundary.

285 Expression (15) can be related to the celebrated Berg and Purcell bound (**Berg and Purcell, 1977**),  
286 which sets the limit on the accuracy of sensing small ligand concentration by a small target, due to  
287 the limited number of binding events during some time  $t$ . This bound puts a physical constraint on  
288 the accuracy of biochemical signaling, and has been shown to be relevant in the context of gene  
289 regulation (**Gregor et al., 2007**). With a mean concentration of ligands  $c$  in the cell nucleus, there  
290 are  $m = (4\pi/3)r_n^3 c$  such ligands, and their rate of arrival at the target is  $m/\tau_a = 4\pi c r_n^3 / (3\tau_a)$ , so that  
291 the number of binding events during  $t$  is equal to  $n \sim 4\pi c r_n^3 t / (3\tau_a)$  on average. Random Poisson  
292 fluctuations of  $n$  result in an irreducible error in the estimate of the concentration  $c$ :

$$\frac{\delta c^2}{c^2} \sim \frac{\delta n^2}{n^2} \sim \frac{1}{n} \sim \frac{3\tau_a}{4\pi c r_n^3 t}. \quad (17)$$

293 Replacing  $\tau_a$  in Eq. 17 with the expression in Eq. 16, we obtain in the limit of large nuclei ( $r_n \rightarrow \infty$ ):

$$\frac{\delta c^2}{c^2} \sim \frac{1}{4\pi c t} \left[ \frac{1}{D_n r_f} + \frac{e^{-A/k_B T}}{D_0} \left( \frac{1}{r_0} - \frac{1}{r_f} \right) \right]. \quad (18)$$

294 One can further check that in the limit of a strong potential, or when there is no focus,  $r_0 = r_f$ , we  
295 recover the usual Berg and Purcell limit for a perfectly absorbing spherical measurement device,  
296  $\delta c/c \sim 1/\sqrt{4\pi D_n c r_f t}$ .

297 Eq. 15 agrees well with simulations in the general case (Fig. 4B), where we used parameters  
298 obtained for Rad52 in a repair focus (**Miné-Hattab et al., 2021**). Eq. 15 typically admits a minimum  
299 as a function of  $r_f$ , meaning that there exists an optimal focus size that minimizes the search time.  
300 Using the measured parameters for Rad52, we find an optimal focus size of  $r_f^* \approx 120$  nm, which  
301 matches the estimated droplet size  $r_f = 124$  nm in these experiments (**Miné-Hattab et al., 2021**)  
302 (dashed line in Fig. 4B). In these experiments, the estimated experimental noise level was  $\approx 30$  nm,  
303 but  $r_f$  could be extracted accurately by fitting the confinement radius as well as gathering statistics  
304 for the radial steady state distribution. We observe that the theoretical curve in Fig. 4B is rather flat  
305 around its minimum, suggesting an optimal range of droplet sizes rather than a single one.  
306 In the limit where  $r_n \gg r_0$ , the optimal size takes the explicit form:

$$(r_f^*)^4 = r_0 r_n^3 \frac{\frac{D_0}{D_n} - e^{-\frac{A}{k_B T}}}{3(1 - e^{-\frac{A}{k_B T}})} = r_0 r_n^3 \frac{\frac{D_0}{D_n} - \frac{p_{\text{out}}}{p_{\text{in}}}}{3 \left(1 - \frac{p_{\text{out}}}{p_{\text{in}}}\right)}. \quad (19)$$

307 This optimum only exists for  $D_0 e^{A/k_B T} > D_n$  or  $D_0 p_{\text{in}} > D_n p_{\text{out}}$ , that is, when the benefit of spending  
308 more time in the focus compensates the decreased diffusion coefficient. Incidentally, in that case  
309 the Berg and Purcell bound on sensing accuracy generalizes to:

$$\frac{\delta c^2}{c^2} \sim \frac{1}{\pi c t} \left( \frac{p_{\text{out}}}{4p_{\text{in}} D_0 r_0} + \frac{1}{3D_n r_f} \right). \quad (20)$$

310 The previous formulas for the search time and sensing accuracy are valid for the general  
311 Langevin equation (1), which describes both the LPM and the PBM in the mean-field regime. Fig. 4C  
312 and D show the search time as a function of the focus size for the specific case of the PBM, where  
313 diffusion and potential are further linked. The relation between  $\tilde{U}$  and  $\tilde{D}$ , given by Eq. 11, imposes  
314  $D_0 e^{A/k_B T} = D_n + D_b (e^{A/k_B T} - 1) > D_n$ , giving the optimal focus size:

$$(r_f^*)^4 = r_0 r_n^3 \frac{D_b}{3D_n}. \quad (21)$$

315 For the physiologically relevant regime of very slow binding sites,  $D_b \ll D_n$ , this optimal focus size  
 316 shrinks to 0, meaning that the focus offers no benefit in terms of search time, because binding sites  
 317 “sequester” or “titre out” the molecule, preventing it from reaching its true target.

318 These results suggest to use the search time, or equivalently the rate for binding to a specific  
 319 target, as another measure to discriminate between the LPM and the PBM. In the case of slowly  
 320 diffusing binding sites, the search time in the PBM does not have a clear local minimum (see Fig. 4D),  
 321 and depends less sharply on the focus size than in the LPM. Therefore, identifying an optimal focus  
 322 size would suggest to rule out the PBM. Conversely, a monotonic relation between the search time  
 323 and the focus size would be consistent with the PBM (without excluding the LPM). Testing for the  
 324 existence of such a minimum would require experiments where the focus size may vary, and where  
 325 reaching the target can be related to a measurable quantity, such as gene expression onset in the  
 326 context of gene regulation.

## 327 Discussion

328 The PBM and the LPM are the two leading physical models for describing the nature of nuclear  
 329 foci or sub-compartments. In this work, we analyzed how the traces of single particle tracking  
 330 experiments should behave in both models. Using statistical mechanics, we derived a mean field  
 331 description of the PBM that shares the general functional form of the LPM (Eq. 1), but with an  
 332 additional constraint linking concentration and diffusion inside the focus: the denser the focus, the  
 333 higher the viscosity. This constraint does not appear to be satisfied by the experimental data on  
 334 Rad52 in repair foci, favoring the liquid droplet hypothesis. We use our formulation of the PBM  
 335 to predict the behaviour of the mean radial movement around the focus boundary, which may  
 336 differ markedly from observation of traces inside a liquid droplet (described by the LPM). We find  
 337 the range of LPM parameters where this difference would be so significant that it would lead to  
 338 ruling out the PBM. This work provides a framework for distinguishing the LPM and PBM, and  
 339 should be combined with modern inference techniques to accurately account for experimental  
 340 noise and limited data availability (for instance accounting for molecules going out of the optimal  
 341 focus). Future improvements in single-particle tracking experiments will allow for longer and more  
 342 accurate traces necessary to deploy the full potential of these methods.

343 The LPM and PBM have often been presented as opposing models (*Miné-Hattab and Taddei,*  
 344 **2019**), driven by attempts to compare the macroscopic properties of different membraneless sub-  
 345 compartments to the original example of liquid-like P granules (*Brangwynne et al., 2009*). The  
 346 LPM is a macroscopic description of a liquid droplet in the cytoplasm (*Hyman et al., 2014*), which  
 347 concentrates some molecules inside the droplet, and alters their different diffusion properties.  
 348 The droplet is formed by a phase transition, which means it will be recreated if destroyed, and will  
 349 go back to its spherical shape if sheared or merged. Conversely, the PBM describes the motion  
 350 and effective diffusion coefficient inside the focus as a result of fundamental interactions, which  
 351 provides an explicit binding mechanism by which a focus is formed. Here we clarified the link  
 352 between the two from the point of view of single molecules. We confirmed mathematically the  
 353 intuition that, in the limit of very fast binding and unbinding, the PBM is a particular case of the LPM  
 354 model. Going further, we show that the PBM imposes a strong constraint between the effective  
 355 diffusion of molecules in the sub-compartment,  $D(\mathbf{r})$ , and the effective potential,  $\tilde{U}(\mathbf{r})$  (Eq. 11).  
 356 The LPM is compatible with this choice, but does not impose it in general, although alternative  
 357 mechanistic implementations of the LPM may impose similar constraints with different functional  
 358 forms. The correspondence between the two models breaks down when binding and unbinding are  
 359 slow. However, for this regime to be relevant, experimental observations need to be fast enough to  
 360 capture individual binding or unbinding events, which is expected to be hard in general, and was  
 361 not observed in the case of repair foci in yeast.

362 We found another way in which the two models behave very differently: in the LPM, the focus  
 363 may act as a funnel accelerating the search for a target inside the focus, and we calculated the  
 364 optimal focus size that minimizes the search time. In the PBM, such an improvement is negligible  
 365 unless binding sites themselves have a fast diffusive motion. This difference between the two  
 366 models could potentially be tested in experiments where the focus size varies. It is not clear whether  
 367 this optimality argument is relevant for DSB: the merger of two foci leads to larger condensates,  
 368 suggesting that the focus size is not tightly controlled. But the argument may be relevant for gene  
 369 expression foci, especially in the context of development where transcription factors need to reach  
 370 their regulatory target fast in order to ensure rapid cell-fate decision making (*Bialek et al., 2019*).  
 371 On the contrary, if a focus is created in order to decrease the probability of specific binding, such as  
 372 in silencing foci (*Brown et al., 1997*), a PBM implementation may be more advantageous. Binding  
 373 sites, which act as decoys (*Burger et al., 2010*), sequester proteins involved in gene activation, thus

374 increasing the time it takes to reach their target and suppressing gene expression. In that picture,  
 375 genes would be regulated by the mobility and condensation of these decoy binding sites. Therefore,  
 376 while this difference between the two models may be hard to investigate experimentally, it provides  
 377 be a very important distinction in terms of function.

378 More generally, foci or membraneless sub-compartments are formed in the cells for very  
 379 different reasons and remain stable for different timescales. For example, repair foci are formed  
 380 for short periods of time (hours) to repair double strand breaks, and then dissolve. In this case the  
 381 speeds of both focus formation and target finding are important for rapid repair, but long term  
 382 stability of foci is not needed. Gene expression foci (*Hnisz et al., 2017; Bing et al., 2020*) can be long  
 383 lived, and their formation may be viewed as a way to “prime” genes for faster activation. However,  
 384 given the high concentrations of certain activators, not all genes may require very fast search  
 385 times of the transcription factors to the promoter. While molecularly the same basic elements  
 386 are available for foci formation – binding and diffusion – different parameter regimes exploited  
 387 in the LPM and PBM may lead to different behaviour covering a vast range of distinct biological  
 388 requirements.

## 389 Materials and Methods

### 390 Simulation of PBM

391 In order to simulate the bridging model we generated  $N$  binding sites of radius  $r_b$ . We simulate a  
 392 diffusing molecule through the free overdamped Langevin equation in 3 dimensions, and at each  
 393 time-step we find the closest binding site to the particle. If the distance of the particle ( $\Delta r$ ) is smaller  
 394 than  $r_b$  we bind the molecule with probability  $p_b = \kappa \sqrt{\pi \delta t / D_n}$ . If the particle does not bind, it is  
 395 reflected so the new distance to the center of the particular binding site is  $2r_b - \Delta r$ . At this new  
 396 position we evaluate the position of all other binding sites (they all diffuse with diffusion coefficient  
 397  $D_b$ , and if the molecule is within the radius of another binding site (happens extremely rarely), it is  
 398 again accepted to bind with the same probability  $p_b$ . If a particle binds, it stays at the position of  
 399 the intersection with the binding site, and at each time step it can be released with probability  $k_- \delta t$ .  
 400 We choose  $\delta t$  small so that  $p_b \ll 1$  and  $\sqrt{2D_n \delta t} \ll r_b$ , which for the considered parameter ranges in  
 401 Table 1 is typically obtained for values of  $\delta t = 10^{-6}$  s.

### 402 Simulation of LPM

403 To simulate the LPM, we use the Milstein algorithm to calculate the motion of a particle. As in the  
 404 PBM, the particle is reflected at the nucleus boundary, and can otherwise move freely in the nucleus.  
 405 We typically choose the same value of  $\delta t$  as the PBM, since the surface potential typically has a very  
 406 steep gradient, given by  $b \approx 1000$  as shown in Table 1.

### 407 Experimental measurements

408 Experimental details about single-particle tracking are given in (*Miné-Hattab et al., 2021*). Briefly,  
 409 the x- and y-values of single particles were sampled at 50 Hz for molecules inside the visible z-  
 410 frame ( $\approx \pm 150$  nm thick). Therefore one cannot separate whether molecules are inside the focus or  
 411 above/below it, but since the radius of the focus is  $\approx 125$  nm, this effect is very small, and statistically  
 412 it is possible to take this effect into account when calculating the radial concentration of molecules.

413 The diffusion coefficient inside the focus was calculated as follows. The distributions of dis-  
 414 placements was fitted by a mixture of two Gaussians corresponding to a slow (inside focus) and a  
 415 fast (outside focus) population. Diffusion inside the focus was extracted from the mean-squared  
 416 displacement of the slow population, taking the confinement and experimental uncertainty into  
 417 account (see text related to Fig. 2G in *Miné-Hattab et al. (2021)*). Free energy differences were esti-  
 418 mated based on the size of the focus and the concentration of particles inside the focus compared  
 419 to outside (see text related to Fig. 7 and Table S1, *ibid.*). These estimates are not sensitive to radial  
 420 effects, such as the definition and size of the focus, or to the issue of some particles being above or  
 421 below the focus.

## 422 Acknowledgments

423 The authors are grateful to Jean-Baptiste Masson, Alexander Serov and Ned Wingreen for valu-  
 424 able discussions. The study was supported by the Agence Nationale de la Recherche (Q-life  
 425 ANR-17-CONV-0005), Centre National de la Recherche Scientifique (80' MITI project PhONeS), the  
 426 European Research Council COG 724208, the Labex DEEP (ANR-11-LABEX-0044 DEEP and ANR-  
 427 10-IDEX-0001?02 PSL), the ANR DNA-Life (ANR-15-CE12-0007), the Fondation pour la Recherche  
 428 Médicale (DEP20151234398), and the ANR-12-PDOC- 0035?01. The authors greatly acknowledge

429 the PICT-IBISA@Pasteur Imaging Facility of the Institut Curie, member of the France Bioimaging  
430 National Infrastructure (ANR-10-INBS-04).

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521 **Appendix 1**522 **Binding rate by a partially absorbing sphere**

523 We consider a particle with diffusivity  $D$ , which can be partially absorbed by a spherical  
 524 binding site of radius  $r_b$  and absorption parameter  $\kappa$ . Its Fokker-Planck equation takes the  
 525 following form, in spherical coordinates projected onto the distance to the center of the  
 526 binding site,  $r$ :

$$527 \quad \partial_t p = \frac{D}{r^2} \partial_r r^2 \partial_r p. \quad (22)$$

528  
 529 The boundary conditions are  $p(r = \infty) = 1/V$ , where  $V$  is the total volume, assumed to be  
 530 much larger than that of the binding site, and the Robin condition:

$$531 \quad D \partial_r p(r_b) = \kappa p(r_b). \quad (23)$$

532  
 533 The solution of Eq. 22 at steady state with these boundary conditions reads:

$$534 \quad p(r) = \frac{1}{V} \left( 1 - \frac{\kappa r_b / r}{\kappa + D / r_b} \right), \quad (24)$$

535  
 536 the total diffusive flux is then given by

$$537 \quad J = 4\pi D r_b^2 \partial_r p(r_b) = \frac{1}{V} \frac{4\pi D r_b}{1 + \frac{D}{r_b \kappa}}. \quad (25)$$

538  
 539 Normalizing by the volume factor gives the association rate for binding,  $k_+ = V J = 4\pi D r_b / (1 +$   
 540  $D / \kappa r_b)$ .  
 541  
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545 **Appendix 2**546 **Searching for a target in a funneling potential**

547 We consider a problem similar to that of the previous appendix. Now the spherical object is  
 548 a target, which is perfectly absorbing. It is at the center of a liquid droplet, which we model  
 549 by a spherically symmetric potential  $U(\mathbf{r})$ .

550 The probability distribution of a molecule is denoted by  $p(\mathbf{r}) = p(r)$ . The probability  
 551 density of being at distance  $r$  from the center,  $q(r)$ , is related to  $p(r)$  through  $q(r) = 4\pi r^2 p(r)$ ,  
 552 accounting for the volume of the sphere. The evolution of  $r$  is described by the stochastic  
 553 differential equation:

$$554 \quad dr = \frac{2D}{r} + \partial_r D - \frac{D}{k_B T} \partial_r U + \sqrt{2D} dW, \quad (26)$$

555 where  $W$  is a 1-dimensional Wiener process. The corresponding Fokker-Planck equation  
 556 reads:

$$557 \quad \partial_t q = -\partial_r \left[ \left( \frac{2D}{r} + \partial_r D - \frac{D}{k_B T} \partial_r U \right) q \right] + \partial_r^2 (Dq) \doteq -\partial_r J. \quad (27)$$

558 At steady state with a non-vanishing flux  $J = \text{const}$ , we have:

$$559 \quad \left( \frac{2D}{r} - \frac{D}{k_B T} \partial_r U \right) q = D \partial_r q - J, \quad (28)$$

560 or equivalently:

$$561 \quad q \partial_r \phi + \partial_r q = \frac{J}{D} \quad (29)$$

562 with  $\phi \doteq -2 \ln(r) + U/k_B T$ . Multiplying both sides of the equation by  $e^\phi$ , we obtain:

$$563 \quad \partial_r (e^\phi q) = \frac{J}{D} e^\phi. \quad (30)$$

564 The general solution to that equation is:

$$565 \quad q(r) = C e^{-\phi(r)} + J e^{-\phi(r)} \int_{r_0}^r \frac{e^{\phi(r')}}{D(r')} dr'. \quad (31)$$

566 We have  $C = 0$  because of the absorbing boundary condition  $q(r_0) = 0$ . The constant  $J$  is  
 567 determined by the normalization  $\int_{r_0}^{r_n} dr q(r) = 1$ , yielding:

$$568 \quad J^{-1} \doteq \tau_a = \int_{r_0}^{r_n} dr e^{-\phi(r)} \int_{r_0}^r dr' \frac{e^{\phi(r')}}{D(r')}, \quad (32)$$

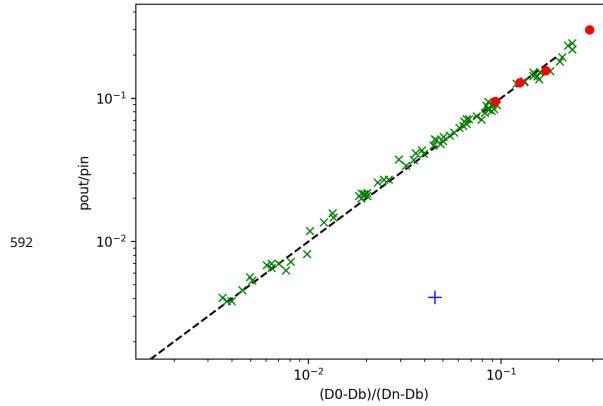
569 This in turns gives the result of the main text after replacing  $\phi(r)$  by its definition.  
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584 **Figure 2-Source Data 1:** Compressed ZIP file containing all the data plotted in the panels of Figure  
585 2 as CSV and TXT files.

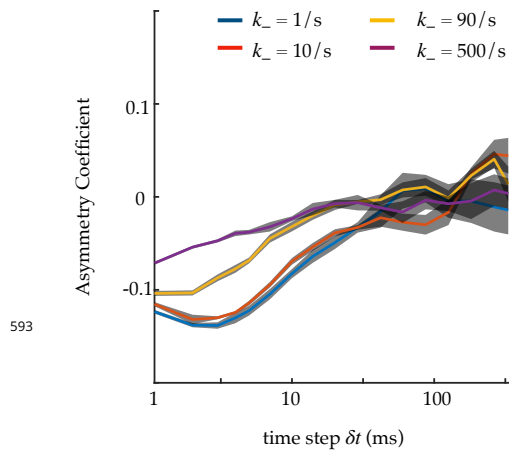
586 **Figure 3-Source Data 1:** Compressed ZIP file containing all the data plotted in the panels of Figure  
587 3 as CSV files.

588 **Figure 3-Source Data 2:** Compressed ZIP file containing all the data plotted in Figure 2-figure  
589 supplement 1 as CSV files.

590 **Figure 4-Source Data 1:** Compressed ZIP file containing all the data plotted in the panels of Figure  
591 2 as CSV files.



**Figure 2-Figure supplement 1.** Same as 2H., but with added red points corresponding to replacing 20% (leftmost), 40%, 60%, and 80% (rightmost) of the binding sites by inert, totally reflecting spheres to simulate crowding. While crowding should decrease the measured  $D_0$ , in practice the effect is negligible.



**Figure 3-Figure supplement 1.** Asymmetry coefficient (following the definition of *Izeddin et al. (2014)*), for an infinite focus, simulated with the PBM, as a function of the time step  $\delta t$ . Asymmetry is due to molecules reflecting off binding sites, causing more consecutive displacements to have 180 degree angles. The faster the binding and unbinding relative to the time step  $\delta t$ , the closer to mean-field limit of standard diffusion, and the more symmetric the angle distribution.  $k_+$  is changed alongside  $k_-$  to keep  $p_u$  constant. The asymmetry coefficient is defined as  $\log_2[\mathbb{P}(|\theta| < \pi/6)/\mathbb{P}(|\theta| > 5\pi/6)]$ , where  $-\pi < \theta < \pi$ . Standard deviation is obtained from 4 independent runs of 1,000 s.