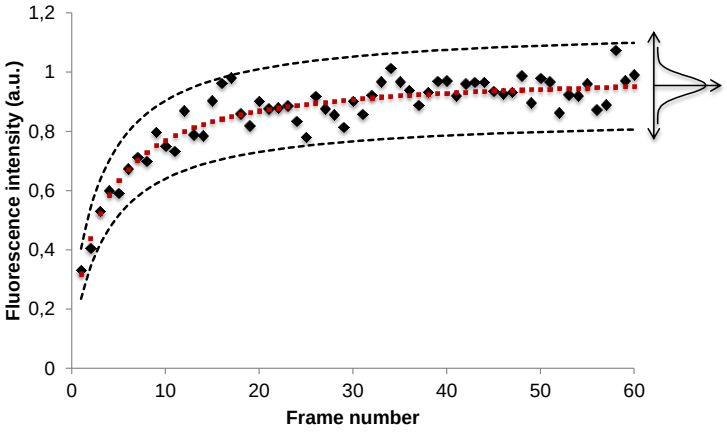
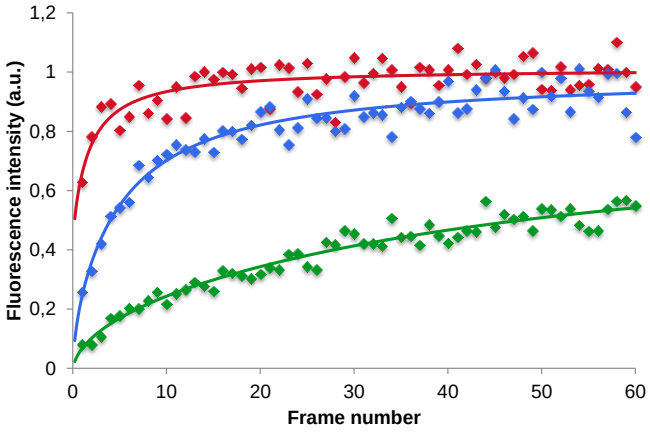
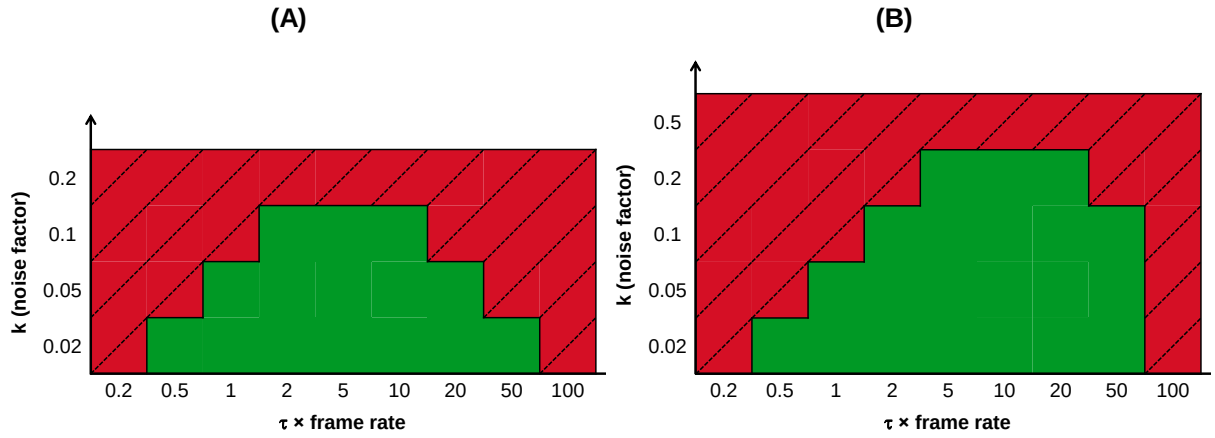


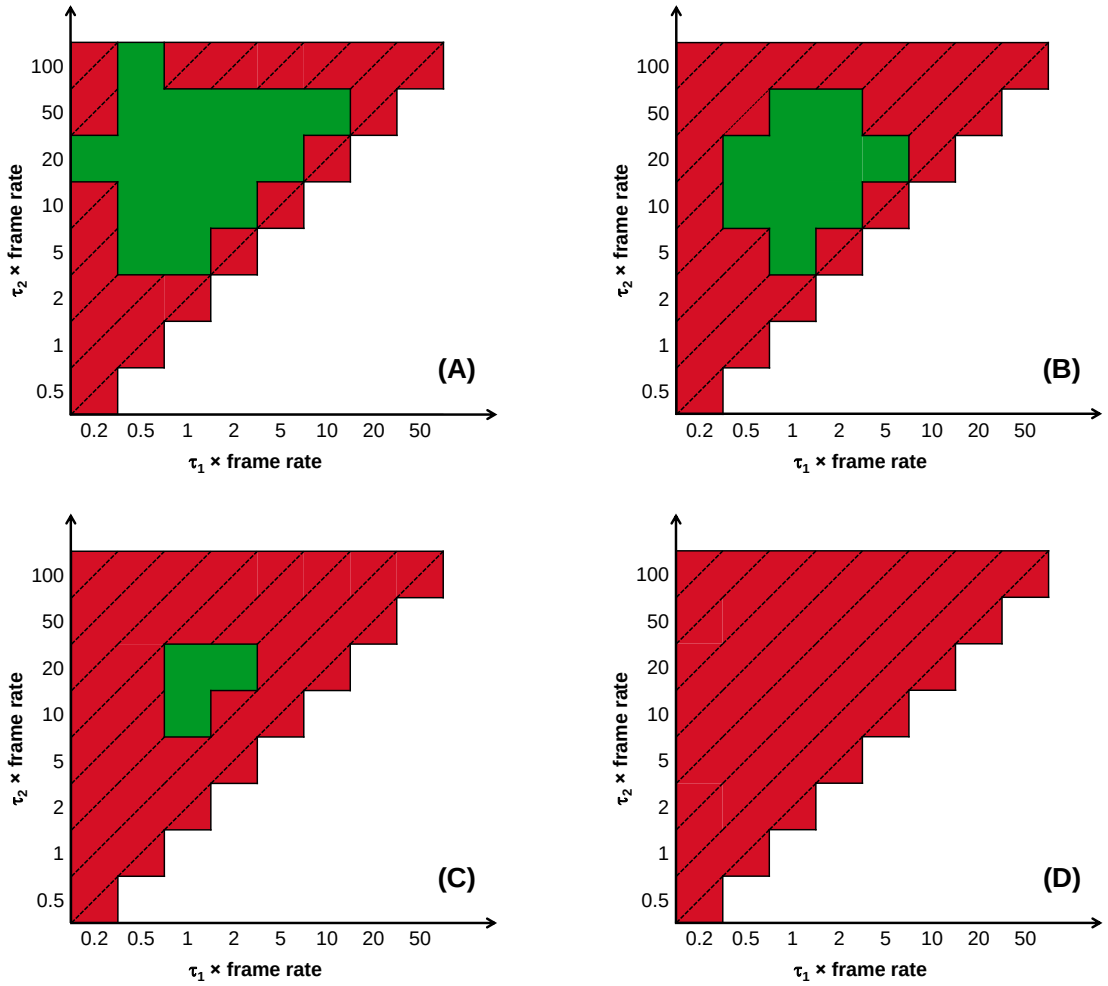
S1 Figure. Typical *in silico* simulated fluorescence recovery curve of a FRAP experiment (*i.e.* with the disk-shaped bleaching geometry). The simulated fluorescence intensity values (black diamonds) were generated by adding Gaussian noise *G(i)* to the theoretical values *I(i)* (red dots) deduced from Equation (1). *G(i)* is a random number with the Gaussian probability distribution centered on 0 with the standard deviation k $\sqrt{I(i)}$ (zone delimited by the two dashed curves). In this example, τ = 3 frame periods and k = 0.05.



S2 Figure. Examples of 3 fitted *in silico* simulated fluorescence recovery curves for FRAP experiments (*i.e.* with the disk-shaped bleaching geometry) with 3 different characteristic diffusion times: τ = 0.5 (red), 5 (blue) or 50 (green) frame periods. The noise factor k used to generate the simulated fluorescence intensity values was 0.05 in all 3 cases. The fits were obtained using Equation (1).

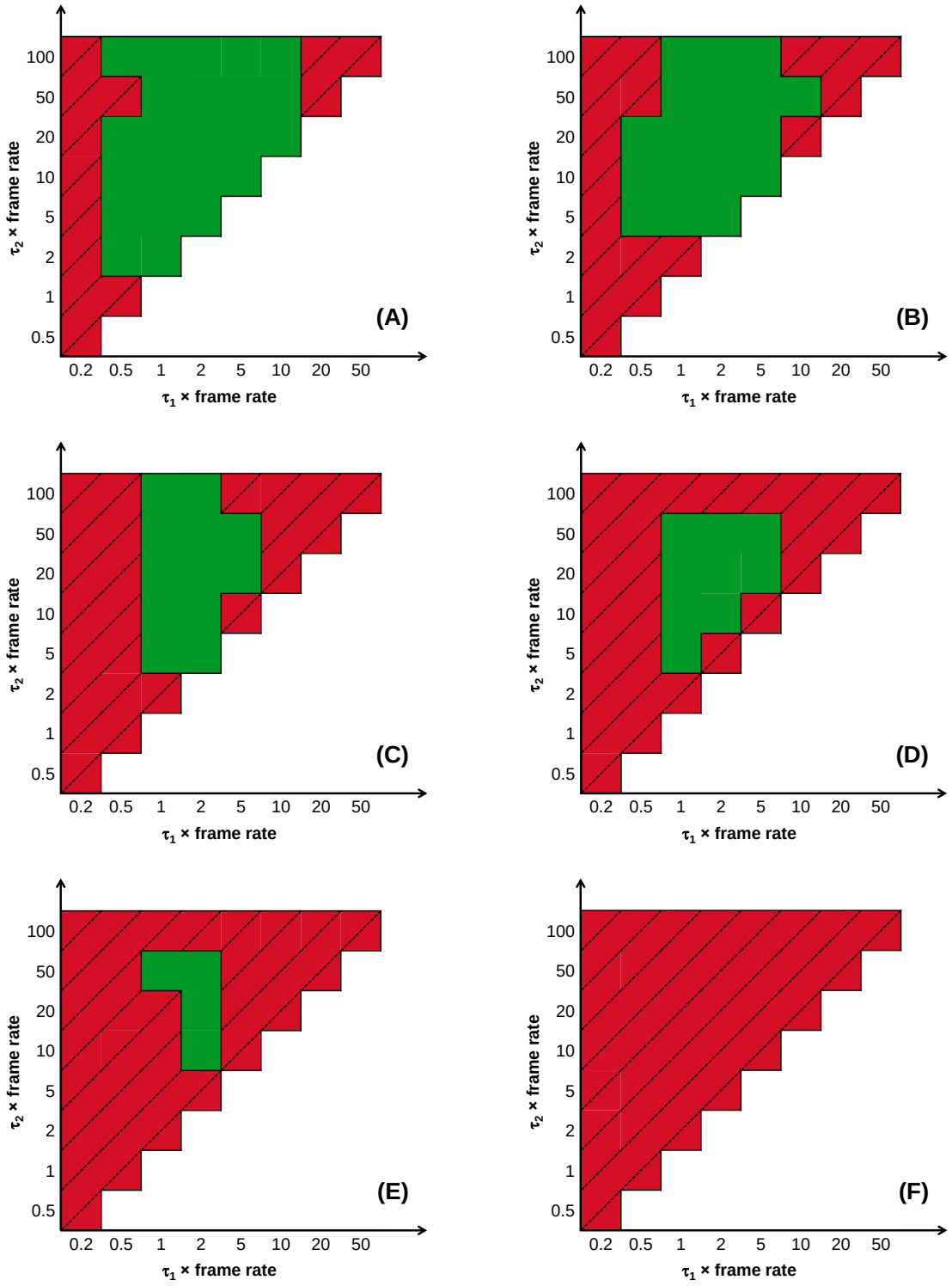


S3 Figure. *In silico* study of FRAP and FRAPP experiments with a single diffusive species. Simulated experimental curves were generated from a normalized theoretical fluorescence recovery curve. These curves were composed of N = 60 intensity data points $I_{sim}(i) = I(i) + G(i)$, where the noise $G(i)$ is a random number with the Gaussian probability distribution centered on 0 with the standard deviation $k\sqrt{I(i)}$ (see methods and the examples in S1 and S2 Figs). The acquisition time was fixed at 60 frame periods. The noise factor k was varied from 0.02 to 0.5 and the characteristic diffusion time τ from 0.2 to 100 frame periods. For each condition, we generated 3000 simulated fluorescence recovery curves and we randomly selected some of these curves in order to form 1000 groups of 3 curves. For each group, the average characteristic diffusion time $\bar{\tau}$ was calculated. The average and the standard deviation on these $\bar{\tau}$, $<\bar{\tau}>$ and $\sigma(\bar{\tau})$, were then calculated across the 1000 groups of 3 curves. The standard deviation $\sigma(\bar{\tau})$ thus reflects the variability on $\bar{\tau}$ if the experimentalist were to reproduce 1000 times the same protocol, each time with 3 independent fluorescence recovery curves. The solvable cases (in green) were arbitrarily defined as the cases where $<\bar{\tau}>$ differs by less than 20% from the theoretical τ value and $\sigma(\bar{\tau}) / \tau$ is lower than 30%. The numerical values of $<\bar{\tau}>$ and $\sigma(\bar{\tau})$ are displayed in S1 Table. The graphs in (A) for FRAP and (B) for FRAPP show the range of τ values that can be accurately measured for any given set of parameters (k, frame rate), and thus allows the experimentalist to optimize the acquisition parameters as a function of the signal-to-noise ratio and the characteristic diffusion time τ. For example, in the case of a typical noise factor k equal to 0.1 (*i.e.* a signal-to-noise ratio of 10 at the plateau), FRAP experiments should be recorded at a frame rate of at least $2/\tau$, and the acquisition time should be between 6τ and 30τ.



S4 Figure. *In silico* study of FRAP experiments with two diffusive species. Simulated experimental curves were generated from a normalized theoretical fluorescence recovery curve as described in the legend of S3 Fig. The acquisition time was fixed at 60 frame periods. The noise factor k was varied from 0.001 to 0.01, the characteristic diffusion time τ_1 from 0.2 to 50 frame periods, and the characteristic diffusion time τ_2 from 0.5 to 100 frame periods (always keeping $\tau_2 > \tau_1$). The fraction of each species was also varied from 10 to 90% (in increments of 10%) and characterized by the fraction of the first species: $R = I_{1\infty} / (I_{1\infty} + I_{2\infty})$. For each condition, we generated 300 simulated fluorescence recovery curves and we randomly selected some of these curves in order to form 100 groups of 3 curves. For each group, we calculated the average characteristic diffusion times $\bar{\tau}_1$ and $\bar{\tau}_2$, and the average fraction $\bar{R}$. The averages $< \bar{\tau}_1 >$, $< \bar{\tau}_2 >$, $< \bar{R} >$ and the standard deviations $\sigma(\bar{\tau}_1)$, $\sigma(\bar{\tau}_2)$, $\sigma(\bar{R})$ on these $\bar{\tau}_1$, $\bar{\tau}_2$ and $\bar{R}$ were then calculated across the 100 groups of 3 curves. These standard deviations thus reflect the variability on $\bar{\tau}_1$, $\bar{\tau}_2$ and R if the experimentalist were to reproduce 100 times the same protocol, each time with 3 independent fluorescence recovery curves. The solvable cases (in green) were arbitrarily defined as the cases where $< \bar{\tau}_1 >$, $< \bar{\tau}_2 >$ and $< \bar{R} >$ all differ by less

than 20% from the theoretical values, and all standard deviations are lower than 20%. The numerical values of $< \bar{\tau}_1 >$, $< \bar{\tau}_2 >$, $< \bar{R} >$, $\sigma(\bar{\tau}_1)$, $\sigma(\bar{\tau}_2)$ and $\sigma(\bar{R})$ are displayed in S2 Table. We show here only the results obtained for R = 0.5 and a noise factor k equal to 0.001 (A), 0.002 (B), 0.005 (C) or 0.01 (D). Compared to the case of a single diffusive species, the signal-to-noise ratio has to be very high in order to obtain accurate values of both τ_1 and τ_2. In the case of a noise factor k equal to 0.001 (*i.e.* a signal-to-noise ratio of 1000 at the plateau), and assuming that τ_1 and τ_2 are already in the measurable range permitted by the acquisition parameters, the experimentalist should be able to distinguish two species by FRAP and to accurately measure their characteristic diffusion time if τ_2 is at least 5 times larger than τ_1 (panel A).



S5 Figure. *In silico* study of FRAPP experiments with two diffusive species. Simulated experimental curves were generated from a normalized theoretical fluorescence recovery curve as described in the legend of S3 Fig. The acquisition time was fixed at 60 frame periods. The noise factor k was varied from 0.001 to 0.05, the characteristic diffusion time τ_1 from 0.2 to 50 frame periods, and the characteristic diffusion time τ_2 from 0.5 to 100 frame periods (always keeping $\tau_2 > \tau_1$). The fraction of each species was also varied from 10 to 90% (in increments of

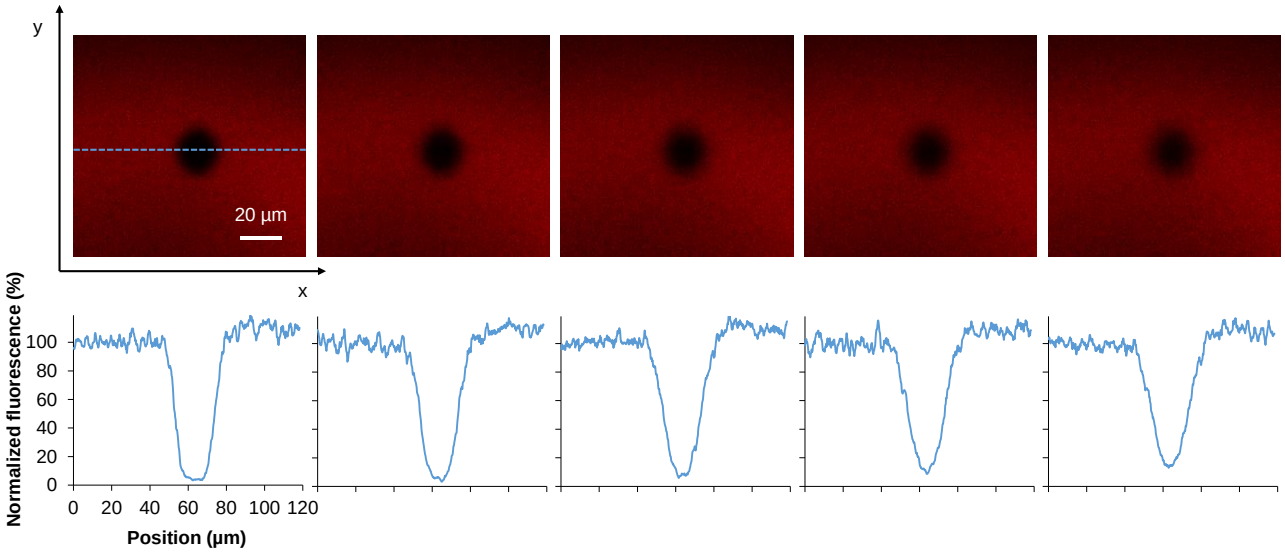
10%) and characterized by the fraction of the first species: R = $I_{1\infty}$ / ($I_{1\infty}$ + $I_{2\infty}$). For each condition, we generated 300 simulated fluorescence recovery curves and we randomly selected some of these curves in order to form 100 groups of 3 curves. For each group, we calculated the average characteristic diffusion times $\bar{\tau}_1$ and $\bar{\tau}_2$, and the average fraction $\bar{R}$. The averages $<\bar{\tau}_1>$, $<\bar{\tau}_2>$, $<\bar{R}>$ and the standard deviations $\sigma(\bar{\tau}_1)$, $\sigma(\bar{\tau}_2)$, $\sigma(\bar{R})$ on these $\bar{\tau}_1$, $\bar{\tau}_2$ and $\bar{R}$ were then calculated across the 100 groups of 3 curves. These standard deviations thus reflect the variability on $\bar{\tau}_1$, $\bar{\tau}_2$ and R if the experimentalist were to reproduce 100 times the same protocol, each time with 3 independent fluorescence recovery curves. The solvable cases (in green) were arbitrarily defined as the cases where $<\bar{\tau}_1>$, $<\bar{\tau}_2>$ and $<\bar{R}>$ all differ by less than 20% from the theoretical values, and all standard deviations are lower than 20%. The numerical values of $<\bar{\tau}_1>$, $<\bar{\tau}_2>$, $<\bar{R}>$, $\sigma(\bar{\tau}_1)$, $\sigma(\bar{\tau}_2)$ and $\sigma(\bar{R})$ are displayed in S3 Table. We show here only the results obtained for R = 0.5 and a noise factor k equal to 0.001 (A), 0.002 (B), 0.005 (C), 0.01 (D), 0.02 (E) and 0.05 (F). Compared to the case of a single diffusive species, the signal-to-noise ratio has to be very high in order to obtain accurate values of both τ_1 and τ_2. In the case of a noise factor k equal to 0.001 (*i.e.* a signal-to-noise ratio of 1000 at the plateau), and assuming that τ_1 and τ_2 are already in the measurable range permitted by the acquisition parameters, the experimentalist should be able to distinguish two species by FRAPP and to accurately measure their characteristic diffusion time if τ_2 is at least 2 times larger than τ_1 (panel A).

Experimental determination of the time t0 of the recovery phase

In order to take into account the contribution of fluorescence recovery that occurs during the photobleaching phase, the time t0 of the recovery phase in all FRAP and FRAPP experiments was set as the time of the last bleaching frame. We will show below that this approximation is valid using the FRAP experiment of Fig 2A as an example.

We will first present a protocol to experimentally determine the actual time t0 of the recovery phase. Then, we will show that our approximation (setting t0 as the time of the last bleaching frame) allows one to estimate the diffusion coefficient with a better than 10% accuracy.

In the following, we set t=0 as the time of the last bleaching frame, t=1 the time of the first post-bleach picture, t=2 the time of the second post-bleach picture, *etc*. The actual time t0 will be determined from the evolution of the post-bleach profile in the first five pictures of the recovery phase (S6 Fig).



S6 Figure. First five post-bleach pictures and corresponding fluorescence profiles following photobleaching of a fluorescent disk of d = 20 µm diameter in the outer monolayer of a supported DOPC:DOPE-Rho (99:1) lipid bilayer (Fig 2A). The post-bleach profiles were obtained by measuring the fluorescence intensity (using ImageJ) along the blue dashed line as illustrated on the first post-bleach picture.

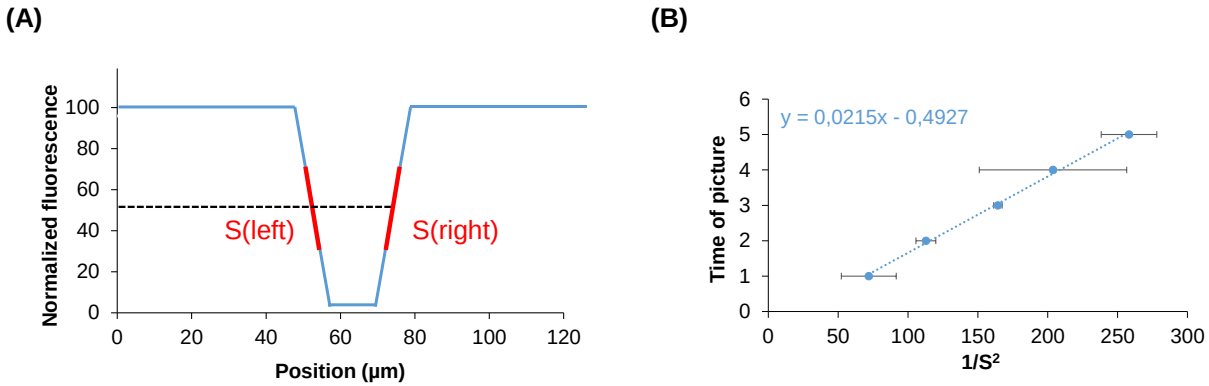
The local fluorescence recovery (at the edge of the bleaching pattern defining x=0) varies initially as:

$$F(x,t) = \frac{1}{2}\left(1 + \mathrm{erf}\left(\frac{x}{\sqrt{4Dt}}\right)\right)$$

Where erf is the error function and:

$$F(x,0) = \begin{cases} 1 & for\ x > 0 \\ 1/2 & for\ x = 0 \\ 0 & for\ x < 0 \end{cases}$$

The slope S defining the post-bleach profile at x=0 (S7A Fig) is therefore proportional to $1/\sqrt{t}$, *i.e.* t varies as $1/S^2$. Plotting t as a function of $1/S^2$ should thus give a straight line, whose intercept with the y-axis is t0: $t = \alpha/S^2 + t0$. The five post-bleach profiles of S6 Fig were used to measure S at the different post-bleach times and to deduce the actual time t0 of the recovery phase. We found t0 = - 0.4 sec (S7B Fig).

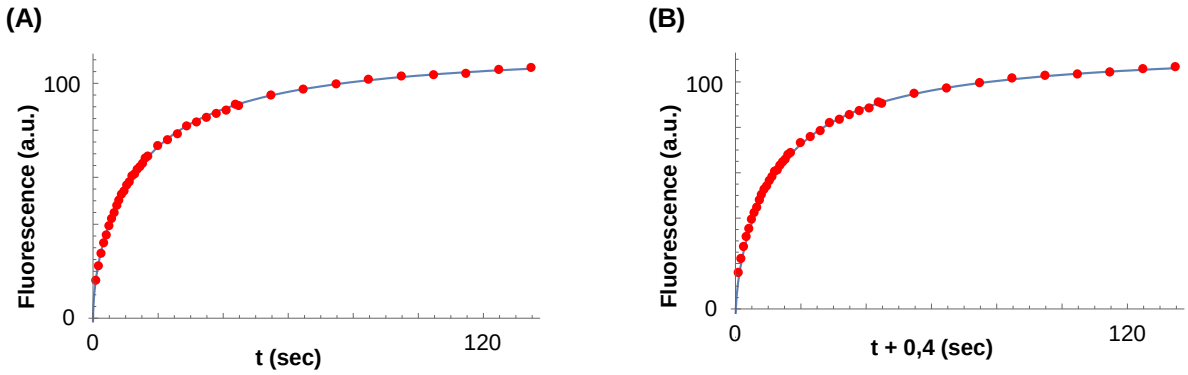


S7 Figure. (A) Schematic representation of the post-bleach profiles of S6 Fig and definition of the two local slopes that are used to characterize them. (B) The slopes on both sides, left and right, were measured and averaged for the five different time points. The linear fit directly provides the actual time t0 of the recovery phase as the intercept with the y-axis. Here, t0 = - 0.5 frame = - 0.4 sec (the scanning rate was 0.8 sec per frame in this FRAP experiment).

In theory, the protocol presented above to determine the actual time t0 of the recovery phase can be applied to any FRAP experiment. However, this renders the analysis quite complicated. Here, we will show that setting t0 = 0 allows one to obtain D with a good approximation and that it is therefore not necessary to systematically apply this protocol.

Let us show that this works on the same example (Fig 2A). Using the approximation t0 = 0, we find D = 2.1 $\mu m^2/s$ (S8A Fig). Using the protocol above to determine the actual time t0 (here t0 = - 0.5 frames = - 0.4 sec), we obtain D = 2.2 $\mu m^2/s$ (S8B Fig).

The difference between the two D values is therefore within the experimental error (1.9 ± 0.3 $\mu m^2/s$) over several experiments (Fig 3 and Table 2).



S8 Figure. Fitting of the experimental data from the FRAP experiment of Fig 2A using (A) t0 = 0 or (B) t0 = - 0.4 sec.

One should note that in most FRAP experiments, the error on t0 will usually be lower than the one measured in the present example. In fact, we have considered here an extreme case in which three bleaching frames were used, whereas a single bleaching frame is typically sufficient for bleaching. Using three bleaching frames obviously increases the error on t0. Since the error on D is negligible in this extreme case, our choice (setting t0 = 0 as the time of the last bleaching frame) will allow a good approximation of D in most cases.