Solute diffusion is hindered in the mitochondrial matrix

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Intracellular chemical reactions generally constitute reaction-diffusion systems located inside nanostructured compartments like the cytosol, nucleus, endoplasmic reticulum, Golgi, and mitochondrion. Understanding the properties of such systems requires quantitative information about solute diffusion. Here we present a novel approach that allows determination of the solvent-dependent solute diffusion constant (Dsolvent) inside cell compartments with an experimentally quantifiable nanostructure. In essence, our method consists of the matching of synthetic fluorescent recovery after photobleaching (FRAP) curves, generated by a mathematical model with a realistic nanostructure, and experimental FRAP data. As a proof of principle, we assessed a given FP, which represents an underestimation of the molecular diffusion constant (Dsolvent) in the mitochondrial matrix within living cells. We chose this organelle for the following reasons (18–23): (i) Many intramatrix reactions involve the conversion of mobile substrates by immobilized membrane-bound enzymes into mobile products and thereby constitute reaction-diffusion systems; (ii) mitochondrial length can be considered (e.g., 50 μm in fibroblasts), making solute diffusion particularly relevant; and (iii) mitochondria possess an interesting nanostructure, in which folds (cristae) of the mitochondrial inner membrane (MIM) protrude into the mitochondrial matrix compartment, potentially hindering intramatrix solute diffusion.

Our hybrid in silico and experimental approach revealed that FP diffusion in the mitochondrial matrix is severely hindered by cristae. Therefore, we propose that regulated alterations in compartment nanostructure allow the cell to control solute diffusion inside cellular compartments. This implies that alterations in mitochondrial nanostructure, as observed during numerous (patho)physiological conditions, can affect the properties of intramatrix reaction-diffusion systems and thereby mitochondrial and cellular function.

Results

A Quantitative Mathematical Model of Protein Diffusion in the Mitochondrial Matrix. To allow extraction of the solvent-dependent diffusion constant (Dsolvent) of a given FP from experimental FRAP curves, we developed a 3D diffusion model. This model was constrained by experimental data as follows: (i) the length (Lmito) and radius (Rmito) of the mitochondrial matrix, (ii) the number (n) of diffusion barriers inside the mitochondrial matrix, (iii) the FP concentration in the mitochondrial compartment (Cp), and (iv) the dimensions of the experimental FRAP region (SRAP).

Our strategy was to match the kinetics of the simulated and experimental FRAP curve, as reflected by their FRAP recovery time constant (TFRAP), by varying the value of Dsolvent in the model (Fig. L4). Structurally, the mitochondrial matrix is modeled as a closed cylinder with Rmito = 0.27 ± 0.035 μm and Lmito = 4.0 ± 0.14 μm (N = 45), which were determined experimentally for the HEK293 cells used in this study (13, 16). To account for possible hindrance of FP diffusion by the mitochondrial nanostructure, we performed electron microscopy (EM) analysis of mitochondria in HEK293 cells (e.g., Fig. L8). This revealed that each mitochondrion on average contained between 6.2–8.2 cristae

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Importantly, experimental constraints are imposed on the model. The solvent-dependent solute diffusion constant ($D_{\text{solute}}$) can be predicted by matching the simulated $T_{\text{FRAP}}$-value with the experimental $T_{\text{FRAP}}$-value through varying $D_{\text{solute}}$ in the model. (B) Typical electron microscopy image revealing cristae nanostructure in an AcGFP1-expressing HEK293 cell. (C) Internal geometry of the model; only part of the mitochondrion is shown. (D) Barrier geometry. (E) Simulated position of FP molecules directly following the bleach pulse at $t = 0$ s (blue circles) and 15 s later (red crosses) in a side view projection. (F) Similar to $\ddot{E}$ but now for a top view projection.

FRAP Analysis of Protein Mobility in the Mitochondrial Matrix of Living Cells. To determine FP mobility in the mitochondrial matrix we used inducible HEK293 cell lines (15). These expressed AcGFP1 or its tandem fusion AcGFP1$^2$ in the mitochondrial matrix (Fig. 2 A and B). A typical FRAP experiment is depicted in Fig. 2C. Following the bleach pulse in the FRAP region (square), the fluorescence signal in the mitochondrion equilibrated within 15 s. Calculation of the FP mobile fraction ($F_m$) revealed values close to one (AcGFP1, 1.02; AcGFP1$^2$, 0.99), demonstrating that both FPs were fully mobile. For AcGFP1, fluorescence recovered with an average time constant $T_{\text{FRAP}}$ of 0.60 ± 0.015 s (Fig. 2D). To rule out interference of processes unrelated to solute diffusion, we investigated how the FRAP kinetics depended on the size of the FRAP region in AcGFP1-induced cells. When the size of the FRAP region was increased twofold, recovery was twofold slower and $F_m$ was not affected (Fig. S1). This demonstrates that $T_{\text{FRAP}}$ reflects authentic FP diffusion (25). For AcGFP1$^2$, fluorescence recovered significantly more slowly than for AcGFP1 ($T_{\text{FRAP}}$ of 1.78 ± 0.038 s; Fig. 2D), indicating that the mobility of matrix-soluble FPs decreases with increasing MW.

Quantifying Protein Diffusion by Matching Simulated with Experimental FRAP Data. Increasing the number of barriers in the model led to a progressive reduction in the rate of fluorescence recovery (Fig. 3A). The effect of fixed barriers on FP diffusion can also be appreciated by quantifying the fluorescence loss in photobleaching (FLIP) in a region distal from the FRAP region (7). Therefore, we also simulated the FLIP signal in a region with size $S_{\text{FLIP}}$, located 1.2 μm away from the FRAP region (i.e., at the other tip of the mitochondrion; Fig. 3A, Inset). The rate of FLIP decreased when the number of barriers increased (Fig. 3B). Kinetic analysis further revealed a delay ($dt$) between the onset of fluorescence recovery in the FRAP region and onset of...
Fig. 2. FRAP analysis of mitochondrial AcGFP1 and AcGFP12 in HEK293 cells. (A) SDS/PAGE fluorogram (top panels) and anti-EGFP antibody immunodetection (middle panels) of mitochondrial matrix-targeted AcGFP1 and AcGFP12 in HEK293 cells in the absence (−) and presence (+) of the expression inducer doxycycline (DOX). The lower panels show the expression level of the CII loading control (detected using an anti-SDHA antibody). Closed arrowheads indicate MW (in kDa; upper panels) and CII (lower panel). (B) Confocal images showing mitochondrial colocalization of AcGFP1/AcGFP12 fluorescence and Mitotracker Red CM-H2XROS following expression induction. (C) Typical example of images acquired during a FRAP experiment in cells expressing mitochondria-targeted AcGFP1. Following a 500-ms bleach pulse (triangle) in the FRAP region (square), AcGFP1 fluorescence rapidly redistributed across the mitochondrial filament. (D) Average fluorescence recovery curve and the fitted single-component exponential model (lines) for AcGFP1 and AcGFP12. R² values of the fit equaled: 0.98 (AcGFP1-induced) and 0.99 (AcGFP12-induced). The inset shows the time constant (T_FRAP) of the recovery curve for the two fluorescent proteins. In this figure, values on the Y-axis represent the average of the indicated number (N) of recorded mitochondria in at least three independent experiments. The error bars represents SE (average curves) or SEM (bar graphs). Statistics: significantly different from AcGFP1 (***, p < 0.001).

Fig. 3. Application of the mathematical model to predict diffusion constants. (A) Simulated FRAP curve for an FP with a D solvent of 23 μm²·s⁻¹ in the presence of 19, 25, or 29 diffusion barriers. A single-component exponential model, yielding a time constant T_FRAP, adequately fitted the data. (B) Simulated fluorescence loss in photobleaching (FLIP) curve for a region at the other tip of the mitochondrion. T_FRAP was obtained as described for A. (C) Magnification of B (each curve starting at t = 0 s) revealing that the time-lag (dt) in the onset (open symbol) of FLIP increases, whereas the rate of FLIP becomes slower, upon increasing the number of barriers. (D) Linear relationship between the number of barriers and dt (open symbols) and the number of barriers and the absolute rate of fluorescence decrease in the FLIP region (filled symbols). (E) Effect of varying D solvent in the model on the recovery time of the simulated FRAP curve (T_FRAP) in the presence of 0, 19, 25, and 29 diffusion barriers. A linear relationship between log(D solvent) and T_FRAP was found. (F) Linear relationship between D solvent and the number of barriers. For both AcGFP1 and AcGFP12, three data points are given. These reflect the three values of D solvent predicted by the model for 19, 25, and 29 diffusion barriers using the T_FRAP determined experimentally. From the fitted lines, D solvent values were determined (dotted lines) using the number of diffusion barriers (cristae) determined by EM analysis. All linear fits in this figure had a p-value < 0.05 and an R-value of 0.95–0.99 (positive correlations) or −0.96 (negative correlations).

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fluorescence decrease in the FLIP region (Fig. 3C). The time delay and the rate of fluorescence decay in the FLIP region linearly increased and decreased, respectively, with the number of barriers (Fig. 3D). For 29 barriers dit equaled 830 ms, meaning that FPs travelled the distance between the FRAP and FLIP region at an average speed of 1.5 μm s⁻¹. The latter value is compatible with the “apparent” linear diffusion velocity of the FP mitoDsRed1 (1.0 μm s⁻¹) in HeLa cells (20) and the rapid FP exchange between fusing mitochondria (e.g., 26).

Next, we determined how varying $D_{solvent}$ affected $T_{FRAP}$ in the presence of 0, 19, 25, and 29 diffusion barriers (Fig. 3E; different symbols). It was found that $T_{FRAP}$ increased linearly with decreasing log($D_{solvent}$) for “unhindered” diffusion (0 barriers) and hindered diffusion (19, 25, and 29 barriers). This allows calculation of the experimental $D_{solvent}$ by inserting the experimental $T_{FRAP}$ values for AcGFP1 and AcGFP1² into the linear fitting equations used to describe the data (Fig. 3E; lines). In this way, four predicted values for $D_{solvent}$ were obtained (i.e., in case of 0, 19, 25, and 29 diffusion barriers) for both AcGFP1 and AcGFP1².

In case of 0 barriers, $D_{solvent}$ values equaled 9.54 μm² s⁻¹ and 0.427 μm² s⁻¹ for AcGFP1 and AcGFP1², respectively. Subsequently, we used the three remaining $D_{solvent}$ values (19, 25, and 29 barriers) for AcGFP1 and AcGFP1² to establish the relationship between the number of diffusion barriers and $D_{solvent}$. This yielded two linear relationships (Fig. 3F). Finally, we combined information about the number of barriers determined by EM analysis (shaded area) with the fitted lines. This predicted (dotted lines) that AcGFP1 and AcGFP1² have a $D_{solvent}$ between 46.6–61.4 μm²/s and 23.0–40.1 μm²/s, respectively. This means (see Materials and Methods) that the solvent-dependent viscosity of the mitochondrial matrix ($\eta_{solvent}$) is between 1.50–1.97 cP (i.e., 1.5-fold to twofold higher than pure water).

Discussion

In living cells, understanding the properties of compartmentalized reactions requires quantitative information about intracomartment solute diffusion. Here a strategy is presented allowing determination of the solvent-dependent diffusion constant ($D_{solvent}$) inside cell compartments with an experimentally quantifiable nanostructure. To demonstrate the feasibility of this approach we determined $D_{solvent}$ for an inert monomeric FP (AcGFP1) and its tandem fusion (AcGFP1²) inside the mitochondrial matrix. To this end we constructed a realistic mathematical FRAP model and quantified $D_{solvent}$ by matching the kinetics of the simulated and experimental FRAP curves.

A combination of FRAP and mathematical modeling was used previously to investigate FP diffusion inside the ER, but these studies did not consider the internal nanostructure of this compartment (27, 28). In the case of mitochondria, we are aware of only two other reports that applied mathematical modeling to analyze submitochondrial FRAP experiments (7, 8). Our strategy differs from these studies as follows: (i) It is constrained by experimental values for the dimensions of the compartment, its internal nanostructure, intracompartment FP concentration, and the size of the FRAP region; (ii) it has a temporal and fluorescence intensity scale that quantitatively match those of experiments without the need for rescaling; (iii) it can be used with data obtained from “classical” FRAP experiments instead of “fast” spot-FRAP techniques, which requires specialized equipment; and (iv) it applies a unique computational approach to account for intracomartment diffusion hindrance (see below).

Our model predicts how intramatrix diffusion barriers affect free FP diffusion. External and internal model geometry was based on experimental data in HEK293 cells concerning mitochondrial length, diameter, and size of the FRAP region. These parameters, as well as matrix GFP concentration ($C_m$), were determined by confocal microscopy and FCS and have a fixed value in the model. Therefore the value of $D_{solvent}$ predicted by the model only depends on the number of diffusion barriers. To compute the synthetic FRAP curves, the model takes into account the increased distance that the FP has to diffuse when it moves around the barriers. This means that the exact topology of the barriers is not of major importance. Therefore our EM analysis can be considered as an estimate of mitochondrial cristae shape and number.

For the 0-barrier model the obtained $D_{solvent}$ values predict (Eq. 6) that the mitochondrial matrix is 10 times more viscous than pure water (i.e., $\eta_{solvent} = 9.7$ cP). A key determinant of matrix solvent viscosity is the concentration of macromolecules. In case of human serum albumin (HSA), an aqueous solution at 20°C with a viscosity of 9.7 cP contains 313 mg/mL of protein (29). Analysis of mitochondrial homogenates revealed a protein concentration up to 500 mg/mL (e.g., 30), compatible with our 0-barrier model. However, in reality mitochondria contain cristae. Moreover, the obtained $D_{solvent}$ values for the 0-barrier model are far below the $D_{app}$ values reported in FRAP and FCS experiments, which already account for effects of diffusion hindrance (e.g., 8, 13). This demonstrates that a 0-barrier model is inappropriate for describing intramatrix FP diffusion.

Our “hindered” models predict a fivefold lower viscosity of the mitochondrial matrix solvent, equivalent with a HSA concentration of 130 mg/mL (29). This means that a substantial part of the mitochondrial proteins are not present as solutes in the mitochondrial matrix. The latter is compatible with the fact that four of the most abundant mitochondrial proteins (ATPSA1, ATP5B, ANT1, and ANT2) are membrane-bound (31). Similarly, key enzymes of the matrix-residing tricarboxylic acid (TCA) cycle are immobilized (32). Also the multiprotein complexes of the mitochondrial oxidative phosphorylation (OXPHOS) system are membrane-bound (23). Taken together, our results predict that (i) the mitochondrial matrix possesses a moderate viscosity (i.e., approximately twofold higher than pure water) and (ii) intramatrix FP diffusion is substantially hindered by diffusion barriers (cristae). These findings argue against the presence of a central mitochondrial “water channel” allowing rapid unhindered FP diffusion (8, 17).

We assumed during our simulations that the 500-ms bleaching pulse in the FRAP region does not alter mitochondrial ultrastructure. This assumption was based on the following data: (i) Intense AcGFP1 illumination is not phototoxic (24); (ii) photobleaching does not alter the morphology of cell surface membranes (33) nor the lateral mobility of plasma membrane proteins (34); (iii) targeted photodamage of mitochondria induces their fragmentation (35), which was not observed in our experiments; and (iv) we did not detect alterations in mitochondrial diameter within the FRAP region. In a worst-case scenario the cristae within the FRAP region would be completely destroyed, reducing the number of diffusion barriers in the model. However, this would not affect the main conclusion of our study, namely that matrix diffusion barriers are essential to quantitatively explain our FRAP results.

The obtained AcGFP1 diffusion constant is approximately twofold lower than in pure water. Importantly, this is the “real” diffusion constant ($D_{solvent}$) and not the “apparent” diffusion constant. The value obtained for $D_{solvent}$ is not directly informative about the absolute speed with which a solute diffuses in the matrix, because it does not account for the effect of diffusion hindrance. This concept is illustrated by our calculation of the (hindered) linear GFP diffusion velocity (Fig. 3C), which is in agreement with previous studies (20, 26).

Our results demonstrate that FP diffusion in the mitochondrial compartment decreases with the MW of the protein and, in sharp contrast to the current biological dogma (17), is severely hindered by the presence of diffusion barriers. These findings are important because changes in mitochondrial ultrastructure, viscosity, and metabolic state often occur in parallel (13, 21, 22, 36, 37).
For instance, matrix solute diffusion differs significantly between different mitochondrial respiratory states (38). Moreover, matrix volume changes causing alterations in the concentration of enzymes, their substrates, and metabolites probably play a role in metabolic control (30). Furthermore, we provided evidence that assembly of an important OXPHOS complex (complex I), involves intramatrix diffusion of subassemblies (15). This suggests that the diffusion properties of the matrix solvent are important during the complex I assembly reaction. In this sense, our findings suggest that changes in compartment nanostructure can greatly affect solute diffusion and thereby the dynamics of diffusion-limited reactions. Similarly, in Dictyostelium reorganization of the cytosolic acting meshwork has been linked to alterations in protein mobility (39). In principle, our approach is applicable to cellular compartments of which the 3D nanostructure can be experimentally accessed, for instance by EM analysis. For such compartments, cell-controlled changes in their internal nanostructure might also function to regulate intracompartment reaction dynamics.

Materials and Methods

Generation of Inducible Cell Lines. Stably transduced human embryonic kidney (HEK293) cell lines conditionally expressing AcGFP1 or its tandem fusion (AcGFP1−2) in the mitochondrial matrix were generated and cultured as described previously (15, 40). AcGFP1 and AcGFP1−2 expression was induced by adding 1 μg/ml doxycycline (DOX; Sigma) to the culture medium, followed by 24 h incubation.

SDS/PAGE and BN-PAGE analysis. Native mitochondrial proteins were isolated and run on SDS/PAGE gels as described before (15, 40). Following electrophoresis, Western blotting was performed as described previously (15, 40).

Colocalization Analysis. Colocalization analysis of AcGFP1 fluorophore and a mitochondrial marker dye (Mitotracker Red CM-H2XROS; Invitrogen) was performed using confocal microscopy as described previously (41).

FRAP Experiments. Fluorescence recovery after photobleaching (FRAP) experiments were performed as described previously (15) using a ZEISS LSM510 Meta confocal microscope (Carl Zeiss). FRAP measurements were carried out at 20 °C to minimize mitochondrial movement (42). Images were acquired at a rate of 10 Hz using a 63x oil immersion objective (N.A. 1.4; Carl Zeiss), a zoom factor of 4 and an optical section thickness of <2 μm. Only single nonmoving mitochondria of constant length and diameter that were fully within the focal plane were analyzed. A FRAP region of 1.4 μm × 1.4 μm was used and FP photobleaching was performed during 500 ms using 488-nm light. Only mitochondria in which FRAP was performed by FLP in a part distal to the FRAP region were considered to possess a continuous mitochondrial matrix and included in the analysis. FRAP was monitored using a HFT488 beam splitter and a LP550 emission filter. Individual FRAP curves (F(t)) were calculated as described previously (11):

\[
F(t) = \frac{F(t)_{\text{FRAP region}} - F(t)_{\text{background}}}{F(t)_{\text{total mito}} - F(t)_{\text{background}}} \times \frac{(F(t)_{\text{total mito}} - F(t)_{\text{background}})}{(F(t)_{\text{FRAP region}} - F(t)_{\text{background}})}. \tag{2}
\]

Here the fluorescence intensity in the bleached mitochondrial region \((F(t)_{\text{FRAP region}})\) and for the total mitochondrion \((F(t)_{\text{total mito}})\) is background-corrected \((F(t)_{\text{background}})\) at each time point. Next, the corrected fluorescence signal in the bleached region is divided by the corrected intensity of the total mitochondrial to correct for the loss of fluorescence during the bleach. The data are normalized to the background-corrected prebleach intensity \((F(t)_{\text{total mito}})\) and \((F(t)_{\text{FRAP region}})\) and multiplied by 100 to yield a percentage of prebleach fluorescence. Mean recovery curves were fitted by a single-component exponential model:

\[
F(t) = y_0 + A \cdot (1 - e^{-t/T_{\text{Frac}}}). \tag{3}
\]

The mobile fraction \(F_m\) was calculated from mean recovery curves according to the equation (11, 12, 15, 43):

\[
F_m = \frac{F_{\infty} - F_0}{F_{\infty} - F_0}. \tag{4}
\]

With \(F_\infty\) being the fluorescence intensity at \(t = \infty\) (\(F_\infty\) was calculated from the fit in Eq. 3: \(F_\infty = y_0 + A\)), \(F_0\) being the starting fluorescence level of fluorescence recovery and \(F_{\text{initial}}\) being 100%.

Electron Microscopy (EM) Analysis. EM analysis was performed as described previously (14).

Numerical Simulations. Mathematical modeling was performed with MATLAB 6.1 (The Mathworks Inc.) using custom scripts. Diffusion described by Eq. 1 was simulated by adding a random displacement (Δx, Δy, Δz) at every time step (Δt = 1 ms), with Δx, Δy and Δz drawn from a normal distribution with zero mean and standard deviation: \(\sqrt{\frac{2D_{\text{solvent}}}{\Delta t}}\). A normalization factor of \(\frac{1}{\sqrt{3}}\) for diffusion along each of the three dimensions x, y, and z was used to ensure that the 3D diffusion is equal to \(D_{\text{solvent}}\). In the model the distance between neighboring barriers (ΔL) is given by: \(ΔL = \frac{L_{\text{mito}}}{n+1}\) and the matrix-protruding length of each barrier equals \(2R_{\text{mito}} - ΔL\). The distance between both sides of the cylinder (Δv) is given by \(n+1\) times the length \((2R_{\text{mito}} - ΔL\) plus \(n\) times the distance (w) between two barriers on the same side of the cylinder (\(w = ΔL\)). Therefore, a cylindrically shaped mitochondrial with length \(L_{\text{mito}}\) and radius \(R_{\text{mito}}\), containing n barriers can be approximated by a new cylinder of length \(L_{\text{mito,new}}\):

\[
L_{\text{mito,new}} = (n + 1)\left(2R_{\text{mito}} - \frac{L_{\text{mito}}}{n + 1}\right) + n \frac{L_{\text{mito}}}{n + 1}.
\]

This means that increasing the number of diffusion barriers is equivalent to a longer cylinder with a smaller radius \(R_{\text{mito,new}} = \frac{L_{\text{mito,new}}}{L_{\text{mito}} + L_{\text{mito,new}}}\). This approximation is valid if \(w\) is smaller than \(2R_{\text{mito}}\) (i.e., when the number of cristae is sufficiently large). Considering the experimental values for \(R_{\text{mito}}\) and \(L_{\text{mito}}\), this implies that the number of barriers in the model should be 9 or larger. By adding barriers (i.e., by increasing the value of \(n\)) the FRAP region has a length of \(L_{\text{mito,new}}\) in the new equivalent cylinder. Because this equivalent cylinder is always longer in the presence (hindered model) than in the absence (unhindered model) of barriers, molecules have to diffuse over a longer distance before entering the FRAP region. Intuitively, this longer diffusion distance will result in a slower FRAP recovery and thereby a larger recovery time constant \((T_{\text{FRAP}})\) in the presence of barriers. The topological equivalence of a mitochondrial cylinder with diffusion barriers to a longer and thinner cylinder is a special case applying to mitochondria. This means that the previously described analytical solution for FP diffusion in a mitochondrial cylinder (7, 8) could also be used to iteratively determine \(D_{\text{solvent}}\).

Calculation of Solvent Viscosity from the Protein Diffusion Constant. A modified version of the Stokes–Einstein relationship can be used to predict the solvent viscosity \(η_{\text{solvent}}\) (in centipoise; cP) for freely diffusing spherical and cylindrical FPs in an aqueous solution (44):

\[
D_{\text{solvent}} = \frac{6.85 \times 10^{-8}}{η_{\text{solvent}} \cdot \sqrt[3]{M^{1/3} \cdot R_G}}. \tag{6}
\]

Here, \(D_{\text{solvent}}\) represents the solvent-dependent FP diffusion constant (in cm²/s⁻¹), \(M\) represents the FP molecular weight (MW); in g mol⁻¹, \(R_G\) represents the FP radius of gyration (in Å) and \(T\) indicates the temperature \(\theta\) (in K). For AcGFP1, \(M\) equals 29149 g mol⁻¹ and the \(R_G\) value can be calculated from the hydrodynamic radius \(R_H\) according to (45):

\[
R_G = \frac{\sqrt{3}}{5} \cdot R_H. \tag{7}
\]

For GFP, an \(R_H\)-value of 20 Å was reported (46), meaning that the \(R_G\) value of AcGFP1 equals 15.5 Å, compatible with molecular modeling results (45).
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Supporting Information

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Fig. S1. Effect of the size of the FRAP region on the AcGFP1 FRAP curve. (A) Cartoon representing the experimental dimensions of the FRAP region. Either the default size of 1.4 μm × 1.4 μm (upper figure), or a size of 1.4 μm × 2.8 μm (lower figure) was used. (B) Value of $T_{\text{FRAP}}$ (larger is slower) for the two sizes of FRAP region. (C) Value of $F_m$, the mobile fraction calculated from the FRAP curve, for the two sizes of FRAP region. In this figure, values on the Y-axis represent the average of the indicated number (N) of experiments. The error bar represents SEM. Statistics: significantly different from the 1.4 μm × 1.4 μm FRAP region (***$p < 0.001$).