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Supporting Methods

FRAP Validation. The FRAP method is based on the following assumptions, which are often implicit and rarely checked: (*i*) bleaching during the bleach pulse occurs in the illuminated volume only, and (*ii*) fluorescence recovery is caused by and proportional to the number of fluorescent molecules coming back into the observation area/volume. The latter assumption implies that (*ii*a) the photobleached region does not move relative to the laser beam; (*ii*b) photobleached molecules do not recover their lost fluorescence, otherwise relaxation could occur with immobile molecules also; and (*ii*c) the intensity level is proportional to the number of fluorescent molecules returning into the excitation volume. To provide quantitative interpretations, these points were carefully evaluated as follows.

(*i*) Bleaching indeed only occurs while molecules reside inside the excitation volume, but bleached molecules can be transported away. If the bleach pulse lasts for a long enough time for that transport to occur, the bleached volume will be larger than the excitation volume. This depletion effect, which is often overlooked, has two major consequences: (*i*) it leads to a shallower bleaching depth, and (*ii*) it interferes with the apparent time scales of the fluorescence relaxation curves. To circumvent these difficulties, one alternative is to make the bleach pulse duration shorter than the shortest expected diffusion time ("shallow-FRAP" bleaching). In that case, rigorous mathematical tools yield precise estimates of diffusion coefficients, provided a number of assumptions are met concerning the geometry and the dynamics of the model: analytical solutions have been given for recovery curves, initially for 2D diffusion (1) and recently for 3D diffusion with two-photon excitation (2).

However, this protocol of shallow-FRAP bleaching leads to such a small amplitude relaxation signal that one must repeat the bleach-relaxation measurement sequence a very large number of times on the point of interest---typically 1,000 times (2). Therefore, this repetitive approach is valid only under the assumption that: (*i*)

successive bleach-relaxation sequences are equivalent; in other words, the relaxation is complete before the next sequence starts; and (*ii*) the point that is repeatedly sampled remains immobile over the whole duration of the cyclic repetition. These two hypotheses are not met in the present situation, because the long relaxation times we found are much longer than the time scale at which microvilli move. Consequently, a 50-ms pulse duration was chosen as a trade-off between long bleaching pulse durations that yield a larger relaxation amplitude and short ones that limit fluorescence depletion and thus give access to short relaxation times. In turn, spatial depletion did occur for mobile proteins. This was taken into account in the numerical simulations (see *Diffusion*, below). An extended description of this effect will be presented elsewhere (F.W. *et al*., unpublished work). An important parameter is the size of the region bleached beyond the boundary of the point-spread function at the end of the bleach pulse: our numerical simulations allow estimation the widening of the full width at half maximum to 0.8 μm for diffusion of $D = 0.8\ \mu m^2 \cdot s^{-1}$, 1.6 μm for diffusion of $D = 10\ \mu m^2 \cdot s^{-1}$.

(*ii*a) Confluent LLC-PK1 cells have very limited overall motion on the time scale of hours, but their microscopic substructures such as microvilli do move on a much shorter time scale, with a typical amplitude that cannot be neglected relative to the size of the two-photon excitation volume. To resolve fluorescence relaxation caused by molecules coming back into the excitation volume from that caused by a motion of the bleach volume relative to the laser beam position, we measured these motions with a temporal resolution of 2 s and a spatial resolution of 0.1 μm, by fast 3D fluorescence microscopy coupled to image deconvolution (3, 4). For LLC-PK1 microvilli, we found a typical amplitude of 0.1 μm over 10 s (Fig. 7). The duration of the fluorescence relaxation observation was thus limited to 10 s, a time scale over which motion amplitude was negligible compared to the focal volume size. An additional indication that microvilli movements did not influence measured ezrin dynamics is that the recovery measured for ezrin was not altered when the cells were treated with jasplakinolide, an F-actin stabilizer that blocks the movement of microvilli.

A detailed study of these motions and their cause will be presented in a separate work (S.C. *et al*., unpublished work). Briefly, our results suggest that microvilli have a constant length over minutes, and that their uncoordinated motions are essentially driven by the basal polymerization--depolymerization of actin in the terminal web.

(*ii*b) A limited recovery (7 % in 10 s) was observed on immobilized proteins in cells fixed for 15 min with 4% paraformaldehyde/0.25% glutaraldehyde, experimental conditions in which no residual motions remain (5). This weak recovery did not modify significantly the interpretation of our data. Such a limited recovery for immobile proteins has already been described (6). This limited spontaneous fluorescence recovery of bleached molecules is possibly related to the reported blinking behavior (7). Arguments for this statement are based on a detailed experimental study of the bleaching kinetics of fixation-immobilized green fluorescent protein (GFP) molecules and a novel theoretical analysis of fluorescence emission statistics (F.A., unpublished work).

(*ii*c) To count the molecules that come back into the focal volume, the measured fluorescence should ideally be proportional to their number, with a constant proportionality ratio. However, the continuous illumination level required to count molecules leads to a limited but unavoidable photobleaching process, so long as molecules remain inside the excitation volume, which was referred to as "observation bleaching" (Fig. 8 *a* and *b*). In the present work, the observation power, 2 mW, was chosen to have a good signal-to-noise ratio with minimal observation bleaching. To quantify the effect of observation bleaching, the postpulse recovery sampled at 10 s only ("end-point sampling") was compared with the value obtained at 10 s with the standard protocol. Because the extent of observation bleaching is related to the average residence time of the choromophore inside the excitation volume, the observation bleaching did not affect the counting of fast-moving proteins, which eventually reach a full recovery. For low-mobility molecules, observation bleaching led to an underestimation of recovery amplitude by typically 15% at 10 s, as assessed by comparison with end-point sampling data. This fact was taken into account, and results were linearly rescaled using end-point sampling data. Observation bleaching was not taken into account in numerical simulations, because we observed that the

photobleaching of GFP did not obey a simple first-order kinetics (not shown), precluding the use of a simple exponential model.

Interpretation Frame, Simulations, and Fitting Procedures

Fluorescence recovery occurs as a consequence of the gradient of fluorescent molecules created by the brief photobleaching pulse. Because the total concentration of the protein remains constant, the chromophore motions underlying fluorescence relaxation are not induced by the bleaching pulse but reflect only the intrinsic motions of the chromophore-bearing molecule. In the case of ezrin, the basic cause of relaxation is protein diffusion, by which, for instance, cytosolic molecules uniformly dispersed in the cytoplasm are efficiently brought into the vicinity of the membrane. In this context, the membrane-bound compartment can be supplied by the cytosolic compartment or depleted by dissociation from the membrane. Depending on which one is the slowest process, either diffusion, association with, or dissociation from the membrane compartment, the fluorescence of the latter compartment will follow distinct kinetics behaviors.

This background led us to propose an interpretation of the FRAP data in the following terms: (*i*) fluorescence relaxation is kinetically limited either by diffusion or reaction, in such a way that only one of the two corresponding uncoupled equations must be considered at a given time scale; and (*ii*) processes at distinct time scales are uncoupled. This scheme corresponds to a small number of adjustable parameters.

Reaction-Limited Exchange. If ezrin binds to a saturable pool of immobile sites at the membrane, the fluorescence of the membrane-bound pool can recover only if bound molecules detach to leave empty sites free for fluorescent molecules. Quantitatively, we assumed here that the replenishment of these sites is allowed by a fast enough diffusion from the cytosol but limited by the rate at which ezrin-binding sites are stochastically emptied. We also assumed that the dissociation can be described as a first-order reaction. Because these assumptions apply to all sites regardless of their location within the excitation region, the exponential relaxation also applies to the measured fluorescence, and the fit of the relaxation curves directly yields a time-constant occupancy lifetime.

The above hypotheses are justified by the following facts: (*i*) the measurements are performed over a time scale at which ezrin and actin distribution remains stationary; and (*ii*) the bleaching is a local perturbation in comparison with the volume of the cell.

Diffusion. In the diffusive case, the position r relative to the excitation volume is a variable in the classical diffusion equation $\partial_t C(r,t) = D\,\Delta C(r,t)$ obeyed by the local concentration of fluorescent molecules with diffusion coefficient D. Analytical treatments available for that equation do not apply to the complete equation that must be solved here: $\partial_t C(r,t) = D\Delta C(r,t) - k_b I^2(r) C(r,t)$ (C, concentration; r, position; D, diffusion coefficient; $I(r)$, illumination intensity distribution; k_b, bleach constant, determined on immobile proteins). The last term corresponds to the photobleaching process modelled as a first-order process. Because significant GFP photobleaching does require a bleach pulse (50 ms) that is long compared to the short diffusion time of a fast-moving protein, diffusion cannot be neglected over that time scale, and the resulting fluorescence depletion in the vicinity of the excitation volume can be assessed only by solving the full nonhomogeneous equation. This was done numerically with the centered difference algorithm (custom program written in C) in 1D geometry with reflective boundary conditions. The solution was computed over a domain much larger than the extent of the fluorescence excitation region. A 1D geometry was taken for these simulations, as a reasonable approximation of a geometry that not only is very complex in shape and dimension (microvilli length for instance) but also is not uniform over the cell surface. For that reason, experiments were carried out over very extensive statistical samples, and the biological conclusions of the present paper essentially depend on the relative characteristics of the different mutants. Note that the first-order term bleaching $k_b I^2(r) C(r,t)$ is an approximation used by all authors that is correct only for short times but quite wrong over long times. Therefore, this term was dropped for simulating relaxation curves.

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