Exchanges Slowly From Pentamers but Rapidly From the SERCA Regulatory Complex

Seth L. Robia, Kenneth S. Campbell, Eileen M. Kelly, Zhanjia Hou, Deborah L. Winters, David D. Thomas

Abstract—Phospholamban (PLB) or the sarcoplasmic reticulum Ca\(^{2+}\)−ATPase (SERCA) were fused to cyan fluorescent protein (CFP) and coexpressed with PLB fused to yellow fluorescent protein (YFP). The expressed fluorescently tagged proteins were imaged using epifluorescence and total internal reflection fluorescence microscopy. YFP fluorescence was selectively bleached by a focused laser beam. CFP fluorescence at the targeted site increased after YFP photobleaching, indicating fluorescence resonance energy transfer between CFP-SERCA/CFP-PLB and YFP-PLB. The increased donor fluorescence relaxed back toward baseline as a result of donor diffusion and exchange of bleached YFP-PLB for unbleached YFP-PLB, which restored fluorescence resonance energy transfer. Requenching of CFP donors, termed Förster transfer recovery (FTR), was quantified as an index of the rate of PLB subunit exchange from the PLB:SERCA and PLB:PLB membrane complexes. PLB subunit exchange from the PLB:SERCA regulatory complex was rapid, showing diffusion-limited FTR (τ=1.4 second). Conversely, PLB:PLB oligomeric complexes were found to be stable on a much longer time scale. Despite free lateral diffusion in the membrane, they showed no FTR over 80 seconds. Mutation of PLB position 40 from isoleucine to alanine (I40A-PLB) did not abolish PLB:PLB energy transfer, but destabilization of the PLB:SERCA complex was apparent from an increased FTR rate (τ=8.4 seconds). Oligomers of I40A-PLB were stabilized by oxidative crosslinking of transmembrane cysteines with diamide. We conclude that PLB exchanges rapidly from its regulatory complex with the SERCA pump, whereas subunit exchange from the PLB oligomeric complex is slow and does not occur on the time scale of the cardiac cycle. (Circ Res. 2007;101:1123-1129.)

Key Words: phospholamban ■ SERCA ■ FRET ■ TIRF ■ crosslinking

The regulatory role of the pentameric form of PLB is not clear. However, shifting the pentamer/monomer ratio toward the monomer form can increase inhibition of the pump,12,14-16 and increasing SERCA/PLB ratio depolymerizes pentameric PLB.17 Taken together, these results suggest that the monomer form is the active species, and the PLB:PLB and PLB:SERCA interactions act as interdependent, balanced equilibria.

The affinity of the regulatory interaction has been estimated from fluorescence resonance energy transfer (FRET) measurements,5 NMR,18,19 and electron paramagnetic resonance.6,18 Less is known about the on/off rates of PLB subunits from the pentamer and regulatory complex, although NMR experiments provide evidence that PLB can unbind from SERCA with rapid kinetics.20 In general, observation of the binding/unbinding rates of membrane protein complexes is hampered by the slow kinetics of membrane fusion; proteins in vesicles cannot be rapidly mixed or diluted. Instead, it is necessary to observe the interactions of proteins preequilibrated in the same membrane structure.
To investigate the dynamics of exchange of PLB subunits from the binding site on SERCA and from the PLB pentamer, we used spatially resolved acceptor photoobleaching to detect FRET between membrane protein binding partners. Subsequent exchange of bleached for unbleached acceptor-labeled PLB subunits was then observed as a recovery of FRET over time, a process we refer to as Förster transfer recovery (FTR). This approach may be generally useful for observing and quantifying subunit exchange of membrane protein complexes.

Materials and Methods

Molecular Biology and Cell Culture
Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were fused to the N terminus of PLB, and CFP was fused to the N terminus of SERCA. Recombinant plasmids were transfected into cultured AAV-293 cells by calcium phosphate precipitation. Please refer to the online data supplement, available at http://circres.ahajournals.org, for additional details.

Crosslinking and Western Blotting
Oxidative crosslinking was accomplished by exposing adhered AAV-293 cells expressing YFP-I40A-PLB to 5 mmol/L diamide for 10 minutes at room temperature. Cells were harvested with a cell scrapper in hys buffer consisting of PBS with 0.5% Triton X-100 and protease inhibitors (Complete Protease Inhibitor Cocktail, EDTA-free, Santa Cruz Biotechnology, Santa Cruz, CA). Cell lysates were subjected to vortexing and then centrifugation at 14,000 rpm for 20 minutes. Supernatant was diluted 10-fold in Laemmli buffer, heated to 40°C for 10 minutes, and subjected to electrophoresis on a 10% polyacrylamide gel. The protein was transferred to poly(vinylidene difluoride) membrane, probed with anti-PLB monoclonal primary antibody 2D12 and peroxidase-conjugated secondary antibody, and developed with dianinobenzidine color reagent.

Imaging
Fluorescence imaging was performed with an inverted microscope equipped with a 1.49 NA × 60 objective. For epifluorescence imaging, illumination was introduced through an excitation filter wheel equipped with 427/10 nm (for CFP) and 504/12 nm (for YFP) narrow band filters and a multiple band dichroic mirror. Emission was detected with a back-thinned electron-multiplying charged couple device camera (iXon 887, Andor Technology, Belfast, Northern Ireland) through an emission filter wheel, 472/30 nm (for CFP) and 542/27 nm (for YFP).

“Prismless” total internal reflection fluorescence (TIRF) used the 457.9-nm Ar laser line, directed through the objective with a multiple band dichroic mirror. TIRF emission was selected with filters described above. For spatially resolved, acceptor-selective photo-bleaching, the Ar laser 514.5-nm line was selected by a laser line filter and directed to the sample with a 10/90 beam splitter. The bleach beam was focused to a spot on the specimen using a Keplerian telescope composed of 2 planoconvex lenses. Laser photobleaching exposure time was controlled by a Uniblitz shutter and was typically 100 to 500 ms. Filter transitions and shutter events were automated with acquisition software to give reproducible timing. Image records from multiple experiments were averaged together for improved signal/noise ratio and to reduce intensity fluctuations caused by membrane organelle structural dynamics. Sample sizes were: CFP-SERCA, n = 15; CFP–wild type (wt)PLB, n = 9; CFP-I40A-PLB, n = 6; CFP-I40A-PLB+diamide, n = 10. Averaged postbleach images from multiple experiments were averaged together for improved signal/noise ratio and to reduce intensity fluctuations caused by membrane organelle structural dynamics.

Results

Localization
Fluorescence microscopy of AAV-293 cells showed that CFP-SERCA was exclusively localized to internal membrane structures, probably the endoplasmic reticulum (ER). YFP-PLB colocalized with SERCA in the ER but was also observed at the plasma membrane (PM). Fluorescently labeled PLB in the basal PM that contacted the glass coverslip culture surface was readily detected by TIRF. In some cells, a portion of the ER was positioned very close to this basal membrane (<1 μm). In these cells, ER-localized proteins were detected by TIRF. Figure 1 (left) shows a TIRF image of CFP-SERCA, which had a reticulated distribution consistent with ER membrane ultrastructure. YFP-PLB (Figure 1, middle) colocalized with this reticulated pattern, and there was an additional YFP-PLB fraction in the basal PM, visible as a smooth, evenly fluorescent area of contact between the membrane and the glass substrate. Colocalized CFP-SERCA and YFP-PLB appeared white in an additive image overlay (Figure 1, right).

The membrane being observed (PM or ER) was selected by adjusting the angle of incidence of laser excitation (θ), which determines the depth of the evanescent field. At extreme laser angles, TIRF illumination was highly selective for the PM, SERCA fluorescence was very dim or undetectable, and the PM fraction of YFP-PLB was selectively illuminated. At smaller angles (closer to critical angle), CFP-SERCA and YFP-PLB in the ER became apparent. This transition away from PM selectivity started to occur at a TIR laser angle of...
≈65° from normal. This value of θ corresponds to a field
depth (d) of 100 nm, according to the relationship d=λ/4π(n² sin²θ−n₀²)½, where λ is the wavelength of the excitation light (457.9 nm).²³

The network pattern of the ER complicated quantitative analysis of TIRF images of CFP-SERCA, particularly because the ER structure was observed to be highly dynamic on the seconds time scale. Spurious intensity fluctuations arising from ER structural remodeling were circumvented by image averaging of many individual experiments. This also smoothed the reticulated fluorescence distribution into a uniform field.

**Regulatory Complex Subunit Exchange**

When 2 fluorescent probes are brought into close proximity (<100 Å), they can undergo fluorescence resonance energy transfer (FRET).²⁴ FRET between CFP-SERCA and YFP-PLB was detected by acceptor-selective photobleaching. Selective photobleaching of YFP by brief exposure to a focused spot of 514-nm laser illumination (Figure 2, arrow) resulted in a local increase in CFP fluorescence at the target site. A CFP-SERCA F/F₀ ratio image (postbleach/prebleach) indicated that the 20% increase in the CFP-SERCA fluorescence was restricted to the target site (Figure 2). Donor enhancement after acceptor photobleaching is diagnostic of FRET.²⁵ Before YFP photobleaching, CFP fluorescence was quenched by FRET and then destruction of YFP relieved quenching of CFP donor. To quantify CFP-SERCA dequenching and subsequent processes, the cells were imaged with TIRF, which simplified the observation volume to an optical section of the near-basal region of the cell. A CFP-SERCA F/F₀ ratio image (prebleach/postbleach) indicated that the average enhancement of CFP-SERCA donor fluorescence at the first postbleach time point (2.5 seconds postbleach) was 8%. The average efficiency of energy transfer from CFP-SERCA to YFP-PLB was estimated from the Y-intercept of a linear regression of a plot of CFP F/F₀ versus YFP F/F₀ (Figure 3B), which was taken to represent donor fluorescence in the absence of acceptor (F₀).

FRET efficiency was 13%, according to the relationship E=1−(F₀/F₀). The profile YFP-PLB fluorescence at 2.5 seconds postbleach exhibited a minimum of 45% of initial fluorescence (Figure 3A, green triangles). Figure 3C shows the evolution of the donor (blue circles) and acceptor (green triangles) fluorescence signals. After photobleaching, the acceptor fluorescence in the bleach spot exhibited fluorescence recovery after photobleaching (FRAP), indicating lateral mobility of the YFP-PLB in the membrane (Figure 3C, green triangles). Concomitant with FRAP in the acceptor channel, the fluorescence of CFP-SERCA, which was dequenched by the photobleaching of its FRET partner, relaxed toward baseline (Figure 3C, blue circles). This exponential relaxation of donor fluorescence may be attributed to 2 distinct processes: (1) lateral diffusion of dequenched CFP-SERCA out of the region of interest; and (2) subunit exchange of PLB from PLB:SERA regulatory complexes, which restores energy transfer (FTR). Line-out Gaussian analysis through the image time series showed that the YFP-PLB bleach spot profile evolved with respect to fit parameter σ² (variance) as a result of lateral diffusion of...
that the enhancement of CFP-wtPLB fluorescence at the 2.5 second time point was 45%. The YFP-PLB bleach spot profile showed a minimum of 60% of initial fluorescence (Figure 4A, green triangles). The average efficiency of energy transfer from CFP-wtPLB to YFP-wtPLB was 54%, as obtained from the y-intercept of a linear regression of a plot of CFP F/F₀ versus YFP F/F₀ (Figure 4B), as described above. Figure 4C shows the evolution of the donor (blue circles) and acceptor (green triangles) fluorescence signals. As in the experiments with the regulatory complex, the fluorescently labeled PLB was laterally mobile in the membrane. YFP-PLB fluorescence in the target region recovered from spot photobleaching (Figure 4C, green triangles). The enhanced fluorescence of CFP-PLB in the region of interest also relaxed exponentially (Figure 4C, blue circles). Notably, this donor fluorescence relaxation was entirely attributable to diffusion of dequenched donors out of the target region.

Figure 5. Immunoblot of YFP fusions of PLB variants probed with anti-PLB monoclonal antibody 2D12 showing pentamer (P) and monomer (M) forms. Lanes from left are: wtPLB (1), oligomer-destabilized PLB (I40A-PLB) (2), I40A+diadime (3), I40A-PLB + Diadime + jIME (4), Cys-null PLB (AFA-PLB) (5), AFA-PLB + diadime (6), and untransfected AAV-293 cell homogenate control (7). The intermediate-sized band (*) present in all samples is a non-PLB cross-reaction.

PLB Pentamer Subunit Exchange

Because of the PM localization of a fraction of PLB expressed in AAV-293 cells, CFP/YFP fusions of PLB could be observed with high signal/noise using TIRF illumination. Spot photobleaching of YFP-PLB resulted in enhanced fluorescence of the CFP-PLB donor, indicating intrapentameric FRET. The peak of the donor Gaussian (Figure 4A, blue circles) obtained from the average (n=9) F/F₀ image showed the time evolution of PLB oligomer-destabilized PLB (I40A-PLB) (2), I40A+diadime (3), I40A-PLB + Diadime + jIME (4), Cys-null PLB (AFA-PLB) (5), AFA-PLB + diadime (6), and untransfected AAV-293 cell homogenate control (7). The intermediate-sized band (*) present in all samples is a non-PLB cross-reaction.
Discussion

Förster Transfer Recovery

The rapid mixing techniques that have proven so useful for protein–protein binding kinetics are difficult to apply to membrane proteins. Separately prepared proteins in vesicles do not encounter one another until their respective vesicles fuse, and once they are trapped in the same membrane structure, they cannot be rapidly diluted. The FTR experiments described here provide a general approach to quantifying membrane protein–protein binding dynamics. By observing the relaxation of a perturbed FRET pair, one gains access to the binding and unbinding of proteins from complexes at equilibrium. Because both the detection (imaging) and perturbation (photobleaching) are optical methods, these experiments can be performed in living cells. For FTR experiments in which the donor-labeled protein is a diffusible species, it is important to account for both the donor brightness and spatial distribution, because diffusion can also result in donor-intensity relaxation in the area of interest. In the present study, our approach was to explicitly measure the intensity and distribution of fluorophores in the target area, but other solutions can be envisioned for the quantification of FTR. For the current experiments, FTR time resolution is limited to a window of seconds to hundreds of seconds. The theoretical rapid limit of this technique is determined by the minimum diffusion time of a diffraction-limited spot, ie, 100 ms for a 200-nm spot. However, a practical limitation is the decrease in signal/noise ratio with decreasing spot size, because of both fewer fluorophores observed and the shorter camera integration times required for faster diffusion/exchange processes. The slow limit is set by the time of equilibration of diffusible membrane probes in the area of contact between the membrane and the glass substrate on the basal surface of a cultured cell, which has an area on the order of 500 μm². Longer time regimes would be accessible for proteins reconstituted into planar lipid bilayers. Such reconstituted systems would also give control of protein concentration, permitting explicit determination of on and off rate constants.

The partial recovery of YFP-PLB fluorescence shown in Figure 3C and Figure 4C is typical; we have never observed YFP fluorescence return to baseline (ie, 100% FRAP), even with soluble, cytosolic YFP (not shown). This is commonly reported in the literature and is usually attributed to an “immobile fraction” of poorly diffusible species. In our experiments, a more significant factor is depletion of the total cell brightness by the photobleaching of a fraction of the fluorescent species. Neither the fractional bleaching of YFP nor its fractional recovery affects the present study, because the analysis is directed at the time dependence of these transients rather than their absolute magnitude.

In our experiments, the FTR method was complimented by TIRF microscopy, which selectively illuminated cells cultured on glass cover slides to a depth of ~100 nm. This method was suitable for imaging CFP-SERCA and YFP-PLB in the PM-adjacent portions of the ER and was particularly effective for detecting PLB in the PM. The PM pool of PLB may be mislocalized “spillover” resulting from overexpression. It is also possible that PLB has undiscovered regulatory roles for PM-localized PLB.

PLB Subunit Exchange

Based on observed differential rates of FTR, we propose that PLB exchanges rapidly from its binding site on SERCA but slowly from PLB pentamers. This kinetic model is summarized in Figure 7, which represents PLB monomers (PLB₁) binding and unbinding from the pentamer (PLB₅) and the
that is not abolished by calcium or phosphorylation. An avid PLB-SERCA interaction has been described previously as dynamic, although usually in the context of a putative binding/unbinding of PLB en masse from the population of pumps in response to calcium binding to the pump and PLB phosphorylation. The present experiments focus on the exchange kinetics of PLB in heterologous cells at rest, in the absence of Ca\(^{2+}\) oscillations or stimulated kinase activity. However, the pump is never static, even during diastole. At a “resting” calcium concentration of 100 nmol/L, calcium forward and reverse fluxes balance at \(\approx 21 \mu\text{mol/L/s}\). With a pump concentration of 15 \(\mu\text{mol/L}\), this implies that the entire pump population undergoes E1–E2 structural transitions every few seconds. This is compatible with the kinetics of SERCA PLB exchange observed by FTR. This study provides direct evidence for a rapidly dynamic interaction in unstimulated heterologous cells in which the cytosolic calcium is below the threshold of pump activation. Using FTR, we have established an upper limit of 1.4 seconds for the time constant of the binding/unbinding of PLB to/from SERCA. Thus, rather than being a static structure, the regulatory complex may be regarded as the steady-state result of continual binding and unbinding of PLB from its target. The perpetual dynamics of this interaction may contribute to the difficulty in obtaining PLB-SERCA cocrystals suitable for x-ray diffraction studies.

Furthermore, the present data do not necessarily conflict with studies that describe a “monomeric” PLB, because, unlike wtPLB, it is not abolished by calcium or phosphorylation. An interaction that is high affinity \(K_d<\text{protein concentration}\) can still support rapid binding/unbinding. The equilibria and the kinetics are related as follows: dissociation constant = \(k_{\text{off}}/k_{\text{on}}\) and exchange rate = \(k_{\text{off}}+k_{\text{on}}\). This means the same equilibrium could be achieved with fast kinetics or slow kinetics. In a simplified example, \([k_{\text{off}}/k_{\text{on}}=1/2]\) and \([k_{\text{off}}/k_{\text{on}}=10/20]\) both yield the same dissociation constant (they have the same affinity) but give different exchange rates of 3 and 30, respectively. Both PLB and SERCA are present in large amounts in the SR membrane at concentrations that greatly exceed the dissociation constant, resulting in a large proportion of pumps being bound to PLB in the steady state. In general, a reversible interaction (with a large off rate), compensated by high ligand concentration, is characteristic of a rapidly responsive system.

It is remarkable, then, that PLB pentamers are stable on the minutes time scale. This indicates that monomers (the active species) cannot exchange from PLB complexes (the inactive pool) during the 1-second time scale of the cardiac cycle. The monomer/pentamer ratio is not expected to follow putative fluctuations of PLB-SERCA binding and would integrate the time-averaged concentration of free monomer into the monomer–pentamer equilibrium. It is possible that the equilibrium is regulated over a longer period by sustained signals, such as phosphorylation. The kinetics of PLB oligomerization is significantly altered by mutation of the isoleucine at position 40 to alanine. This mutant has been described as a “monomeric” PLB, because, unlike wtPLB, it runs as a monomer on SDS–polyacrylamide gels. However, the present FRET results indicate that the I40A mutant is still able to form oligomers. This is consistent with the observations of Stokes et al, who detected pentamers of I40A-PLB by electron microscopy of 2D crystals. Similarly, the Cys-null mutant Ala-PLB has been shown to form oligomers in DMPC bilayers. Cornea et al showed that several weakly pentameric PLB mutants are progressively dissociated by increasing concentrations of SDS. Together with the present study, these reports underscore the importance of measuring membrane protein–protein interactions in the membrane environment. Detergents used to solubilize membrane proteins for coimmunoprecipitation or SDS-PAGE experiments may disrupt physiologically important interactions.

The rapid FTR observed for I40A (Figure 4) suggests that these complexes exhibit much faster subunit exchange kinetics compared with wtPLB. If both bind with a diffusion-controlled off rate, then the pentamer-to-monomer off rate must be increased for I40A. Importantly,-crosslinking of I40A-PLB transmembrane cysteines by diamide stabilizes the complex and prevents FTR. This demonstrates that the FRET signal arises from protein–protein interactions, rather than nonspecific energy transfer between highly concentrated donors and acceptors. Furthermore, it shows that oxidation is an important determinant of I40A-PLB oligomerization. Wild-type PLB is also subject to cysteine oxidation; presumably crosslinking would further stabilize the wtPLB pentamer. This may be an important mechanism in conditions of oxidative stress, such as myocardial ischemia.

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Disclosures

None.

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Förster Transfer Recovery Reveals That Phospholamban Exchanges Slowly From Pentamers but Rapidly From the SERCA Regulatory Complex

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Supplemental Materials and Methods

**Molecular Biology and Cell Culture:**

Cyan/Yellow Fluorescent Protein fusions to the N-terminus of PLB were created by subcloning canine PLB into ECFP-C1 and EYFP-C1. A Cyan Fluorescent Protein fusion to the N-terminus of SERCA was created by subcloning rabbit SERCA1a into ECFP-C3 (frame-shifted variant of commercial vector ECFP-C1). SERCA1a can functionally substitute for SERCA2a, the predominant cardiac isoform, and is regulated by phospholamban\(^1\)\(^-\)\(^3\). In particular, the regulation by PLB and its analogs is quantitatively identical for SERCA1a and SERCA2a, as shown by co-expression\(^4\) and reconstitution\(^5\). Mutagenesis of fluorescent proteins (A206K monomerization mutation) and phospholamban (I40A) was performed with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and custom oligonucleotide primers. Mutations were confirmed by sequencing.

AAV-293 cells were cultured on polylysine coated glass-bottom dishes in complete DMEM growth medium with 10% fetal bovine serum (heat inactivated), 2mM L-glutamine, 5% penicillin streptomycin. AAV-293 cells were transfected according to manufacturer’s instructions, using MBS Mammalian Transfection Kit (Stratagene, La Jolla, CA). 6% modified bovine serum was used instead of 10% fetal bovine serum during the three hour incubation of AAV-293 with the CaPO\(_4\)-DNA precipitate. For FRET experiments, cells were co-transfected with plasmids encoding CFP/YFP-fusion proteins with 5-fold or 20-fold molar excess of acceptor plasmid.
Imaging:
For “prismless” total internal reflection excitation through the objective, the 457.9 nm line of a multi-line argon laser (Newport, Irvine, CA) was selected with a laser line filter of 1.7 nm bandwidth (Semrock, Rochester, NY), coupled to a single-mode fiber with an achromatic fiber coupler (Oz Optics, Ottawa, Ontario, Canada), and introduced into a Nikon TIRF II illuminator. To achieve sufficient laser power, it was necessary to remove the non-selectable neutral density filter that is integrated into the beam path of the TIRF II illuminator. Laser excitation for TIRF was directed to the objective with a multiple band dichroic mirror (z457/514rpc, Chroma Technology Corp., Rockingham, VT). TIRF emission was selected with a 463.9 nm long pass filter and a 472/30 nm filter (for CFP) or a 542/27 nm filter (for YFP). The depth of the TIRF illumination was controlled by adjusting the angle of incidence of the beam on the interface between the coverglass and specimen. The incident angle during TIRF was estimated by calibrating the beam adjustment actuator position to the angle of the beam exiting the objective over a range of sub-critical angles. By this method, the transition point at which total internal reflection began to occur (the critical angle, $\theta_c$) was estimated to be $61.2^\circ$ from normal. This value is in good agreement with the expected critical angle, predicted to be $61^\circ$ according to $\theta_c = \sin^{-1}(n_w/n_g)$, where $n_w$ and $n_g$ are the refractive indices of water and glass, 1.33 and 1.52, respectively. Pseudocolor representation of monochrome image data was performed with the public domain software ImageJ.

Förster Transfer Recovery
Subunit exchange was quantified by analyzing the donor fluorescence intensity and spatial profile at the site of acceptor spot photobleaching. The fluorescence image of the target site can
be visualized as a 3-dimensional “volume”, with image dimensions of x,y (microns) and normalized fluorescence intensity in the z-dimension (F/F₀). Supplemental Figure S1 shows 3D representations (left) of a square region of interest rendered from donor and acceptor image data as a surface plot using ImageJ (plugin by Kai Uwe Barthel). The fluorescence of the acceptor (lower left) is depleted in the center of the target region, while the fluorescence of the donor (upper left) is enhanced, giving concave and convex surface plots, respectively. This “dequenching” of the donor is indicative of FRET. The widths of the donor and acceptor distributions increase over time due to diffusion of the donors in the membrane plane. The total volume of the donor distribution may also evolve if there is a change in the intrinsic fluorescence intensity of the CFP fluorophores. This could occur if subunit exchange of bleached for unbleached YFP reestablishes FRET, “requenching” the donor (FTR). To illustrate how the donor fluorescence profile may evolve with respect to width (diffusion), or with respect to the total amount of fluorescence (FTR), a cross-section through a target region is shown as a plot of fluorescence (F/F₀) versus position (Figure S1, right). The donor fluorescence distribution immediately after the bleach is well described by a Gaussian fit (black line). If the rate of diffusion is much greater than the rate of subunit exchange, the distribution will widen, but the integrated fluorescence under the curve (the volume of a 3D Gaussian) will not change over time. For this scenario, a single post-bleach time-point is simulated in Figure S1 (red profile). Contrariwise, if the subunit exchange process dominates, donor requenching (FTR) will cause a decrease in the integrated total fluorescence (volume of a 3D Gaussian), shown in Figure S1 as a green profile. This volume evolution is quantified as a measure of subunit exchange. If diffusion and subunit exchange occur with comparable kinetics, both the width and volume will
change (not shown), and these processes can be independently quantified as separate parameters of a Gaussian fit.

References:

Supplemental Figure Legend:

Figure S1: (Left) The CFP donor (blue) and YFP acceptor (yellow) fluorescence profiles immediately following acceptor-selective spot photobleaching, rendered as 3D surfaces. (Right) A cross-section through the 3D “volume” yields a 2D plot of CFP fluorescence (F/F₀) vs. distance (µm) across the target site. The image data (blue circles) are well-described by a Gaussian fit (black line). Diffusion causes widening of the Gaussian (red profile), while subunit exchange resulting in FTR decreases the total fluorescence integrated under the curve (green profile).
Supplemental Figure S1