Improving the Accuracy and Scope of Quantitative FRAP Analysis

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Abstract

New mathematical models are presented for analysis of fluorescence recovery after photobleaching (FRAP) data to account for the conical geometry of photobleaching, for spatial variations in binding of fluorescent molecules, and for directed transport of fluorescent molecules. A fast computational method for calculation of fluorescence recovery with this conical geometry is presented, and is shown to be applicable in calculations with spatial variations. Some of the new models are applied to experimental data from a previously published paper (Biophys. J. 95:3048) and are found to reinforce the existing conclusions, and furthermore to suggest that the protein γ-tubulin is likely to be uncomplexed in the cytoplasm.

Introduction

Fluorescence recovery after photobleaching (FRAP) is a powerful technique used to measure the dynamics of mobile fluorescent molecules in vivo, particularly diffusion, binding to non-diffusing structures, and directed transport due to the motion of those structures. Many mobile cellular proteins can be made fluorescent by appending the DNA for a fluorescent protein such as green fluorescent protein (GFP) to their genes, and such GFP fusion proteins are the molecules most studied by FRAP, as they typically do not differ functionally from their wild-type (non-fused) equivalents. FRAP involves photobleaching (i.e. deactivating fluorescence in) microscopic areas of samples with intense laser pulses and then monitoring fluorescence in those areas as unbleached molecules enter and the equilibrium level of fluorescence is reestablished. Many studies involving FRAP have been carried out without quantitative analysis, to compare the general rates of some protein motion, such as diffusion or directed transport, in a pair of systems (e.g. Ellenberg 1997). However, FRAP also shows promise for quantitative determination of kinetic parameters (Sprague 2005), provided that sufficiently accurate mathematical models can be developed.

Early work in quantitative FRAP models centered on photobleaching molecules confined to a membrane (Soumpasis 1983); such models were used to measure diffusion coefficients for 2D diffusion. More recently, applications to finding rate constants for binding of the proteins to immobile cellular structures have been explored (Bulinski 2001), as have been models that combine reaction and diffusion parameters in 3D (Sprague 2004). Such studies, all based on reaction-diffusion equations, have provided useful and computationally efficient solutions, but their approaches have often involved approximations that may be inconsistent with experimental conditions and thus induce significant errors, in many cases with regard to the geometry of the photobleaching beam (Hallen 2008a). In particular, they have tended to work in the same geometry as in a membrane, thus assuming for 3D systems a "cylindrical approximation" in which variables depend only on r in cylindrical coordinates and thus the bleaching beam is cylindrical, while in practice the bleach beam has more of a double-cone shape and thus there is significant dependence on z. These difficulties with geometry were addressed to some extent by (Braeckmans 2003); however, the applicability of their approach is
largely limited to large bleach spots and small-numerical-aperture lenses, conditions not satisfied by and not particularly convenient for many FRAP assays. Furthermore, though (Sprague 2006) have investigated the effects of having different binding and diffusion parameters inside and outside the bleach spot, we know of no model to date that accounts for spatial inhomogeneity of binding and diffusion within the bleach spot, which may have significant impacts when FRAP is used to study small cellular structures like centrosomes. Similarly, many problems in which FRAP is relevant involve dynamics in cellular structures that are a few times the size of a bleach spot. The effects of inhomogeneity outside the bleach spot have not, to our knowledge, been investigated before. Finally, recent work (Hallen 2008b) has extended FRAP modeling beyond reaction-diffusion conditions to a reaction-advection scenario, in which the protein binds to a substrate that is moving nonnegligibly; this model helped shed light on cytoskeletal dynamics in an assay with tight-binding protein, including the velocity of transport of the protein, i.e. the velocity of the cytoskeletal fibers to which it was bound. However, the model in this study was applicable only to the specific assay’s cytoskeletal motion, photobleaching, and fluorescence monitoring geometries and used a somewhat crude empirical approximation for the distribution of microtubule velocities. A more general development of the solution would increase versatility and likely accuracy.

This paper seeks to improve the accuracy and scope of quantitative FRAP modeling by taking these issues—optical geometry, spatial inhomogeneities of diffusion and binding, and reaction-transport systems—into account. Mathematical models are developed for reaction-diffusion and reaction-transport scenarios.

Models and Analytical Results

Two models were used for FRAP recovery: a reaction-diffusion model and a reaction-transport model. The timescale of the FRAP recovery is determined in large part by the tightness of the binding involved: less tight binding will lead to faster recoveries in which the substrate moves negligibly, making the reaction-diffusion model applicable, while tighter binding will allow free protein to nearly equilibrate diffusionally before significant bound protein recovery has occurred, causing the recovery of bound protein to fit better to a reaction-transport model if there is significant transport of that protein in the cell. Recovery in two phases (reaction-diffusion followed by reaction-transport) may also be observed (Hallen 2008b).

In each of the models, the reaction is the binding of free protein to a substrate, such as the cytoskeleton, where the binding rate is equal to a pseudo-first-order binding rate constant $k_{\text{on}}$ times the concentration of free protein, and the dissociation rate is equal to a dissociation rate constant $k_{\text{off}}$ times the concentration of bound protein, as in (Bulinski 2001) and (Sprague 2004).

Reaction-Diffusion Model

Assuming that the protein is either freely diffusing or bound to a stationary structure, the concentration of the protein that is unbleached (i.e. still fluorescent) is described by reaction-diffusion equations (Sprague 2004):
\[
\frac{\partial c}{\partial t} = k_{\text{on}} f - k_{\text{off}} c \\
\frac{\partial f}{\partial t} = D \nabla^2 f - k_{\text{on}} f + k_{\text{off}} c
\]

where \(c\) is the concentration of bound protein, \(f\) is the concentration of freely diffusing protein, and \(D\) is the diffusion coefficient. Fluorescence may be substituted for concentration because of the direct proportionality of fluorescence to concentration. We let the initial time (i.e. \(t=0\)) correspond to the time of bleaching. Initial conditions are determined by the bleaching. Boundary conditions are specified based on the observation that the bleaching is localized. The center of the bleached zone is taken to be the origin; \(g\) and \(b\) are assumed to decay to 0 along any line leading away from the origin.

The equilibrium values of \(c\), \(C_{eq}\), and of \(f\), \(F_{eq}\), are determined by the thermodynamics of the reaction:

\[
\frac{C_{eq}}{F_{eq}} = \frac{k_{\text{on}}}{k_{\text{off}}}
\]

with \(F_{eq}\) being the same everywhere in space, making the diffusion coefficient irrelevant. Define now the bound protein depletion \(b=C_{eq}-c\) and the free protein depletion \(g=F_{eq}-f\); these new quantities decay to 0 at large times and away from the origin, facilitating the use of Fourier transforms (Sprague 2004). The reaction-diffusion equations with the changed variables are

\[
\frac{\partial b}{\partial t} = k_{\text{on}} g - k_{\text{off}} b \\
\frac{\partial g}{\partial t} = D \nabla^2 g - k_{\text{on}} g + k_{\text{off}} b
\]

**Initial Conditions**

The initial conditions are determined by the equilibrium values as well as the illumination provided by the bleaching. FRAP bleaching is performed by scanning a laser beam over a region in the plane of focus; circular regions (or “bleach spots”) are a common shape that work well for most experimental systems. A laser beam focused at a single point illuminates a double-cone shape, with the shared tip of the cones at the focal point and the cone sides at an angle of

\[
\alpha = \sin^{-1} \left( \frac{NA}{n} \right)
\]

from vertical, where \(NA\) denotes the numerical aperture and \(n\) denotes the index of refraction (see Fig. 1). Convolution of this conical profile with the focal plane illumination profile allows the illumination profile to be calculated above and below the plane, thereby accounting for the effects of vertical diffusion:

\[
I(r, z) = I_{\text{focal plane}}(r) \otimes \begin{cases} 
0 & r > |z| \tan\alpha \\
1 & r < |z| \tan\alpha \\
\pi z^2 \tan^2\alpha & r \approx |z| \tan\alpha
\end{cases}
\]

where \(I\) denotes intensity of illumination, and \(I_{\text{focal plane}}(r)\) is the illumination intensity in the focal plane for a given \(r\) value.
Fig. 1. The conical bleach profile geometry. Bleaching is most intense near the focal plane. (A) A schematic diagram of a bleach profile. (B) Optical profiles, without diffraction, as a function of r and z. Left: Initial distribution of fluorescence (i.e. of unbleached protein), as given by equation (8). Right: P, as given by equation (10), with white indicating the highest value and black indicating 0.

The focal plane illumination is itself the convolution of the region scanned over (a larger circle) with the diffraction-limited point illumination. The latter is a Fraunhofer diffraction pattern known as the Airy disk. So given an intended bleach pattern in the focal plane, and letting $B=0$ outside this intended bleached zone and $B=1$ inside,
\[ I_{\text{focal plane}}(r) \propto \left( \frac{J_1 \left( \frac{2\pi r (NA)}{\lambda} \right)}{\frac{2\pi r (NA)}{\lambda}} \right)^2 \otimes B \]

(6)

where \( \lambda \) is the light wavelength, which is determined by the laser used to bleach, and \( J_1 \) is a Bessel function of the first kind. For the circular bleach spot of radius \( w \), \( B(r) = 1 \) if and only if \( r < w \).

Bleaching due to an illumination profile is governed by

\[ \frac{c_{\text{post-bleaching}}}{c_{\text{eq}}} = e^{-k_B I} \]

(7)

where \( c_{\text{post-bleaching}} \) is the concentration of unbleached protein after bleaching, \( c_{\text{eq}} \) is the concentration at equilibrium, \( I \) is the illumination at that point, and \( k_B \) is a space-independent constant. Equation (7) applies at every point in space to both the free and the bound protein concentrations.

A simpler profile that retains the conical geometry and has negligible error throughout most of space may be obtained by assuming that diffraction is negligible (Hallen 2008a). This is because the diffraction effects are noticeable only within a few tenths of a micron of the bleach spot edge. In this case the bleaching is described by

\[ \frac{c_{\text{post-bleaching}}}{c_{\text{eq}}} = e^{-k \left( \frac{w}{w + z \tan \alpha} \right)^2} \quad r < w + |z| \tan \alpha \]

\[ = 1 \quad r \geq w + |z| \tan \alpha \]

(8)

where \( w \) is the bleach spot radius (Fig. 1A) and \( k \) is a constant. The effect of diffraction is primarily to smooth out the sharp edge of this profile, which has very little effect on recovery since diffusion does the same thing for free protein in negligible time.

**Data Collection**

Experimental monitoring of the concentration is achieved by taking a timeseries of images that include the region of interest (ROI), which is typically the bleached area, and then averaging over the ROI. Thus, the observed data is

\[ \text{frap}(t) = 1 - \iiint_{\text{whole medium}} (P)(g + b)dV \]

(9)

where \( P \) is a function of space representing the effect of concentration at a given point in space on the imaged ROI fluorescence. When imaging and detection are performed under the same conditions, the light paths for bleaching a pixel (the laser scans through all the pixels that need to bleached) and measuring its fluorescence intensity are essentially identical and thus \( P \) is proportional to \( I \). However, it is useful in a confocal microscope to narrow the pinhole for detection but keep it wide open for bleaching, because in bleaching one needs to maximize power and in detection one needs to maximize resolution—without narrowing the pinhole the resolution will probably be inadequate for obtaining meaningful results. Narrowing the pinhole will exclude non-vertical rays as each point in a raster scan is imaged, resulting in selective imaging of the bleach spot in the focal plane. In the no-diffraction approximation, this can be approximated with axial resolution \( z_0 \) as
As noted above, the choice of concentration units is arbitrary, but it is most convenient to normalize the data so that \( \text{frap}(t) \) is 1 at equilibrium (Sprague 2004); any data set can be thus normalized, by choosing the normalization factor as a fit parameter as necessary (Hallen 2008a). In such a case, one can take a measurement of fluorescence before photobleaching, divide all later values of fluorescence by this value, and then let the equilibrium fluorescence be the "extent of recovery" (which is partially influenced by experimental error in the original measurements, and possibly by errors in the model used to analyze the data as well). It is also convenient to let \( F_{eq} + C_{eq} = 1 \); this provides a normalization for \( P \):

\[
\biggl\{ e^{-\left(\frac{z}{z_0}\right)^2} \quad r < w \\
0 \quad r \geq w
\biggr. \]

(10)

Note that when the ratio \( k_{on}^{*}/k_{off} \) varies spatially, \( F_{eq} + C_{eq} \) must vary as well since \( F_{eq} \) is the same everywhere; thus, one can choose some point at which \( F_{eq} + C_{eq} = 1 \) and normalize \( P \) accordingly.

The circular scanning region and corresponding conical illumination profile are symmetrical with respect to \( \theta \) in polar coordinates. We assume also that the reaction and diffusion parameters exhibit the same cylindrical symmetry; thus the differential equations may be reduced to two dimensions, \( r \) and \( z \):

\[
\frac{\partial b(r,z)}{\partial t} = k_{on}^{*}(r,z)g(r,z) - k_{off}(r,z)b(r,z)
\]

\[
\frac{\partial g(r,z)}{\partial t} = D(r,z)\left(\frac{\partial^2 g}{\partial r^2} + \frac{1}{r} \frac{\partial g}{\partial r} + \frac{\partial^2 g}{\partial z^2}\right) - k_{on}^{*}(r,z)g(r,z) + k_{off}(r,z)b(r,z)
\]

(12)

This model was derived with confocal microscopy in mind because of the substantial difficulty in quantitative imaging of a large, homogeneously fluorescent medium without some method of removing light from outside the focal plane, and the prevalence of confocal microscopy for that purpose. However, the model may also be useful in analyzing FRAP data obtained by TIRF microscopy, if the bleaching is performed by an external laser rather than by the evanescent wave (the penetration of the evanescent wave would then determine the axial resolution for image acquisition). If bleaching is performed by the evanescent wave, then the bleaching geometry is localized axially similarly to the acquisition geometry, suggesting the greater applicability of the model of (Bræckmans 2003).

A very useful approximation here is one that may be called the diffusion phase-binding phase approximation, derived by Sprague (2004) for their model but highly applicable to many bleaching systems and binding scenarios. In this approximation, recovery consists of a diffusional term plus a binding term with the diffusional term dominating in the first phase of recovery and then the binding term dominating in the ensuing second phase. The approximation is valid in the regime where binding takes place much more slowly than diffusional recovery, i.e. \( k_{on}^{*}w^2/D << 1 \) in their geometry. In such cases, binding has little effect on the recovery of the unbound protein, and
likewise the bound protein recovers essentially as if \( g=0 \) because \( g \) declines so quickly relative to the reaction timescale. Thus (3) becomes

\[
\frac{\partial b}{\partial t} = -k_{off} b \\
\frac{\partial g}{\partial t} = D \nabla^2 g
\]

(13)

Note that the second equation in (13) and its associated initial conditions have the solution \( F_{eq}(1 - \text{frap}_d(t)) \), where \( \text{frap}_d(t) \) is the fluorescence recovery for the equivalent FRAP system with no binding (i.e. with \( k^*_{on} \) changed to 0 everywhere but all other parameters kept the same). \( b \) decays exponentially; setting the extent of photobleaching to \( B=1 - \text{frap}(0)=1 - \text{frap}_d(0) \), we have \( b(0)=BC_{eq} \), and this gives the fluorescence recovery under the diffusion phase-binding phase approximation:

\[
\text{frap}(t) = F_{eq} \text{frap}_d(t) + C_{eq} (1 - Be^{-k_{off}t})
\]

(14)

For example, in the 2D model, \( B=1 \) and \( \text{frap}_d(t) \) is given by the Soumpasis equation,

\[
\text{frap}_d(t) = e^{-\frac{w^2}{2D}t} \left( I_0 \left( \frac{w^2}{2Dt} \right) + I_1 \left( \frac{w^2}{2Dt} \right) \right)
\]

(15)

with the second equation being the same as Eq. 8 from (Hallen 2008a), the diffusion phase-binding phase solution in the Sprague et al model. The diffusion phase-binding phase approximation allows a FRAP recovery curve, if it is in the right parameter region, to be rapidly evaluated for many values of \( k^*_{on}, k_{off}, \) and \( D \) given \( \text{frap}_d \); since \( \text{frap}_d \) for one diffusion coefficient can be obtained from \( \text{frap}_d \) for another diffusion coefficient just by changing the time units, this allows a spline of \( \text{frap}_d \) to be used in very rapid fitting (with no geometry-specific calculations in the iterations) for any bleaching and image acquisition geometry. It is also necessary in some of the more complicated FRAP systems described below.

The reaction-dominant approximation (Bulinski 2003) differs from the diffusion phase-binding phase approximation only in that it does not contain a diffusional phase, assuming it to be over negligibly fast, i.e. \( g=0 \); this is most accurate when \( C_{eq} \) is large. Applying (14) to reaction-dominant behavior gives

\[
\text{frap}(t) = 1 - BC_{eq} e^{-k_{off}t}
\]

(16)

which, in the traditional approximation \( B=1 \) (complete bleaching), is identical to the previous reaction-dominant model. If bleaching is incomplete, then this will create errors in the calculated \( C_{eq} \) but not in \( k_{off} \); incompleteness is a matter of setting a tolerance rather than absolute accuracy, since, based on (7), it is impossible to completely photobleach any area, and furthermore imperfect axial resolution will cause the acquired image to include some light from outside the focal plane, where bleaching is not quite as complete.

**Fast computation**

To allow a more rapid evaluation of the integrated fluorescence over time, an exact solution was derived using Fourier and Hankel transforms for the reaction-diffusion
recovery with spatially invariant parameters; the method is implemented for the conical bleach profile in the routine \texttt{frap\_ffi\_homogeneous} (see Supplemental Code).

Let
\[
\tilde{g}(\omega, q, t) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} g(r, z, t) J_0(qr) e^{-i\omega z} r dr dz
\]
be the Hankel transform with respect to \( r \) and unitary Fourier transform with respect to \( z \) of \( g \); similarly, let
\[
\tilde{b}(\omega, q, t) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} b(r, z, t) J_0(qr) e^{-i\omega z} r dr dz
\]
Then the reaction-diffusion system (3) becomes
\[
\frac{d}{dt} \begin{bmatrix} \tilde{b} \\ \tilde{g} \end{bmatrix} = \begin{bmatrix} -k_{\text{off}} & k^*_{\text{on}} \\ k_{\text{off}} & -D(\omega^2 + q^2) - k^*_{\text{on}} \end{bmatrix} \begin{bmatrix} \tilde{b} \\ \tilde{g} \end{bmatrix}
\]
which may easily be solved:
\[
\begin{bmatrix} \tilde{b} \\ \tilde{g} \end{bmatrix} = \exp \left( \begin{bmatrix} -k_{\text{off}} & k^*_{\text{on}} \\ k_{\text{off}} & -D(\omega^2 + q^2) - k^*_{\text{on}} \end{bmatrix} t \right) \begin{bmatrix} \tilde{b}_0 \\ \tilde{g}_0 \end{bmatrix}
\]
thus providing the spatially and transformed depletions at any point in time in terms of the spatially transformed initial conditions. The eigenvalues and eigenvectors of the matrix can be calculated analytically before beginning timesteps and then used for each step. This solution may easily be converted into the FRAP recovery curve using spatial averaging:
\[
\text{frap}(t) = 1 - \int_{0}^{\infty} \int_{-\infty}^{\infty} P(r, z) \left( g(r, z, t) + b(r, z, t) \right) r dz dr
\]
\[
= \int_{-\infty}^{\infty} \int_{0}^{\infty} \bar{P}(\omega, q) \left( \tilde{g}(\omega, q, t) + \tilde{b}(\omega, q, t) \right) q d\omega dq
\]
where
\[
\bar{P}(\omega, q) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} \int_{0}^{\infty} P(r, z) J_0(qr) e^{-i\omega z} r dr dz
\]
is the spatial Hankel and Fourier transform of the weighting function.

Use of this method relies on knowing the spatial transforms of the initial conditions as well as of the weighting function. The Hankel transform may be approached analytically; if, as in the zero-diffraction approximations noted above, the initial conditions are piecewise constant with respect to \( r \):
\[
g(r, z, 0) = \begin{cases} g_{\text{bleached}}(z) & r < r_{\text{bleach}}(z) \\ 0 & r > r_{\text{bleach}}(z) \end{cases}
\]
Then the Hankel transform of \( g \) is given by
\[
\int_{0}^{\infty} g(r, z, 0) J_0(qr) r dr = \frac{g_{\text{bleached}}(z) r_{\text{bleach}}(z) J_1(q r_{\text{bleach}}(z))}{q}
\]
and the Fourier transform can be calculated from this without problems using an FFT. P or b may be substituted for g in (23) and (24), so both initial conditions as well as the bleaching function may be handled analogously.

Spatial variation in parameters

Sometimes the reaction and diffusion parameters \(k_{on}, k_{off},\) and \(D\) are spatially invariant; this tends to be a good approximation for the cytoplasm or another medium much larger than the bleach spot. Spatial variance is also possible, and real-life situations may approximately retain the cylindrical symmetry: for example, when the bleach spot is concentric with a centrosome. The problem of spatial variance can be considered in three dimensions with conical bleaching geometry; it can also be considered in 2D (e.g. in a membrane), or equivalently in the cylindrical approximation (i.e. the reaction and diffusion parameters, like \(b\) and \(g\), depend only on \(r\) in cylindrical coordinates).

A useful 2D model where parameters differ inside and outside the bleach spot has been derived in (Sprague 2006); however, two additional approximations can be used to greatly extend the range of FRAP systems treatable under conditions of parameter spatial variance: an application of the diffusion phase-binding phase approximation and a new "small-body" approximation. First off, the diffusion phase-binding phase approximation can be applied directly in cases where the diffusion coefficient \(D\) is the same everywhere and is fast compared to the reactions—in other words, given \(k_{on}\) and \(k_{off}\) from any point in space, the recovery curve for \(k_{on}, k_{off},\) and \(D\) without any spatial variance can be calculated sufficiently accurately using the diffusion phase-binding phase approximation. In these cases, equation (14) applies at each point that is being monitored, giving

\[
frap(t) = \left( \iiint P F_{eq} dV \right) frap_d(t) + \left( \iiint P C_{eq}(1 - Be^{-k_{off} t}) dV \right)
\]

where \(frap_d(t)\) is calculated from \(D\) without considering any issues of spatial variance.

For example, suppose that the kinetic parameters are invariant within the bleach spot but vary outside, for example if the bleach spot is in some cytoskeletal structure like a spindle with special binding properties for the protein of interest. Then equation (25) simply reduces to (14) where \(C_{eq}, F_{eq},\) and \(B\) have their values inside the bleach spot. As another example, suppose \(B=1\) (a common scenario, see Fig. 2C) and the reaction is characterized by \(C_{eq1}, F_{eq1},\) and \(k_{off}\) inside a circle with radius \(r_c < w\) and \(C_{eq2}, F_{eq2},\) and \(k_{off2}\) outside (we are monitoring the bleach spot, which has radius \(w\)). Then a fraction \(r_c^2/w^2\) of the fluorescence exhibits the first set of parameters while a fraction \(1 - r_c^2/w^2\) exhibits the second set, and so

\[
frap(t) = \left( \frac{r_c^2}{w^2} F_{eq1} + \left( 1 - \frac{r_c^2}{w^2} \right) F_{eq2} \right) frap_d(t) + \left( \frac{r_c^2}{w^2} C_{eq1}(1 - e^{-k_{off1} t}) \right) - \left( \frac{r_c^2}{w^2} C_{eq2}(1 - e^{-k_{off2} t}) \right)
\]

However, the requirements for this approximation can be relaxed somewhat. If a small area—say, a small fraction of the inside of the bleach spot—has parameter values that seem to deviate from the diffusion phase-binding phase approximation, the approximation can nevertheless still be applied because diffusion across such a small area is very fast. Likewise, if the diffusion coefficient in such a small area differs from that elsewhere, this difference can be ignored for most purposes. Consequently, it becomes possible to consider some cases in which the diffusion phase-binding phase does not
actually apply outside the bleach spot either—namely, those in which effective diffusion applies throughout most of space. Systems exhibiting effective diffusion recover as if they had no reactive behavior and had diffusion coefficient
\[
D_{\text{eff}} = \frac{D}{1 + \frac{k_{\text{on}}}{k_{\text{off}}}}
\]  
(Crank 1975) so one may simply calculate recovery as if \(D_D_{\text{eff}}\) and \(k_{\text{on}}\) outside the inhomogeneity.

The small-body approximation is useful for analyzing the binding properties of small structures at the center of the bleach spot; to be experimentally useful this structure must bind enough fluorophore to be resolvable over noise but not so much that it depletes the medium around it of fluorophore over the course of recovery (or else it must exhibit slow enough binding kinetics that it takes up fluorophore slowly enough for this depletion to be avoided). Structures that fit these requirements are not unusual: a centrosome is a good example, as are many other small organelles or microtubule-organizing centers like oocyte pole bodies. The approximation is more applicable in 3D than 2D systems because 3D systems tend to have a monitored area localized in the focal plane but diffusion from all directions, allowing a structure to take up a greater portion of bleach spot fluorescence without significantly depleting recovery. Let \(\text{frap}_c(t)\) the fluorescence in the center of the bleach spot not due to the structure, which can be calculated by \(\text{frap}_c\) or a similar routine by monitoring a spot with a small radius (though this is not likely to be very different from the mean fluorescence of the entire bleach spot; see Fig. 2E). Also, let \(B_c\) be the extent of bleaching of the structure (which could also be calculated using such a routine). Then, the medium other than this structure recovers normally, while the structure recovers its fluorescence \(F(t)\) by binding kinetics with an association rate constant \(k_{\text{on}}\) and a dissociation rate constant \(k_{\text{off}}\):
\[
\frac{dF}{dt} = k_{\text{on}} \text{frap}_c(t) - k_{\text{off}} F
\]  
giving the equilibrium value (noting that \(\text{frap}_c = 1\) at equilibrium)
\[
F = \frac{k_{\text{on}}}{k_{\text{off}}}
\]  
so
\[
F(0) = \frac{k_{\text{on}}}{k_{\text{eff}}} (1 - B_c)
\]
\[
F(t) = \left( k_{\text{on}} \int_0^t \text{frap}_c(t')e^{k_{\text{off}}t'} dt' + \frac{k_{\text{on}}}{k_{\text{eff}}} (1 - B_c) \right) e^{-k_{\text{off}}t}
\]  
Unlike \(\text{frap}_c\) and other homogeneous-medium solutions (e.g. the Soumpasis equation), \(F\) does not suddenly increase at the beginning of recovery. Consequently, if significant fluorescence is observed coming from the structure in the first images (i.e. fractions of a second) after bleaching, then \(B_c\) must differ significantly from 1, and \(B_c\) can be calculated experimentally by comparing the background-corrected fluorescence of the structure before and after photobleaching. This may in fact be a useful way to determine bleaching parameters for a given FRAP system. Likewise, the fraction of bleach spot fluorescence
at equilibrium that is due to the structure can be calculated experimentally by taking the ratio of the background-corrected structure fluorescence to the total bleach spot fluorescence before photobleaching. Let this ratio be $Q_c$. (The background correction can be achieved by calculating the average fluorescence per pixel in the structure, likely over different images in a time series, and then subtracting the average fluorescence per pixel for nearby points outside the structure, or even for larger sections of the image). Then, given the FRAP recovery curve in the absence of the structure $frap_0(t)$, one has a FRAP recovery curve for a bleach spot containing the structure:

$$frap(t) = Q_c F(t) + (1 - Q_c) frap_0(t) = Q_c e^{-k_{off}t} \left( k_{on}^* \int _0 ^t frap_0(t')e^{k_{off}t'} dt' + \frac{k_{on}^*}{k_{off}} (1 - B_c) \right) + (1 - Q_c) frap_0(t)$$

(31)

$F$ and thus $frap(t)$ here are normalized based on the average equilibrium fluorescence in that medium and thus may exceed 1. Based on (29) and (31),

$$\lim _{t \to \infty} frap(t) = (1 - Q_c) + Q_c \frac{k_{on}^*}{k_{off}}$$

(32)

An alternate choice of fluorescence units (i.e. normalization) may sometimes be desirable to allow an equilibrium value of 1; this applies as well in non-small-body cases of spatially varying binding parameters, since there also the equilibrium fluorescence in one area of space may differ from that in another area. Using the extent of recovery as a fit parameter (i.e. fitting $Rfrap(t)$ to the data, where $R$ is the extent of recovery and $frap(t)$ is the recovery predicted by the model; see Hallen 2008a) resolves this problem because the renormalization is built into the factor $R$.

**Numerical Methods for Reaction-Diffusion**

The model equations (12) were integrated using a fractional time-stepping method. For each time step, a half-time-step was first performed neglecting diffusion, thus allowing an analytical solution. Then, a full time-step was calculated neglecting the reaction and thus only involving $g$; a second-order implicit algorithm was used, and the Laplacian was approximated to second order in space using centered differences in cylindrical coordinates. Finally, a second reaction half-time-step was performed.

The mesh points were evenly spaced with respect to $z$; in the $r$-direction, adaptive mesh refinement was used: each $z$ value could be assigned a different spacing in $r$. This accommodated closer spacing of points in $r$ at the edges of the bleach spot, reducing error from the steep initial conditions there, despite the fact that the radius of the bleach spot depends on $z$. In cases where the mesh with respect to $r$ is different for consecutive values of $z$—that is, where a mesh point does not have another mesh point directly above or below it—linear interpolation was used to approximate the derivative.

Computer routines were written to apply this strategy with adaptive timesteping; each timestep was chosen based on the maximum absolute value of $\delta^2g/\delta r^2$ at the focal plane immediately prior to the step. One routine was written for spatially invariant reaction and diffusion parameters, and another was written to accommodate spatial differences.
**Reaction-Transport Model**

As previously noted, in systems with tight binding and small bleach spots, diffusion is sufficiently fast compared to the reaction that free protein largely equilibrates before the FRAP recovery curve begins. If the effect of diffusion is noticeable at all, it is in the first few seconds of a >1-minute FRAP recovery curve, because only then does the free protein concentration significantly depart from equilibrium (Sprague 2004, Hallen 2008a). A relatively accurate model has previously been constructed by neglecting such departures; then, the spatial dependence of the recovery is due only to the bleaching in the focal plane itself and to spatial variation in k*on and k*off (Bulinski 2001).

Recoveries that take place on a timescale much slower than diffusion may be affected by the motion of the cytoskeleton, which often moves on this much slower timescale. Cytoskeletal motion is most significant during major cellular rearrangements, such as cell division and the formation of pseudopodia, and specialized processes like muscle contraction. The recovery has been modeled for the case of cytoskeletal motion along a single axis in a region of constant k*on and k*off; this approximates recovery for microtubule-binding proteins during mitosis. In this case, let the velocity of the microtubules along the axis of motion be distributed according to the probability density function p(v). (During cell division, this axis will be the line connecting the spindle poles). p(v) is assumed here to be constant over space and time. Define c(v) as the density function of the bound protein concentration with respect to velocity; this function varies over space and time. The concentration of free protein is always F eq due to the high speed of diffusion, so

$$\frac{dc(v,x,t)}{dt} = -v \frac{dc(v,x,t)}{dx} + k_{on}^* F_{eq} - k_{off} c(v,x,t)$$  

(33)

Letting b(v,x,t) be the depletion of bound concentration from equilibrium, i.e. (choosing units such that equilibrium fluorescence at any x, t is 1)

$$b(v,x,t) = c_{eq}(v,x,t) - c(v,x,t) = \frac{k_{on}^*}{k_{on}^* + k_{off}} p(v) - c(v,x,t)$$

(34)

The time-derivative relation for the depletion is

$$\frac{\partial b(v,x,t)}{dt} = -v \frac{\partial b(v,x,t)}{dx} - k_{off} b(v,x,t)$$

(35)

which has the solution

$$b(v,x,t) = b(v,x-vt,0)e^{-k_{off}t}$$

(36)

The initial conditions b(v,x,0) can be calculated from the equilibrium concentrations, the illumination profile in the focal plane, and the velocity distribution. By the time diffusion has equilibrated, the effects of diffusion are largely erased, so it is appropriate to assume that the initial concentration is constant (0 if one assumes near-complete bleaching) inside the bleach spot (e.g. a circle or rectangle) and is equal to the equilibrium value outside. This reaction-transport model thus gives the recovery

$$frap(t) = 1 - \int_{focal\ plane} \int_{0}^{\infty} P(x,y)b_0(v,x-vt,y)e^{-k_{off}t} \, dx \, dy$$

(37)

where b_0(v,x,y) denotes the initial bound depletion with velocity v at a given point (x,y). The space integral is only over two dimensions, i.e. in the focal plane, because this model only deals with transport in this plane (with the x-axis defined as the direction of
transport). As such, $P(x,y)$ and $b_0(v,x,y)$ should be relatively simple functions: $P(x,y)$ should be constant inside the monitored zone and 0 elsewhere, while $b_0$ should be equal to $p(v)$ inside the bleach spot and 0 elsewhere. The bleach spot and the monitored zone need not be the same, nor of any particular shape, for this model to apply, but obviously $P$ and $b_0$ must match the experimental data.

Many systems may be best modeled by a combination of the reaction-diffusion and reaction-transport model, using a diffusion phase-binding phase approximation except with $b$ recovering as in (36) rather than as in (13). The rationale is the same: one may assume the reaction-diffusion model to apply until the minimum of $g$ with respect to space reaches a certain threshold; if the system is suitable (primarily this means if binding is tight enough), very little transport will have occurred in this time, and then one may switch to the reaction-transport model without significant error. A FRAP recovery curve fitting to the reaction-transport model at most points but deviating in a brief, high-slope initial region will likely benefit from this refinement. This is similar to how a curve fitting at most points to a reaction-dominant model with a brief, high-slope initial deviating region can be better fit by a diffusion phase-binding phase model than a reaction-dominant one (Hallen 2008a). The recovery in this case is

$$\text{frap}(t) = F_{eq} \text{frap}_d(t) + C_{eq} \left( 1 - \int_{0}^{\infty} \int_{0}^{\infty} P(x,y) b_{0}(v,x-vt,y) e^{-k_{off}t} \, dv \, dx \, dy \right)$$

A few special cases are of particular interest. For a circular bleach spot in a region where the microtubule motion is homogeneous spatially, e.g. inside one half of a mitotic spindle, the bleach spot fluorescence recovery, including the diffusional term, is

$$\text{frap}(t) = F_{eq} \text{frap}_d(t) + C_{eq} \left( 1 - e^{-k_{off}t} \int_{0}^{\infty} p(v) \left( \frac{2}{\pi} \cos^{-1}\left( \frac{vt}{2w} \right) - \frac{vt}{\pi w^2} \sqrt{w^2 - v^2 t^2} \right) \, dv \right)$$

To neglect diffusion, one may simply set the first term (i.e. the first line of the expression) to $F_{eq}$. A useful approximation for $p(v)$ in systems with microtubule flow all in one direction is a half-Gaussian, i.e.

$$p(v) = \begin{cases} 
\frac{1}{\sigma \sqrt{2\pi}} e^{-\frac{v^2}{2\sigma^2}} & v \geq 0 \\
0 & v < 0
\end{cases}$$

where $\sigma$ is a parameter obtained by fitting (this distribution is useful in that it only has one parameter, thus improving the conditioning of fitting).

For a circular bleach spot at the center of a mitotic spindle or another structure in which protein is being transported from both directions toward some line or plane, e.g. at the center of the spindle, the recovery takes the form

$$\text{frap}(t) = F_{eq} \text{frap}_d(t) + C_{eq} \left( 1 - e^{-k_{off}t} \int_{0}^{\infty} p(v) \left( 1 - \frac{2}{\pi} \left( \sin^{-1}\left( \frac{vt}{w} \right) + \frac{vt \sqrt{w^2 - v^2 t^2}}{w^2} \right) \right) \, dv \right)$$

The reaction-diffusion-transport model for a circular bleach spot at the center of a mitotic spindle presented without derivation in (Hallen 2008b) was derived by plugging a half-Gaussian for $p(v)$ and the Soumpasis equation (15) for $\text{frap}_d(t)$ into (41).
Computational Results

Parameter Values

The simulations in this study were performed with bleach spot sizes on the order of 5 µm and, if applicable, light wavelengths of ~500 nm (appropriate for GFP). Bleaching was set to be relatively intense for most of the simulations, resulting in nearly complete bleaching of the monitored area, though other circumstances may have significant experimental application and are tested as well (this factor is important in the Experimental Results and Discussion below, for example). (Sprague 2004) identified several regions of parameter space with qualitatively different behaviors in FRAP recovery for the reaction-diffusion model; parameter values were chosen from these regions to investigate how they change with application of the new bleach profile model and with the incorporation of inhomogeneities. Some equivalent FRAP curves can be obtained from disparate values; for example, changing the bleach spot radius and diffusion coefficient so that $w^2/D$ stays constant will have negligible effect on recovery since, neglecting diffraction (since the excitation wavelength is generally difficult to change), it amounts to changing distance units. This is important in analyzing FRAP recoveries with larger bleach spots. Similarly, uniformly scaling $k_{on}, k_{off}$, and D will uniformly scale the curve in time. Some other regions of parameter space where quite different sets of parameters yield nearly equivalent curves have been identified by (Sprague 2004) as well (e.g. when only an effective diffusion coefficient, which is a function of multiple parameters, can be determined).

The reaction-transport model is of course not included in previous schemes but will correspond to $k_{on} w^2 D$ and nonnegligible velocities of the medium (on the order of a bleach spot radius per minute).

Reaction-diffusion model: Optical geometry

Calculated fluorescence recoveries using the reaction-diffusion model with a conical bleach profile and confocal image acquisition were compared to the results from the cylinder model and to each other in a number of regimes (Fig. 2). Results differed significantly from the cylindrical results; recoveries generally started more quickly but then completed more slowly. This is expected based on the fact that the conical bleach profile bleaches a larger volume but mostly less intensely: recovery in the conical profile is first accelerated by vertical diffusion that is impossible with a cylindrical profile, but then is slowed down by the large volumes of partially photobleached space above and below the focal plane. Fits of the cylindrical model to recoveries simulated using the conical model were not, however, extremely inaccurate as long as the extent of recovery was used as a fit parameter. This was especially true for recoveries where the reaction (as opposed to diffusion) played an important part. For a pure-diffusional case (Fig. 2A), the Soumpasis equation for the cylindrical profile was fit, yielding a diffusion coefficient (11 µm²/s) approximately 2-fold off from the original diffusion coefficient (5 µm²/s), consistent with estimates from (Hallen 2008a), at 84% recovery. For a case where the reaction plays a more important role in recovery (Fig. 2B), the fit was even better (fitting to the cylindrical model by the inverse Laplace transform method from Sprague 2004),
yielding a fit \( k_{\text{on}}^* = 0.098 \text{ s}^{-1}, k_{\text{off}} = 0.25 \text{ s}^{-1}, D = 9.2 \text{ \( \mu \text{m}^2/\text{s} \)} \) with less than 20% error in each parameter, at 90% recovery. This suggests that many conclusions drawn using the cylindrical model are accurate, and furthermore that at least in some cases, the conical bleach profile may be at least partly responsible for the apparent failure of fluorescence in many experimental FRAP systems to recover fully to the fluorescence observed before photobleaching. One must also note that the fit of the cylindrical model to the conical bleach profile simulation is extremely good, and thus it would not be feasible to experimentally distinguish between the two bleach profiles based on FRAP recovery data in this system. The distinction would most likely become readily apparent only if fluorescence were monitored very quickly after photobleaching (on the tens of milliseconds scale, not accessible to most fluorescence microscopes).

Variation with the extent of bleaching, i.e. with \( k_B \), was observed as well (Fig. 2C), though it was quite limited over a large range of bleach parameters. In particular, there was a large range of parameters for which the bleach spot in the focal plane was bleached essentially completely, and the effects of increased bleaching came in the form of slight delays later in recovery. However, much lighter photobleaching did result in significantly higher fluorescence throughout recovery. Some variation in fluorescence recovery with different cone angles (Fig. 2D) and axial resolutions was observed as well, but is unlikely to be a problem because these parameters should be well known for a given instrument (the axial resolution is often adjustable by changing the pinhole size; the cone angle is largely set by the numerical aperture of the objective and the index of refraction). Note that as the cone angle approaches vertical (\( \tan \alpha = 0 \)), the recovery approaches that of a cylindrical bleach, as expected. Recoveries were also simulated for portions of the bleach spot (smaller circles concentric with the bleach spot); the effect of such variation was small or even insignificant (Fig. 2E).

Both the effective-diffusion and the diffusion phase-binding phase approximations are still of use in the conical approximation. The diffusion phase-binding phase approximation applies for \( k_{\text{on}}^* w^2 / D < 1 \) (Sprague 2004) in the cylindrical approximation, but because recovery is more drawn out for conical bleaching, there is minor deviation from the approximation with parameters in that range (Fig. 2B). Still, the approximation is quite close (within a few percent) and is certainly appropriate for use in the more complicated systems discussed below (spatial variance of parameters and reaction-transport).

Fits of the conical model were performed to simulated experimental data, generated by taking simulations and adding zero-mean Gaussian noise with standard deviation 0.01. Two data sets differing only in bleach spot size were used, and three fits were performed: one for the small bleach spot, one for the large, and one for the two concurrently (Hallen 2008a). The best accuracy was obtained by fitting concurrently; this was especially advantageous relative to fitting the large bleach spot alone (Table 1, Fig. 2F). The three fits together took 2 h 35 min., with 100 points; boxcar averaging (i.e. averaging of subsequent experimental data points to produce a less noisy curve with less points) could reduce this time. Boxcar averaging is especially useful later in recoveries, when real recovery varies slowly; it could impede fitting if used for early points where recovery is significant from each point to the next. On a Gateway laptop, the routine \texttt{frap fft homogeneous} takes approximately 40 s to evaluate a 100-point recovery, but
usually under 10 s for short recoveries (<20 points); it has a constant computational cost plus a cost per point.

A consistency check was performed by solving the reaction-diffusion equations with the same method in the traditional cylindrical approximation (i.e. working in polar rather than cylindrical coordinates); the resulting routine, \textit{frap fft homogeneous 2D}, agreed very closely with the Soumpasis equation for pure diffusion and with the Laplace transform-based method of (Sprague 2004) for several other parameter combinations.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>“Real” values</th>
<th>Small spot fit</th>
<th>Large spot fit</th>
<th>Concurrent fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{on}$ (s$^{-1}$)</td>
<td>2</td>
<td>1.8545</td>
<td>1.5304</td>
<td>1.938</td>
</tr>
<tr>
<td>$k_{off}$ (s$^{-1}$)</td>
<td>2</td>
<td>1.9593</td>
<td>1.7401</td>
<td>1.9755</td>
</tr>
<tr>
<td>D (µm$^2$/s)</td>
<td>2</td>
<td>2.016</td>
<td>0.8204</td>
<td>1.9989</td>
</tr>
<tr>
<td>Extent of recovery (small)</td>
<td>1</td>
<td></td>
<td>0.9974</td>
<td></td>
</tr>
<tr>
<td>Extent of recovery (large)</td>
<td>1</td>
<td></td>
<td>1.0802</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Fitting of simulated experimental data to the conical approximation.
Figure 2. Calculated FRAP curves displaying effects of optical geometry. (A) A purely diffusional recovery with $D=5 \, \mu m^2/s$: conical model (blue), cylindrical model (pink), fit of Soumpasis' cylindrical model to simulation from conical model (yellow). (B) A recovery showing significant reaction effects ($k_{on}=0.1 \, s^{-1}$, $k_{off}=0.3 \, s^{-1}$, $D=10 \, \mu m^2/s$): conical model (blue), values calculated by diffusion-phase-binding-phase approximation with conical bleaching (pink), fit of Sprague’s cylindrical model to conical model (yellow). (C) A recovery ($k_{on}=k_{off}=5 \, s^{-1}$, $D=5 \, \mu m^2/s$) calculated with different bleaching parameters ($k_B$): 2 (purple), 10 (blue), 100 (cyan), 500 (yellow). (D) A recovery ($k_{on}=0.6 \, s^{-1}$, $k_{off}=0.3 \, s^{-1}$, $D=10 \, \mu m^2/s$) with different cone angles: $\tan \alpha=0.5$ (blue), 1 (pink), 2 (yellow). (E) A recovery ($k_{on}=0.01 \, s^{-1}$, $k_{off}=0.3 \, s^{-1}$, $D=5 \, \mu m^2/s$) with monitoring of the entire bleach spot (blue, 1-µm radius), a 0.75-µm-radius spot concentric with the bleach spot (yellow), and a 0.5-µm-radius spot concentric with the bleach spot (pink). (F) Concurrent fits (green, brown) of the conical model to simulated experimental data with the same parameters except differing bleach spot sizes (1-µm radius, blue; 3-µm, pink). See Table 1.

**Reaction-diffusion model: Spatial variation in reactions and diffusion**

Recoveries were calculated in both the diffusion phase-binding phase and small-body approximations. As expected, inhomogeneities in binding had significant effects on recovery with a strong dependence on their spatial extension and in the extent of their deviation from the ambient medium (Fig. 3A, 3B). However, it is possible to fit such
data to a model with no spatial variance (Fig. 3C); for this data, the resulting diffusion coefficient was similar to that in the simulation (8 μm²/s as opposed to 10 μm²/s), while the binding parameters were intermediate between those of the two zones but much closer to those of the ambient medium than to those of the smaller inhomogeneity. The small-body approximation yielded recovery of a quite distinctive shape (Fig. 3D); note the slow initial recovery compared to spatially invariant models (at least for the structure fluorescence F; this effect becomes less apparent when F is averaged with the ambient medium's fluorescence).
Figure 3. Effects of spatial variation in reaction and diffusion. (A) Recoveries in the diffusion phase-binding phase approximation where $k_{\text{off}}$ is decreased fourfold inside radius $r_c$: $r_c=0$ (blue), 0.5 (pink), 0.75 (cyan), 1 (yellow). (B) The same system, with $r_c/w=0.75$, but with $k_{\text{off}}$ inside $r_c$ varying from 1/16 the value outside (yellow) to 1/4 (pink) to the same value (blue) to 4 times the value (cyan). (C) A fitting experiment in 2D: a fit (pink) of a spatially invariant model to a spatially variant simulation with $r_c/w=0.5$ (blue). (D) Recoveries in the small-body approximation with different structures placed in the same homogeneous medium. F for a fast (yellow) and slow (blue) reaction, with the average bleach spot recoveries with $Q_c=0.5$ (cyan and pink respectively).

**Reaction-diffusion model: Numerical calculations**

Numerical calculations extend FRAP analysis to more general geometries than in Fig. 2 and 3. In particular, neither `frap_fft_homogeneous` nor most previous models, like the Soumpasis equation, take into account diffraction. However, they are justified in this neglect, as diffraction causes the depletion of fluorescence due to photobleaching to deviate only slightly from a sharp drop-off at the edge of the bleach spot (Fig. 4A), across the visible spectrum. This is similar to how light microscopy is a perfectly valid tool for
directly imaging cellular structures on the scale typically probed using FRAP. At any rate, diffusion quickly erases the inaccuracies due to diffraction (Fig. 4B). Note that a sharp drop-off would in effect be the result of photobleaching by an extremely low-wavelength source, and would similarly converge to resemble the other wavelengths.

Numerical simulation is also useful in investigating the effects of spatial differences in diffusion coefficients (or other parameters) not well handled by the methods described above. For example, it allows accounting for the presence of additional viscosity or another reason for slowed diffusion in the bleach spot (Fig. 4C), or for exotic diffusional geometries like very slow diffusion outside of a sphere concentric with the bleach spot (Fig. 4D). Note that unlike in the recoveries calculated under more typical conditions, the time derivative of the fluorescence in this latter case is not monotonically decreasing.
Figure 4. Numerical calculations. (A) Initial fluorescence depletions (g) with the effects of diffraction at 300 nm (yellow), 500 nm (blue), and 700 nm (purple) illumination wavelengths (these are the colors of the points on the graph, not the actual light). (B) Recovery quickly erases the effects of diffraction: after 3 ms, recovery with 300-nm photobleaching (blue) is smoothed out (yellow) in a manner resembling the 700-nm initial conditions (pink). (C) The same 300-nm recovery with impeded diffusion in the bleach spot (pink), contrasted with the original (blue). (D) Recovery with impeded diffusion outside the bleach spot.

**Reaction-Transport Model**

Examples of reaction-transport curves are shown in Fig. 5. These were all calculated with circular bleach spots that were also used as the monitored zone, and unless otherwise stated, a constant flow across the bleach spot is assumed. Reaction-transport curves were found to be reasonably well (and, of course, completely erroneously) fit by the 2D reaction diffusion model (Fig. 5A); concurrently fitting with two bleach spot sizes helped to expose the fallacy somewhat, but with real experimental
data the deviation could be difficult to identify. However, the parameters obtained by this fit are likely to be unreasonable for the protein (or, more generally, the FRAP system) that is generating the actual reaction-transport behavior, and the presence of an observable diffusional component in recovery (Fig. 5B) would compound the deviation. Fitting of different parts of a bleach spot, or different bleaching geometries designed with the direction of transport in mind (possibly with concurrent fitting of multiple geometries), would also reveal deviation.

As expected, transport across a bleach spot in a single direction causes slower recovery than transport in from both directions (as in the center of a spindle, see Hallen 2008b) (Fig. 5C, 5D). Transport with a single velocity, i.e. with a delta function for p(v), generates a distinctive recovery curve that rises steeply to 1 and then abruptly levels off since unbleached protein has completely filled the monitored zone (Fig. 5D).
Figure 5. FRAP recovery in the reaction-transport model.
(A) Two recoveries with the same parameters except for the bleach-spot size (2 µm, blue; 5 µm, pink), with concurrent (green, brown) fits to the 2D reaction-diffusion model (Sprague 2004). (B) Combination of reaction-transport and reaction-diffusion recovery: A recovery with (pink) or without (blue) a Soumpasis (2D) diffusional term. (C) The effect of transport from both sides: recovery in a bleach spot with transport across it (blue) or with transport in from both sides (pink). (D) A recovery with a delta-function velocity distribution, with transport across (blue) or from both sides of (pink) the bleach spot. Parameters are identical between the two curves in each of B, C, and D unless otherwise specified.

Analysis of Experimental Data

The new models derived in this paper were used to reanalyze experimental data from (Hallen 2008a), which used FRAP for the fusion protein γ-tubulin-GFP. The study was performed in Drosophila embryos with γ-tubulin37C, an oocyte- and embryo-
specific form of γ-tubulin. γ-Tubulin plays an important role in nucleating microtubules, and the GFP-tagged version is able to perform the same biological function as the wild type. For this protein in the cytoplasm, an excellent fit was obtained (Fig. 6A), with recoveries close to 1 for both bleach spot sizes (1.00 and 1.05 for \( w=1.3 \, \mu m \) and 2.655 \( \mu m \) respectively) and with other parameters as listed in Table 2. The parameters obtained were similar in order of magnitude to those previously obtained with the cylindrical model and published in that paper; a bleaching parameter was also obtained, and using it as a fit parameter was essential to the quality of this curve fit, even though bleaching of the monitored zone was almost complete (\( B=0.93 \)). Notably, the diffusion coefficient obtained was significantly higher than the result from the paper, suggesting that the protein occurs in the cytoplasm in a smaller form. In particular, the previous diffusion coefficient was at the high end of a range of values, 6-18 \( \mu m^2/s \), predicted for the protein occurring as the γ-tubulin small complex (γTuSC), which consists of two γ-tubulin polypeptides and two others (Oegema 1999); it was thus unclear whether the protein was in this form or in an uncomplexed form. The new data strongly support the latter conclusion: cytoplasmic γ-tubulin37C in Drosophila embryos diffuses too quickly to be part of a multi-protein complex.

The paper also provides data for the monitoring of a centrosome in the middle of a bleach spot and fits it to the same 2D model. For this study, these data were re-fit using (30) to the structural fluorescence in the small-body approximation; the fit yields \( k^*_{on} R_c \), \( k_{off} \), and \( B_c \), which were 0.049 s\(^{-1}\), 0.054 s\(^{-1}\), and 60% respectively. Note that the structure and the entire monitored zone are of different shapes and thus it is feasible for \( B \) and \( B_c \) to differ even in the same bleach. The only one of these values directly comparable to the cylindrical-model fit from the original paper or to the cytoplasmic fit is \( k_{off} \), which was originally found to be 0.0272 s\(^{-1}\). The new value, approximately twice that from the cylindrical model, is still significantly less than the cytoplasmic \( k_{off} \), supporting the conclusion from the paper that γ-tubulin binding to the centrosome takes a tighter form than its binding to ordinary microtubules (most likely because it is incorporated into the γ-tubulin ring complex, a microtubule-nucleating structure, in the centrosome).

Table 2. Parameters obtained from fitting \( frap_{fit\_homogeneous} \) (conical bleach profile) to recovery of γ-tubulin-GFP in the cytoplasm (Hallen 2008a), compared to cylindrical model fits from that paper.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cylindrical model fit</th>
<th>Conical bleach fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k^*_{on} ) (s(^{-1}))</td>
<td>0.028</td>
<td>0.012</td>
</tr>
<tr>
<td>( k_{off} ) (s(^{-1}))</td>
<td>0.14</td>
<td>0.16</td>
</tr>
<tr>
<td>( D ) (( \mu m^2/s ))</td>
<td>19</td>
<td>34</td>
</tr>
<tr>
<td>( k_B ) (bleach constant)</td>
<td>n/a</td>
<td>37</td>
</tr>
</tbody>
</table>
Figure 6. Fits to experimental data from (Hallen 2008a). (A) Fits (yellow, w=1.3 µm; cyan, w=2.655 µm) using `frap_fft_homogeneous` to recovery of γ-tubulin-GFP in the cytoplasm (blue, pink respectively). (B) A fit (pink) of the small-body model to centrosome fluorescence data (blue).

**Conclusions**

The models presented in this paper extend the range of FRAP systems to which accurate quantitative models can be fit. As noted throughout the paper, fitting of one model to another system that differs geometrically often yields a fairly good fit and may produce approximately correct results, but it is difficult to determine the accuracy of such procedures, and sometimes, as in the case of the cytoplasmic diffusion coefficient for γ-tubulin, the extra accuracy has definite experimental value.

FRAP, especially studies that seek quantitative information from curve fits, has traditionally been primarily a membrane technique. Models like those in (Sprague 2004) have helped establish it for use in 3D environments like the cytoplasm or the inside of the nucleus to determine the binding and diffusion parameters in these environments. However, it also shows promise for studying the properties of small structures like centrosomes (Hallen 2008a), transport rates (Hallen 2008b), and other systems; it is hoped that the models here can contribute to its applicability in the wide range of systems that could benefit from this powerful, non-invasive technique. It is also important to note that quantitative modeling may be useful even in FRAP studies that only seek to compare diffusion, binding, or transport rates between systems qualitatively, because quantitative models can separate the many factors that are involved in FRAP recovery. For example, comparisons of binding in (Liang 2009) were aided by a quantitative model from (Sprague 2004) that separates binding into two bound states with different kinetics.

The complexity of biological systems has often deterred the extensive use of quantitative methods in experimental studies of them, especially *in vivo*. Yet quantitation of observed phenomena has been essential to the development of technology in non-biological systems, and the same benefit will likely apply in future biological
technologies, as well as in advances in mechanistic understanding of biology. For this reason, quantitative modeling of FRAP, which allows quantitative measurement of chemical processes in vivo, is of great practical relevance today.

**Summary**

1. Using a new computational routine, the reaction-diffusion equations describing FRAP (with binding to a stationary structure) can be solved for more accurate initial conditions, which involve a "double-cone" bleach profile; the resulting simulations allow more accurate diffusion coefficients and binding rate constants to be determined from FRAP data. These parameters differ from those obtained with previous, 2D models particularly in cases where diffusion, rather than binding, dominates recovery. Other parameters such as the strength of bleaching can also have a significant effect on recovery.

2. The diffusion phase-binding phase and small-body approximations allow analysis of spatial variation in binding.

3. The reaction-transport model allows more accurate analysis and velocity determination if the protein binds to a structure that is moving nonnegligibly.

4. The new routine, as well as the small-body approximation, were successfully used to fit experimental FRAP data from (Hallen et al 2008a) and upheld the basic conclusions of that paper.

**Acknowledgements**

I wish to acknowledge Sharyn Endow for advice and experimental data and for introducing M.A.H. to FRAP. This project was funded by Duke University’s PRUV program.

**References**


**Supplemental Code**

```matlab
function frap=frap_fft_homogeneous(t,kon,koff,D,w,talph,k,z0,wmon)
%function frap=frap_fft_homogeneous(t,kon,koff,D,w,talph,k,z0,wmon)
%Returns fluorescence recovery at times t, with pseudo-first-order association rate constant kon, dissociation rate constant koff, diffusion coefficient D, bleach spot radius w, talph=tangent of angle between side %of bleach cone and the normal, k=bleaching constant, z0=axial resolution %for image acquisition (Gaussian profile assumed). wmon (set equal to w if %not otherwise specified) is the radius of the monitored circle (concentric %with the bleach spot)

D=D/w^2;%Using w as the length unit for calculation
if(nargin==8)
    wmon=1;
else
    wmon=wmon/w;
end
z0=z0/w;

dq=0.002;%Note: dq=0.001 or less seem to crash MATLAB on my computer, but it shouldn't make much difference (at least in the regions I tested)
q=0:dq:2.99;
ng=length(q);
domega=5e-5*(1:ng);
```
nomega=1000;
Feq=koff/(kon+koff);
q=ones(nomega,1)*q;
omega=(0:(nomega-1))'*domega;
domega=ones(nomega,1)*domega;
wzt=2*((0:(nomega-1))'*ones(1,nq))*(pi*talph/nomega)/domega+1;%Bleach cone radius at a given z
g0=zeros(size(wzt));
bj=zeros(size(q)-[0,1]);
interpx=sort([q(:,2).*wzt(:,2);diag(q(:,3:(end-1))).*wzt(:,3:(end-1));q(:,end).*wzt(:,end)]);
pp=interp1(interpx,besselj(1,interpx),['linear','pp']);%Using besselj directly takes a very long time.'nearest' works OK here but linear is a little more accurate
for a=1:size(bj,2)
    bj(:,a)=ppval(pp,q(:,a+1).*wzt(:,a+1));
end
g0(:,2:end)=Feq*(1-exp(-k*((1./wzt(:,2:end)).^2))).*wzt(:,2:end).*bj./q(:,2:end);%Hankel transformed initial g
g0(:,1)=Feq*(1-exp(-k*((1./wzt(:,1)).^2))).*wzt(:,1).^2/2;
g0=2*sqrt(2*pi)*real(fft(g0))./(domega*nomega);%Account for just using nonnegative-z points.
if(wmon==1)
    gpsf=exp(-(0:(nomega-1))'*ones(1,nq)*2*pi/nomega./domega).^2/2).*ones(nomega,1)*[0.5,bj(1,:)/q(1,2:end)];%Weighting function for image acquisition
else
    gpsf=exp(-(0:(nomega-1))'*ones(1,nq)*2*pi/nomega./domega).^2/2).*ones(nomega,1)*[0.5*wmon*besselj(1,q(1,2:end)*wmon)./q(1,2:end)];
end
gpsf=real(fft(gpsf))./domega;
gpsf=g0.*gpsf;% Might as well do this here
gpsf=gpsf.*q.*domega*dq;%Weight for integration
f0=quad(@(x)((1-exp(-k./(1+x*talph)).^2)).*exp(-x/z0).^2),0,1e6)*2/sqrt(pi)/z0;%1-frap(t=0)
gpsf=gpsf*f0/(1+kon/koff)/sum(sum(gpsf));%Normalization based on initial value
while(1)%dealing with a divide by zero issue
    frap=zeros(length(t),1);
    qos=D*(omega.^2+q.^2);%D(omega.^2+q.^2)
    V=sqrt((koff+kon+qos).^2-4*koff*qos);
    L1=(-koff-kon-qos+V)/2;
    L2=L1-V;
    W1=(0.5/koff)*L2.*(koff+kon-qos+V)/(L2-L1);
    W2=(0.5/koff)*L1.*(koff+kon-qos-V)/(L1-L2);
    div0test=sum(sum((W1+W2).*gpsf));
    if(isnan(div0test)||isinf(div0test))
        D=D+1e-6;%D-1e-6 would be essentially indistinguishable
    else
    end
break
end
end

for tv=1:length(t)
    frap(tv)=1-sum(sum((exp(L1*t(tv)).*W1+exp(L2*t(tv)).*W2).*gpsf));
end
end