# PRACTICAL MANUAL FOR FLUORESCENCE MICROSCOPY TECHNIQUES

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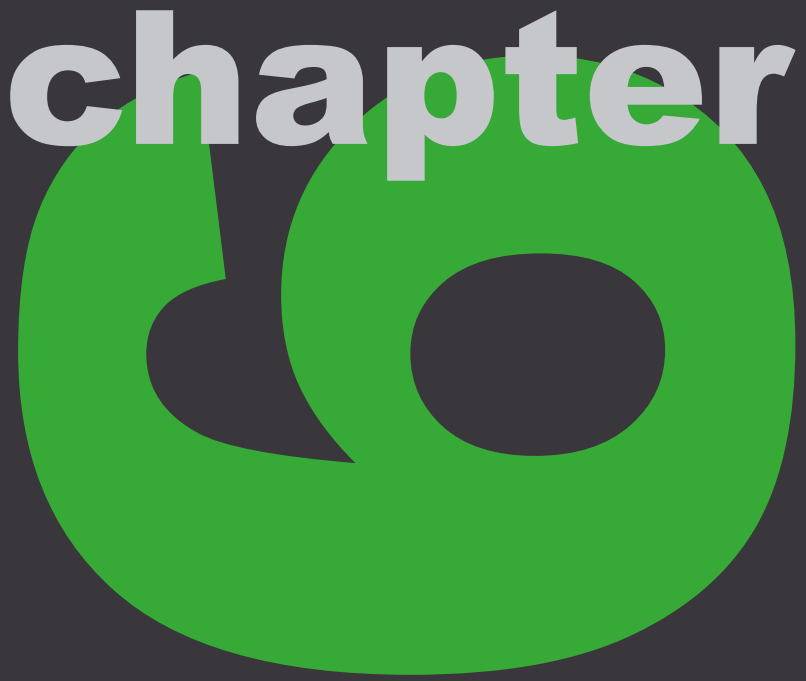
chapter

# Fluorescence Recovery After Photobleaching (FRAP)

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## Abstract

Fluorescence recovery after photobleaching (FRAP) is a microscopy technique capable of quantifying the mobility of molecules within cells. By exploiting the phenomenon of photobleaching, fluorescent molecules within a region of interest can be selectively and irreversibly 'turned off'. The analysis of the fluorescence recovery within the same region, due the redistribution of the molecules, provides information on their diffusion- and binding-dependent mobility. Both qualitative and quantitative analysis can then be applied to decipher the dynamic behavior of the molecules of interest.


## 1. Principle of FRAP

Fluorescence recovery after photobleaching (FRAP) is a popular fluorescence microscopy technique used to quantify the mobility of molecules within cells. The mobility is determined by the molecules' properties of transport, diffusion and binding to immobile sites. Since the initial development by Axelrod et al.[1] and Peters et al.[2] in the 1970's, the technique has been widely used in biological research to study cell membrane diffusion, protein interactions and protein dynamics[3-12].

Photobleaching is a natural phenomenon that manifests itself as decreasing intensity of fluorescence over time during fluorescence imaging. Exposing a fluorophore to a high level of light intensity in the presence of molecular oxygen causes permanent and irreversible chemical changes to that molecule, rendering it non-fluorescent[11],[13],[14]. Under a constant absorption of light, the fluorescence intensity will decrease over time following an exponential decay law:

$$I(t) = I_0 e^{-Kt}$$

where $I_0$ represents the initial fluorescence intensity and K the bleaching rate constant of the fluorophore including the flux of illumination photons.

Generally, photobleaching is considered a problem for time-lapse and 3D imaging, leading to unwanted loss of the signal and resultant degradation of the signal-to-noise ratio during acquisition. In FRAP experiments, however, the photobleaching phenomenon is exploited to selectively 'turn off' a subset of the fluorescent molecules in the sample usually at a spot or in a specific area of interest using a short pulse of

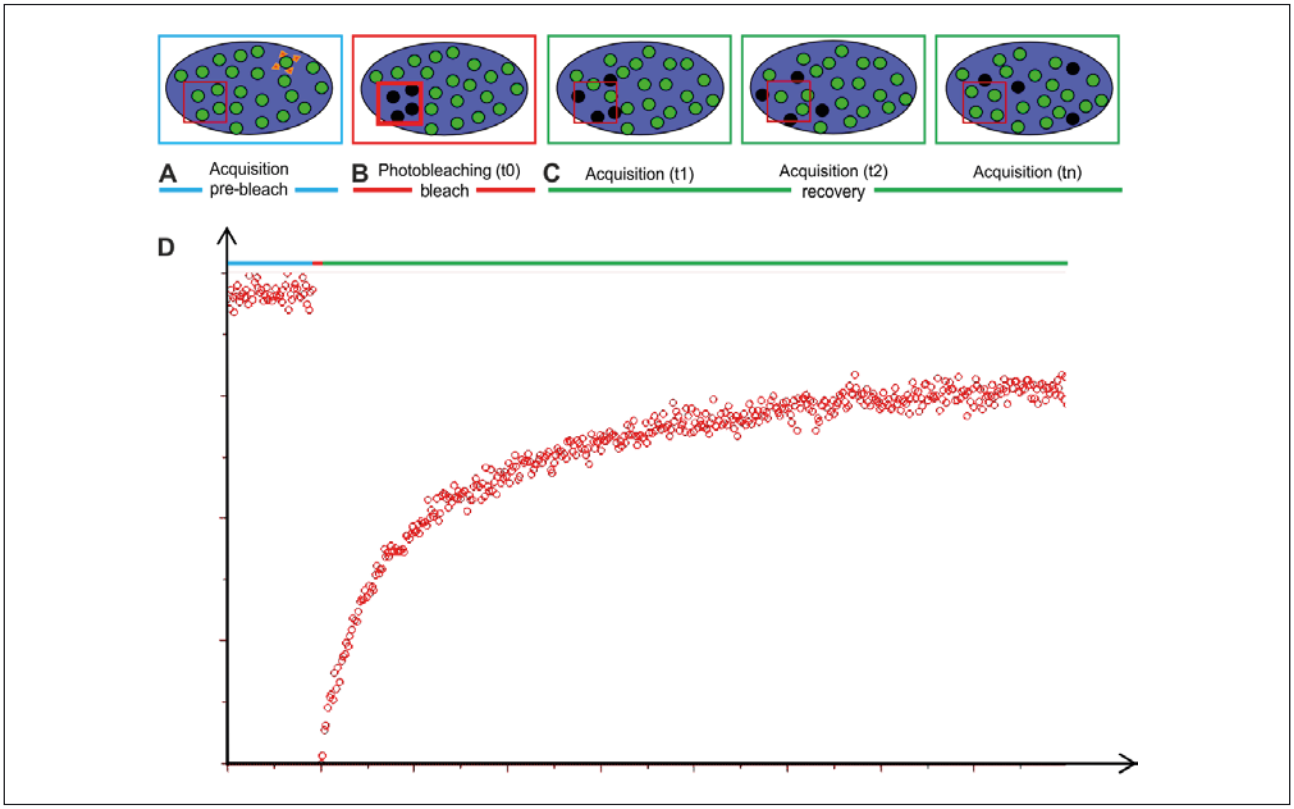


***Figure 1*** *Principle of FRAP experiments.* ***A*** *A cell or organelle is uniformly labeled with a fluorescent tag and a pre-bleach series of images collected.* ***B*** *A ROI is selectively photobleached using a short pulse of intense laser light.* ***C*** *A post-bleach recovery time series of images is collected and the intensity within the ROI monitored as the bleached dye diffuses out and new dye diffuses in.* ***D*** *Intensity changes within the ROI are measured, corrected, normalized and plotted on a graph for further quantification.*

high intensity laser light that can be positioned to, or scanned over, the region of interest (ROI). Monitoring the recovery of intensity in the bleached ROI yields information on protein mobility (Figure 1).

The choice of fluorophore is an important consideration for all fluorescence imaging based experiments, and FRAP is no exception. Ideally it should be stable enough to undergo minimal photobleaching during the imaging phases - the pre- and post-bleach acquisitions – but bleach quickly and permanently during selective ROI photobleaching. Genetically encoded fluorescent proteins, like GFP, are generally used to label a molecule of interest with very high specificity[3],[14],[15]. They provide enormous power and scope to study biomolecules in living cells through transient expression or through producing stable transgenic cell lines. Importantly, it must be taken into consideration that these modifications may change the properties of the tagged molecule and it is noteworthy that high intensity illumination creates free radicals which are highly reactive and thus cytotoxic[16]. The induced photodamage may affect cell viability and result in artifactual results. It is therefore important to minimize the extent of bleaching, even during the bleaching step.

In practice, a FRAP experiment is a 3 step acquisition process, followed by data analysis, as follows (also refer to Figure 1)[11],[17],[18]:

1. **Pre-Bleach:** A time-lapse acquisition containing the cell or sample of interest and ideally some empty background area. It is important to tune the acquisition settings to reduce the amount of photobleaching resulting from the imaging itself (Figure 1A)
2. **Bleach:** A ROI is selectively photobleached using a short pulse of intense laser light, the intensity modulation is typically controlled through an acousto-optical tunable filter (AOTF). The ROI may be a single focused spot, or the laser beam may be scanned (in a raster or a whirlwind pattern) over the area through the action of galvanometric-mirrors. To avoid damaging the cell, just a fraction of the overall pool of fluorescent molecules should be turned off (Figure 1B)
3. **Recovery:** A time-lapse acquisition with similar parameters as the pre-bleach phase, but longer in duration, is performed. When appropriate, this can be done in up to 3 phases with a series of fast acquisitions to cover the fast dynamics of recovery, followed by phases of less frequent imaging as the recovery continues. Again, it is important to reduce the amount of photobleaching caused by the imaging itself to a minimum (Figure 1C).
4. **Data analysis:** Following correction and normalization steps, the intensity fluctuations during the recovery phase within the ROI are analyzed quantitatively (Figure 1D). This allows the extraction of the recovery rate as a measure for molecular mobility as well as mobile and immobile fractions. Advanced fitting using biophysical models can be used under certain, precisely defined boundary conditions, to quantify the diffusion coefficient or chemical exchange rate.

FRAP can be used qualitatively to identify whether a particular molecule is turning over, thereby undergoing exchange with its environment, by basic analysis of the recovery curve. It allows the determination of:

1. the half-time of recovery ($t_{½}$), and
2. the mobile (*M*) and immobile (1-*M*) fractions (Figure 4).

More advanced quantitative models allow the precise determination of the kinetics and molecular properties driving such dynamics. Fitting the experimental curve with advanced theoretical models allows the determination of additional parameters[19-27]:

1. the ratio between mobile (*M*) and immobile (1-*M*) fractions (Figure 4),
2. the *effective* diffusion coefficient (*D*),
3. the binding time of proteins to sufficiently immobile macromolecular complexes ($k_{on}$, $k_{off}$), and
4. the interconnection of intracellular organelles.

A closely related technique to FRAP is Fluorescence Loss in Photobleaching (FLIP[11]; Figure 2). Again the experiment consists of a pre-bleach acquisition, but then a ROI in the cell is repeatedly bleached whilst a second ROI is analysed for the consequent loss of fluorescence. This technique gives information about

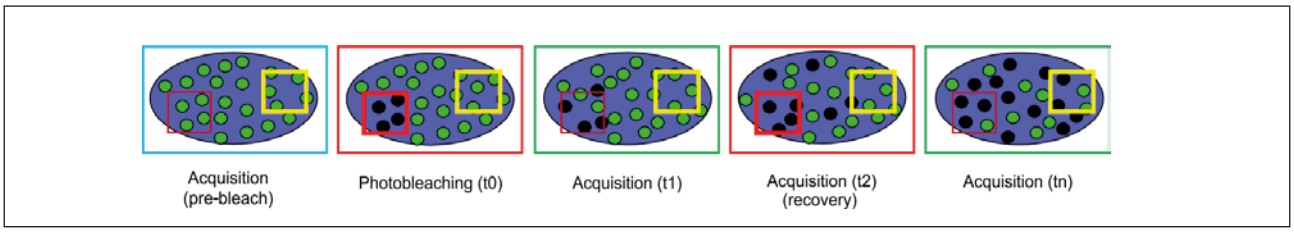


***Figure 2*** *Principle of FLIP experiments. A cell or organelle is uniformly labeled with a fluorescent tag and a pre-bleach series collected. A ROI is selectively photobleached using a short pulse of intense laser light repeatedly whilst a second ROI is analysed for the loss of fluorescence.*

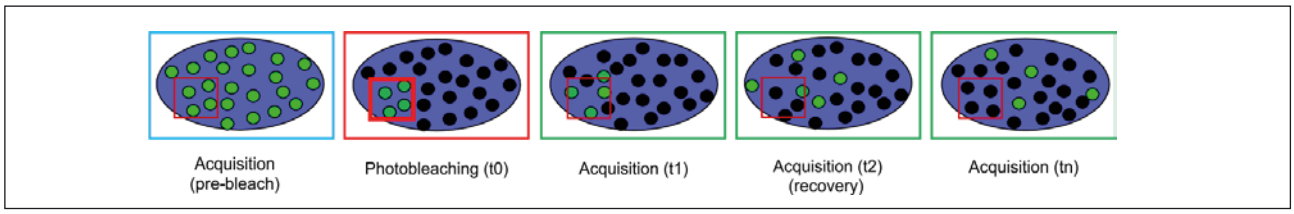


***Figure 3*** *Principle of iFRAP experiments. A cell or organelle is uniformly labeled with a fluorescent tag and a pre-bleach series collected. A ROI is selected and the area outside of it photobleached using a scanned pulse of intense laser light. The ROI is then analysed for the loss of fluorescence over a post-bleach acquisition. Due to the high likelihood of inducing phoodamage on live cells, this technique has largely been replaced by photoactivation-type experiments.*

molecules mobility and interconnection between cellular compartments. It will not be covered further within the scope of this chapter. This technique has to be used with caution to avoid photodamage due to repeated bleaching steps.

iFRAP (inverse-FRAP; Figure 3[28]) consists of bleaching everything but the ROI, essentially the reciprocal experiment of FRAP. The sample's fluorescence, save for a small area, is photobleached and the analysis concentrates on the loss of fluorescence from the ROI, rather than the recovery. This technique is typically more damaging to the sample due to the large areas that are exposed to high laser power. As a result it has been largely replaced by photoactivation (PA) experiments since the discovery of photoactivable GFP and development similar proteins[13],[29-31]. With PA experiments the fluorescence can be selectively "turned on" in an ROI through a pulse of shorter wavelength light (typically 405 nm). These techniques will not be covered further within the scope of this chapter.

## 2. Qualitative Determination of Protein Dynamics

It is common, and quite straightforward, to characterize molecule dynamics from FRAP experiment by the half-time of recovery ($t_{1/2}$) and the mobile (*M*) and immobile (1-*M*) fractions. Even if it has no direct relation with biophysical parameters, they provide a general semi-quanitative estimate on molecule dynamics and can be used to compare various biological conditions. $t_{½}$ gives information on the average dynamics of moving molecules, whereas *M* quantifies the fraction of molecules which are moving and 1-*M* describes the fraction of immobile molecules within the bleached area during the experiment. Immobile molecules strongly interact with a structural component of the cell or be 'trapped' within a multi-component protein complex, preventing them from moving away from the bleached ROI.

From the recovery curves, $t_{½}$ can easily be extracted (see Figure 4), provided the post-bleach recovery imaging segment was sufficiently long and appropriate intensity corrections have been performed. This value needs to be used with caution, since the

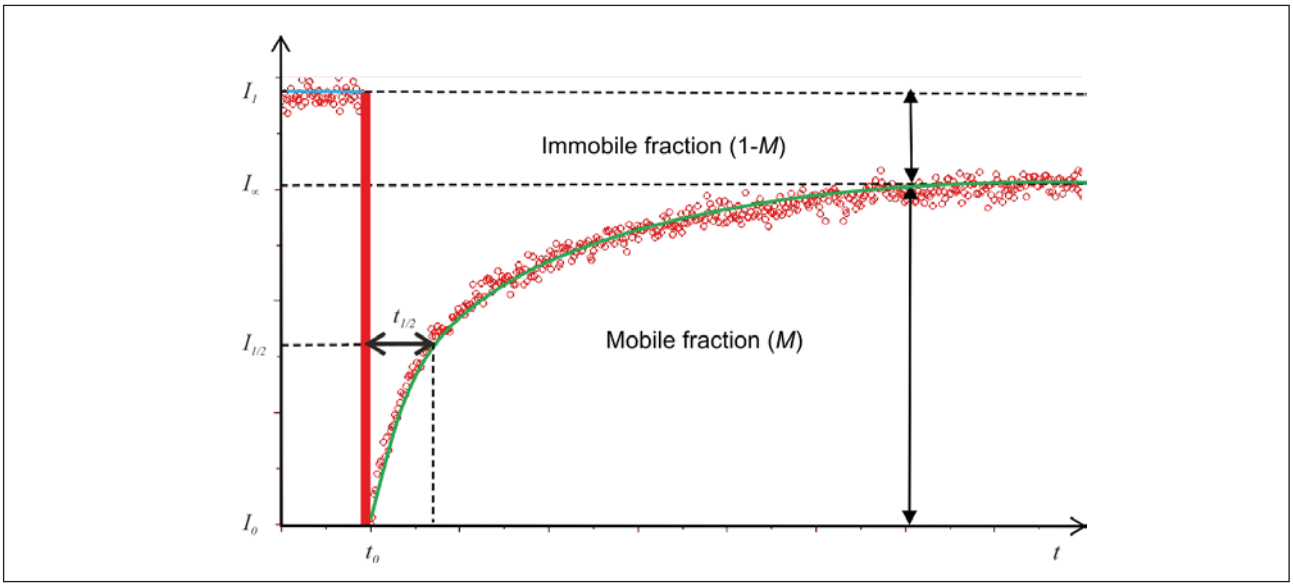


***Figure 4*** *Recovery curve of a FRAP experiment and determination of half-time of recovery ($t_{½}$), mobile (M) and immobile (1-M) fractions.*

cell geometry and bleached area properties (size, position with respect to the cell) can strongly influence $t_{½}$ in addition to the molecules' behavior[11].
The photobleaching depth B is given by the fraction between the remaining signal and the original signal in the bleached ROI. It is given by

$$B=\frac{I_1-I_0}{I_1}$$

This fraction is important for quantitative analysis and has to be less than 80% in practice.
The mobile fraction is given by

$$M=\frac{I_\infty-I_0}{I_1-I_0}$$

and gives information on proteins that are mobile or interact transiently with immobile binding sites during the observation time of the experiment. Again, caution needs to be taken since *M* may depend on acquisition parameters and bleaching dimensions.

## 3. Models for Quantification of Diffusion and Chemical Exchange

Molecular mobility is mainly due to diffusion, flow or chemical reaction (association/dissociation to an immobile molecular complex). The general equation of the fluorescence recovery is given by

$$\frac{\partial c(r,t)}{\partial t}=D\nabla^2 c(r,t)-V\frac{\partial c(r,t)}{\partial r}+(k_{off}-k_{on})c(r,t)$$

The first term describes the diffusion, the second the flow and the last term the association/dissociation processes. Unfortunately, this equation has no analytical solution, and it is easier to investigate each contribution individually. It therefore may be necessary to perform multiple experiments, bleaching for example areas with different sizes.

**Diffusion**
The diffusion coefficient of a molecule is given by the Stokes-Einstein relation:

$$D=\frac{kT}{6\pi\eta R}$$

Where *k* is the Boltzmann constant, *T* the temperature, *η* the viscosity and *R* the hydrodynamic radius of the molecule.

In most FRAP experiments, *D* is defined as

$$D=\frac{w^2}{4\tau_D}$$

where *w* is the waist of the bleached area, $\tau_D$ a characteristic time constant extracted from mathematical model fitting, and *n* the number of spatial dimensions. In general $\tau_D$, has no direct relation with $t_{1/2}$ defined in Figure 4.

**Point Bleaching with Gaussian Profile (Axelrod Model)**
It is possible to extract the diffusion coefficient by single-point bleaching with a Gaussian profile, using the Axelrod model[1] (Figure 5). Recovery curves are reconstructed by averaging the pixel intensity values within a circular region of interest of *w* in diameter, *w* being the waist of the laser at 1/e² (13.5%) of the peak intensity of the Gaussian. Using imaging information, the recovery sequence can be corrected by normalizing mean pixel intensities in another ROI located far from the bleaching area. This step makes it possible to take into account intensity fluctuations due to observational photobleaching or laser instabilities. Once corrected, the recovery curves are fitted with a 10th order limited development of the following equation.

$$F(t)=(1-M)\frac{1-e^{-K}}{K}+M\sum_{n=1}^{\infty}\left[\frac{(-K)^n}{n!}\right]\left[1+n\left(1+\frac{2t}{\tau}\right)\right]^{-1}$$

where *M* is the mobile fraction (accounting for the ability of the molecule to diffuse during the duration of the experiment), *K* is a bleaching constant parameter and  is the characteristic diffusion time. The diffusion coefficient *D* can be correctly estimated as follows:

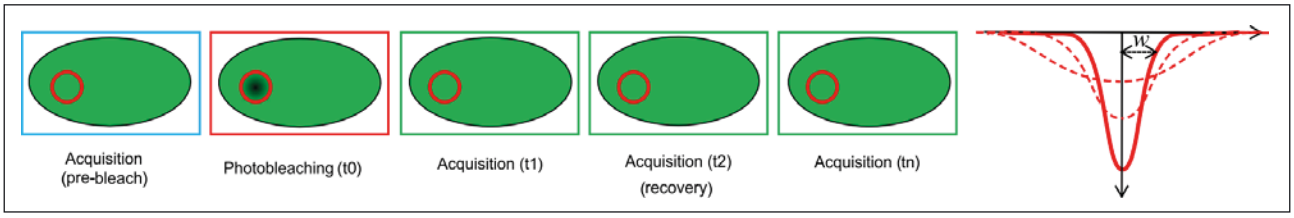


***Figure 5*** *Schematic and model of point bleaching with a Gaussian profile and recovery.*

$$D=\frac{w^2}{4\tau}$$

*K* can be obtained by fitting the following laser intensity distribution on the bleaching profile:

$$I(r,t=0)=I_0 e^{-KB(r)} with\, B(r)=B(0)e^{-\frac{r^2}{2w^2}}$$

**Point Bleaching with Rectangular Profile (Soumpasis Model)**

Bleaching with a rectangular profile (Figure 6) allows avoiding the complexity to measure the bleaching constant K required to solve the Axelrod equation[1],[32]. In this case, the recovery curve can be fitted with the following equation:

$$I(t)=e^{\frac{-2\tau}{t}}\left(J_0\frac{2\tau}{t}+J_1\frac{2\tau}{t}\right)$$

Where *J* is the Bessel function and $\tau = w^2 / 4D$.

**Line Bleaching**

The Soupmasis model requires shaping of the beam with a square profile, which is not commonly available on standard FRAP equipments. A simpler solution consists of performing a line bleaching with a Gaussian beam profile[33],[34] (Figure 7).

In this case, the recovery curve can be fitted by the following equation, a 1D approximation of the diffusion process:

$$I(t)=I_\infty\left(1-\sqrt{\frac{w^2}{w^2+4\pi Dt}}\right)$$

This model displays an accuracy of about 30%.

**Chemical Interaction**

FRAP recovery curves of chemical reactions are the simplest case, since they can be solved by the following single exponential recovery function:

$$I(t)=A(1-e^{-t/\tau}) \quad \text{with} \quad \tau=\frac{1}{k_{on}+k_{off}} \quad \text{and} \quad A=\frac{k_{off}}{k_{on}+K_{off}}$$

where $k_{on}$ and koff are the association and dissociation rates of the bleached molecules to their ligand. In practice, this case is very rare in living cells, and diffusion always plays a role[20]. Nevertheless, it is important to notice that in the case of pure binding, the recovery rate is independent of the bleaching radius, while it is a function of w in the case of Brownian diffusion.

## 4. Methods – FRAP Experiments

**Instrumentation**

The hardware required for FRAP experiments comprises a fluorescence microscope equipped with light sources (arc lamps, LEDs or lasers) and filter sets for imaging as well as a light source for bleaching (typically lasers) with some method of selectively bleaching a region of interest (ROI). Of course, a sample labeled with a fluorescent molecule attached to the protein of interest is required and is one of the challenges the biologist is facing. The fluorophore used should be selected both for its spectral properties in order to match the laser lines and filters available, as well as for having favourable photophysical properties, i.e., negligible bleaching probability at low illumination intensities and high bleaching probability at high intensities. As most biological experiments involve living cells, there is a prerequisite for the

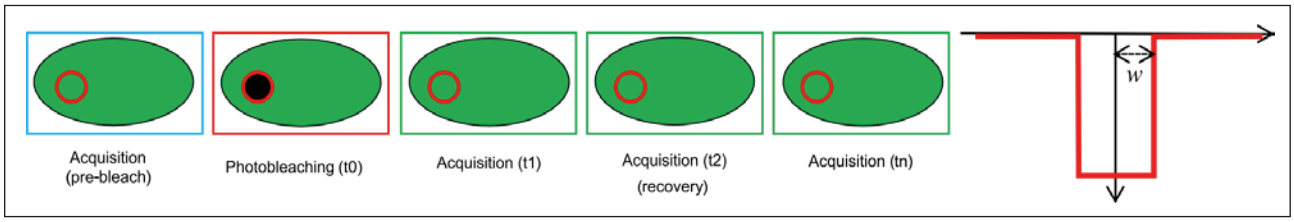


***Figure 6.*** *Schematic and model of point bleaching with a rectangular profile and recovery.*

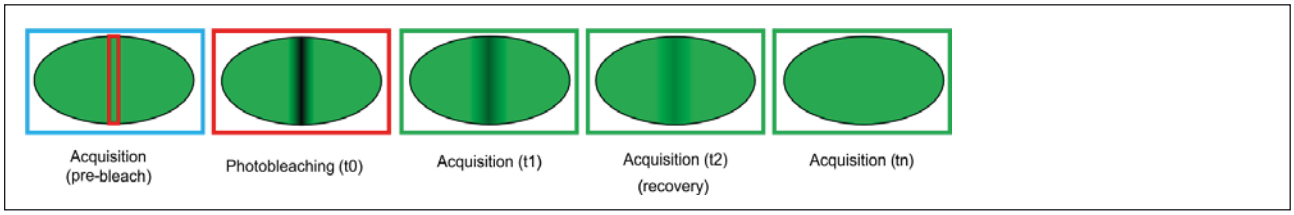


***Figure 7.*** *Schematic of line bleaching and recovery.*

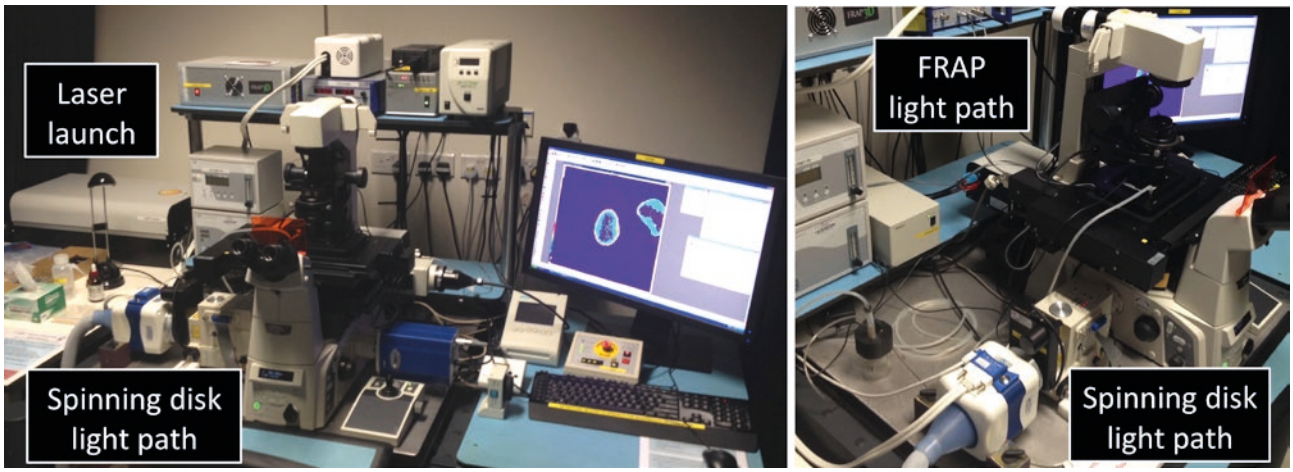


***Figure 8*** *Photographs of an implementation of the Roper iLas2 FRAP-3D system at the Institute of Medical Biology, A*STAR, Singapore. The microscope stand is an inverted Nikon Eclipse Ti equipped with a Yokogawa CSU-22 spinning disk confocal head and a liquid-cooled Photometrics Evolve EM-CCD camera. It is operated through the MetaMorph and iLas2 acquisition software. The lasers (405/491/561 nm) inside the laser launch are used for both imaging and FRAP, with an AOTF to rapidly control the intensity of laser reaching the sample, and a galvo mirror to rapidly switch between the spinning disk (mounted on the left of the microscope) and FRAP light paths (mounted at the rear of the microscope).*

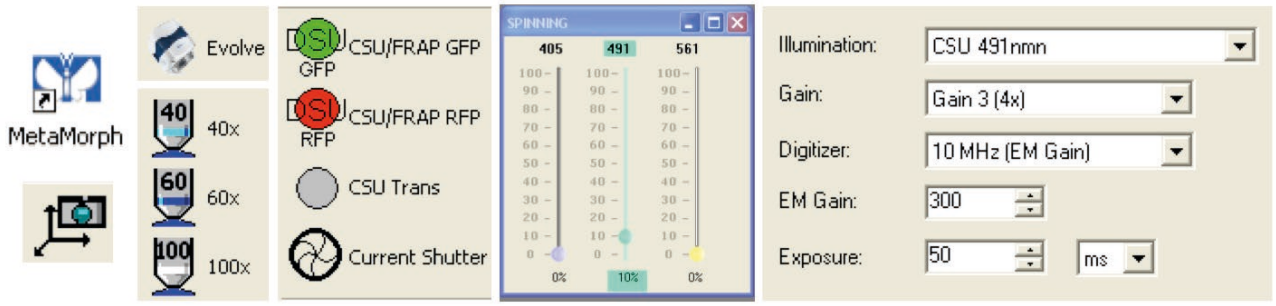


***Figure 9*** *Establishing and refining the imaging conditions in the MetaMorph software through selection of the correct camera mode, objective lens, filter combination, laser power, camera gain and exposure conditions appropriate for the sample. Photobleaching and photodamage, as a consequence of tuning these imaging conditions, should be reduced to a minimum – lower laser power and shorter exposures, whilst maintaining sufficient dynamic range in the images for quantification and imaging frequency for sampling of the recovery.*

microscope to be equipped with an incubator and a supply of humidity and $CO_2$, as dictated by the cell type.

The most common way to achieve selective photobleaching is to use galvanometer-driven mirrors to steer the laser beam, which is momentarily switched to a higher intensity, to a diffraction limited spot or to a scanned region (in raster or whirlwind pattern) over a pre-selected ROI. For this reason, many FRAP experiments have been carried out on confocal laser scanning microscopes (CLSMs) as they are equipped with high-power lasers and galvo mirrors [35-37]. FRAP scanning heads are now also commercially available for widefield fluorescence systems (e.g. DeltaVision Elite from GE), spinning disk confocal or TIRF systems (e.g. iLas2 FRAP-3D from Roper (Figure 8), UltraView VoX from PerkinElmer and Revolution XD from Andor). In these setups the microscope's excitation illumination light-path and the FRAP illumination light-path are independent from one another, offering very fast switching between, or even simultaneous, photobleaching and imaging. Typically, such systems will have some calibration routine to correlate a given pixel's position with the corresponding galvo position. Some of the CLSM manufacturers have also developed systems with dual scanners to enable this (e.g. the SIM scanner on the Olympus FV1000 and FV1200 confocal systems).

The short-lived increase in the laser intensity for photobleaching is achieved either by using an acousto-optical tunable filter (AOTF), by modulating the laser output power directly, or by using a fast switching mirror to steer the beam into an independent light-path with reduced attenuation.

**Data Acquisition**

Acquisition and photo-perturbation parameters need to be carefully adjusted in order to minimise errors

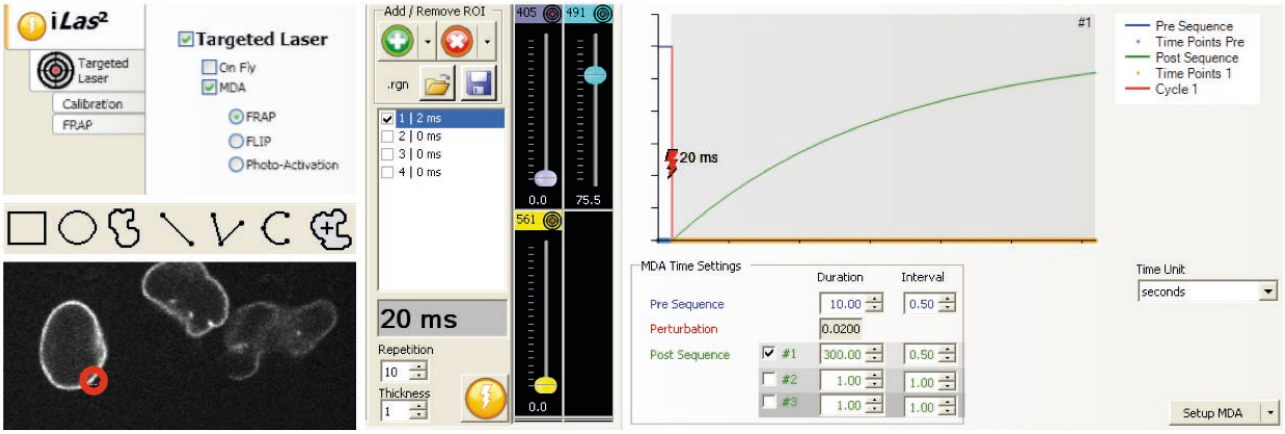


***Figure 10*** *Establishing and refining the FRAP conditions in the iLas2 module through selection of the appropriate mode of operation (On-Fly or MDA), ROI shape, size and location, duration/repetitions of bleach and laser intensity to be used, as well as the temporal frequency and number of images in the pre- and post-bleach acquisition steps. They have to be adjusted according to the protein dynamics and cell and fluorophore stability. This data, once established, is then sent back to the Multi Dimensional Acquisition tool in MetaMorph.*

in quantification and interpretation[27]. This is a rule of thumb for qualitative and quantitative FRAP experiments. Prior to carrying out a FRAP experiment, one should perform empirical tests to establish time-lapse image acquisition settings that minimise photobleaching and match protein dynamics. This usually requires a compromise between sufficient dynamic range for quantification and sufficient imaging speed to properly sample the recovery dynamics. Acquisition frequency and photo-perturbation duration need to be adjusted according to the recovery speed as well. This will involve selection of the appropriate objective lens, fluorescence filter set, laser power, camera settings, exposure duration (or corresponding confocal acquisition settings) and imaging frequency according to the sample preparation and protein dynamics (Figure 9). Subsequent empirical tests should be carried out to tune the FRAP settings like the ROI shape, size and location, bleaching duration and laser intensity (Figure 10). It is important that the duration of the photobleaching step should be short enough to prevent any molecules from entering or leaving the ROI during the bleach. In addition, acquisition of the post-bleach sequence must be fast enough to capture the fluorescence recovery. Finally, the delay between the end of the bleaching and the beginning of the recovery must be as short as possible. In a general manner, one can use the rule of ten:

- bleaching duration must be at least 10 times faster than the half time of recovery ($t_{1/2}$).
- delay between the end of the bleaching step and the beginning of the recovery sequence must be shorter than a 10$^{th}$ of $t_{1/2}$.
- acquisition frequency of the recovery sequence must be at least 10 times faster than $t_{1/2}$ (at least until t = $t_{1/2}$, then the acquisition frequency can be reduced to avoid observational photobleaching).
- recovery sequence duration must be about 10 times longer than $t_{1/2}$.

When the size of the bleaching ROI affects the half time recovery, which is the case for example for diffusional or flow-induced mobility, it can be adjusted for the rule of ten to hold (e.g., bleaching a larger area in a fast mode will increase the $t_{1/2}$, allowing slower acquisition frequency).

In the following example, we show the data acquisition parameters that require consideration for the iLas$^2$ FRAP-3D system (Roper, France), integrated onto the MetaMorph acquisition platform (Molecular Devices; Figure 9). Whilst the considerations are similar on other hardware/software platforms, the terminology and implementation may differ.

Once the appropriate imaging and FRAP conditions are established, single or multiple ROIs are selected in a preview image and the iLas$^2$ software sends the data as a journal (macro) to the MetaMorph Multi Dimensional Acquisition (MDA) tool. This handles the complex time-lapse acquisition routine established in the iLas$^2$ window. When the time resolution of the experiment is critical the FRAP-On-Fly method can be used instead of the MDA. In this case a stream acquisition is setup in which the hardware is pushed to its limits to maximize frame-rate. During the acquisition, the user can then manually select the position of the FRAP ROI with the mouse whilst the stream acquisition continues. The frame rate is then limited by the exposure time and read-out speeds of the camera/computer system.

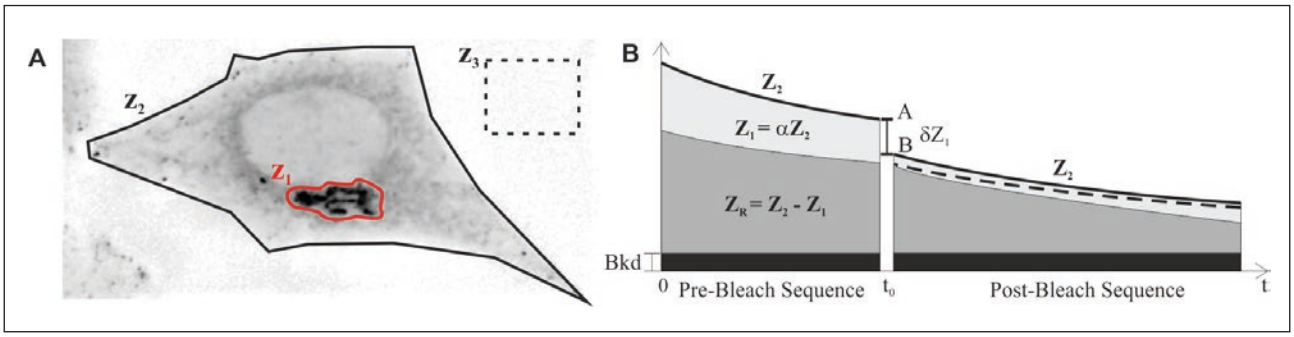


***Figure 11*** *Image correction prior quantification. All the information for a given cell is used to measure the cell fluorescence eliminated by bleaching and to compensate for observational bleaching over time throughout the experiment.* ***A*** *Three distinct ROI are defined: $z_1$ is the region targeted by the laser bleaching pulses; $z_2$ includes the whole cell and is assumed to contain a fixed number of fluorophores, regardless of whether those fluorophores are bleached or fluorescent; $z_3$ is defined for the estimation of background level, mostly due to the CCD dark signal.* ***B*** *The average level over $z_3$ is subtracted from all the images. Signal levels $Z_1$ and $Z_2$, in regions $z_1$ and $z_2$, are analysed after background subtraction. They are processed to account for observational bleaching and for the limitation of recovery in $z_1$ by the total loss over $z_2$.*

**Data Processing Prior to Quantification**

It is of major importance to process the acquired data before quantification in order to correct for observational photobleaching and avoid artifacts in the quantification[27]. Image sequences can be corrected for observational photobleaching using the fact that a closed volume ($Z_2$ in Figure 11A) contains a finite number of fluorescent molecules[5]. Changes in the average intensity inside this volume over time results from both bleaching pulses and observational photobleaching, following a first-order decay with time constant τ. Observational photobleaching can be assessed with $Z_2$ before $(t<t_0)$ and after the pulse $(t>t_0)$, and corrected by multiplying $Z_2$ by $e^{(t-t0)/\tau}$. In this normalization process, the step points A and B are fixed points, and the normalized $Z_2$ curve (second plot) has two constants: $Z_2(t<t0) = A$ and $Z_2(t>t0) = B$.

Let α be the ratio $Z_1/Z_2$, with $Z_1$ the average intensity over the volume $z_1$ (see Figure 11A). Before the bleaching pulse, at $t<t_0$, α remains constant at steady state. $Z_1$ (light grey) and the difference signal $Z_R=Z_2-Z_1$ over the complementary region (dark grey) therefore both decay like $Z_2$. Consequently, $Z_1$ and $Z_R$ are constant before the pulse on the normalized graph. Immediately after the photobleaching of $z_1$, at $t=t_0$, $Z_1/Z_2$ no longer equals α, but instead falls to $\alpha - \partial Z_1 / Z_2$. If full recovery to the initial steady state occurs during the experiment, this proportion then returns to $\alpha_1$, and $Z_1$ recovers at the expense of $Z_R$. With the exception of very limited bleached areas and/or bleaching depths, full recovery in $z_1$ is attenuated by a loss factor $B/A$. Recovery curves are thus compared to their asymptotic limit $Z_1(B/A)$, so that the mobile fraction can still be measured correctly. To enable comparisons between multiple experiments a normalisation step is typically carried out.

Once the data are processed and normalized, recovery curves can be analyzed following the procedure introduced earlier.

## 5. Conclusion

FRAP is carried out on microscopes equipped with components that allow the user to selectively expose regions of interest (ROI) to intense pulses of laser light. This is usually possible on research grade microscopes such as confocal instruments. In doing so the fluorescent molecules (usually proteins of interest tagged with fluorophores like GFP) within that region are photobleached into an 'off' state. By subsequently analysing that ROI the dynamics of the recovery of that fluorescence can be used to give a greater understanding of the molecule being studied like: (1) the half-time of recovery, (2) the ratio between mobile and immobile fractions, (3) the effective diffusion coefficient, (4) the binding time of proteins to immobile macromolecular complexes, and (5) the interconnection of intracellular organelles and the existence of protein complexes.

## Acknowledgements

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## Further Reading

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