A glimpse at mRNA dynamics reveals cellular domains and rapid trafficking through granules

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Chapter 6

Photobleaching based techniques to study molecular dynamics

The discovery of the green fluorescent protein (GFP) [1] caused visualization of movements and dynamics of various cellular components to become within hand reach [2,3].

The GFP technique is based on the fusion of the GFP gene to the gene of a protein of interest and expressing this construct in living cells to study its localization and dynamics. The fluorescently tagged protein can be found either in the nucleus or in the cytoplasm, can be present diffusely throughout the cell or restricted to specific cellular structures.

Continuous efforts have been made to broaden the color palette of the available fluorescent proteins and to improve or modify their intrinsic properties [4-8].

Available colors now range from blue to far red and almost each nuance has a monomeric variant, whereby artificial protein aggregation induced by the fluorescent tag is avoided.

Today, with the concurrent development of advanced imaging systems, researchers are provided with a wide range of tools for live cell imaging. In the past three decades photobleaching techniques have been developed to qualitatively and quantitatively describe protein movements and dynamics within the cell [9,10]. While fine-tuning the various photobleaching protocols for our own purposes, it has become undoubtfully clear that these powerful tools only produce valuable results if they are well controlled. In this chapter, some of the experimental conditions are critically reviewed on the basis of both our own experience and reported literature. Finally, a list of

guidelines and recommendations for the design and performance of photobleaching experiments is provided.

Fluorescence Recovery After Photobleaching (FRAP)

FRAP is a method to measure the apparent mobility of a protein in living cells [11]. A GFP-tagged construct is transiently or stably expressed in a cell, a cellular region expressing this protein is bleached and fluorescence recovery of the same area is subsequently measured (Fig. 1). In fact "fluorescence redistribution" would be a more appropriate term to use than "fluorescence recovery"since photobleaching is mostly irreversible [12].

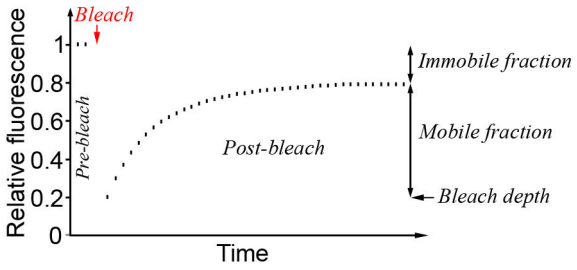

Figure 1: FRAP recovery curve. A few pre-bleach images are taken before bleaching a small area of the cell with a short laser pulse. The number and rate of the post-bleach images taken varies with the protein of interest. The curve represents the relative fluorescence intensity in the bleach area through time. Bleach depth, mobile and immobile fraction are directly visible on this graph. The time scale of the experiment varies with the type of tagged protein but typically is in the order of a few minutes.

The fluorescence intensity reached after bleaching is called the "bleach depth". If the fluorescence intensity does not fully recover after photobleaching, this indicates the presence of an immobile fraction of the protein of interest. The recovery rate reflects a combination of diffusion and particularly transient or stable binding interactions of the protein of interest.
In fact, during a FRAP experiment, the sample is submitted to two types of bleaching. The first type of bleaching is due to image acquisition and will be referred to as "scan bleaching". Each time a fluorescent protein is excited with the appropriate wavelength, it irreversibly loses part of its ability to emit. Each fluorescent protein has its own bleaching characteristics and one intends to use a fluorescent protein variant that is the least affected by scan bleaching. This artificial reduction of fluorescence intensity is not related to any movement of the tagged protein and should be minimized and corrected for.

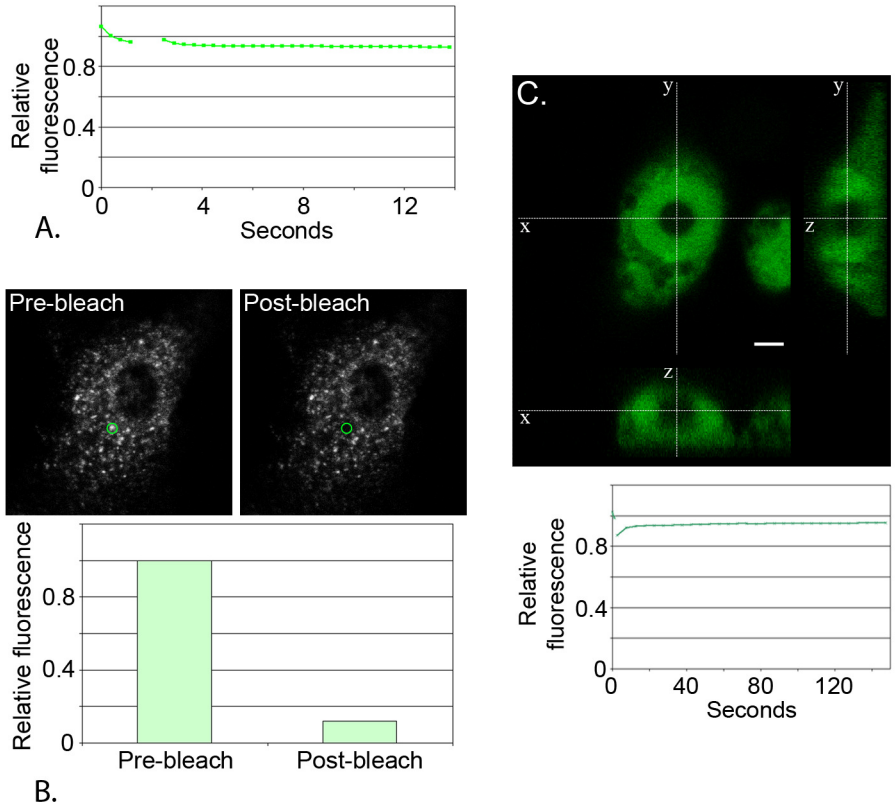

Figure 2: Scan and bleach parameters. **(A)** Scan-bleach test on EGFP fusion protein expressing cells. Fluorescence intensity is measured in the whole cell and normalized to pre-bleach values. In this example, scanning only induces less than 10% loss of fluorescence intensity. **(B)** Bleach depth determination. A region of interest is chosen in a fixed EGFP fusion protein expressing cell (green circle). The area is bleached using one frame full power of the 488 nm argon ion line. Quantification of the fluorescence intensity in the region of interest shows that bleach depth is almost 90%. **(C)** Volumetric bleaching with one bleach event. An EGFP transfected cell was bleached once (bleach area 3µm in diameter on a single focus plane) in the centre of the nucleus and a 3-dimensional view of the effective bleached volume is given. It is clearly seen that the cell is also significantly bleached above and beneath the bleach focal plane. The difference between the maximum recovery point and 100% recovery in the corresponding graph indicates the total amount of EGFP molecules bleached by the FRAP routine.

Therefore, we ensured that the entire data acquisition process did not induce more than 10% loss of fluorescence intensity by performing a scan-bleach test: a “sham” control FRAP experiment was performed on cells expressing a GFP-fusion protein with the bleach laser power set at 0% to only determine the bleaching due to scanning of the sample (Fig. 2A) [13]. So, when applying FRAP to living cells, one should determine the optimal frame rate that in principle allows observation of full recovery of fluorescence with a maximum of 10% scan bleaching.

The second type of bleaching corresponds to “active bleaching” of a defined area of the cell that will be used to monitor fluorescence recovery. Ideally, fluorescence intensity should be reduced by at least 70% (bleach depth) in the bleach area with the adequate bleach parameters to obtain sufficient information on the recovery [13]. To determine the bleach depth, fixed cells expressing the fluorescent fusion protein of interest were used (Fig. 2B), as no molecular movement can hinder the measurement of the real bleach depth in such a situation. Obviously, depending on the dynamics of the protein of interest, the bleach depth determined in fixed cells could be higher than the bleach depth achieved in live cells, indicating that, in case of a very dynamic protein, some fluorescence recovery already took place before the first post-bleach image was acquired. Although the bleaching properties of fixed GFP-tagged proteins may slightly differ from unfixed proteins, comparing the bleach depth in live and fixed cells is a proper tool to determine the proportion of the fast moving fraction of the protein of interest [13].

Moreover, one should realize that a full recovery of 100% is theoretically not possible, not even after full redistribution, as a certain fraction of the fluorescent molecules has been bleached during the FRAP procedure. This should not create an “artificial immobile fraction” of significant proportions. When performing FRAP experiments, cells are imaged and bleached in one focal plane. However, most samples are not flat enough to be considered as pure 2D objects, and therefore considerable bleaching occurs in the Z direction due to optics limitations [14]. In some cases, this three-dimensional bleached volume can be quite large and thus prevent 100% fluorescence recovery, even for a fully mobile protein. This “artificial immobile fraction” might interfere with the evaluation of the real immobile fraction. Therefore, it is advised to keep the size of the bleached area as small as possible and the bleach time (or number of bleach iterations) as short as possible. Note that the unwanted 3D artificial bleaching holds for regular single photon excitation. Selective bleaching in 2D only is possible when multi-photon excitation is performed, as here bleaching outside the focal plane is negligible.

To check for the highest estimation of the total bleached volume, we performed FRAP on EGFP transfected cells (Fig. 2C). As EGFP is very mobile, this test gives an indication of the total amount of proteins that can possibly be bleached with the chosen microscope settings. This value should be taken into account when measuring the immobile fraction of the protein of interest.

When collecting FRAP data, imaging and bleach settings should be fine-tuned to minimize the effect of scan bleaching and optimize active bleaching for each experiment.

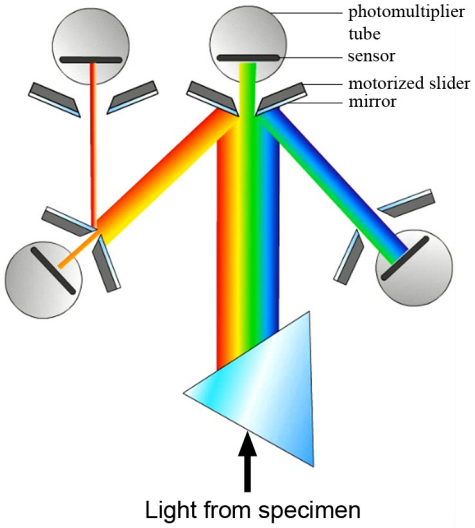

Figure 3: Leica SP5 spectral detector (adapted from the Leica Microsystems brochure "Sensitivity is everything…", 2005). Light emitted from the specimen goes through a prism and is separated by setting physical windows (pinholes) into several channels by the SP detector; accuracy is as small as a few nanometers.

Fluorescence emitted by the sample is collected and enhanced by a photomultiplier tube (PMT). Most confocal microscopes are equipped with several PMTs to allow simultaneous recordings of different colors; in the Leica SP5 each PMT is coupled to sliders that can be adjusted to collect light of the desired wavelengths (Figure 3). Depending on the distance of the PMT from the main light path, loss of detectable signal varies. For instance, the Leica SP5 confocal microscope has four different PMTs. When imaging ECFP-tagged proteins in cultured cells, the four PMTs were tested with same laser intensity and the sliders set to measure a signal between 470 and 525 nanometers (Fig. 4A). The first PMT detected about half of the signal compared to the other three PMTs. Indeed, the first PMT was located further away from the light path in this particular system. This difference in the amount of collected signal does not represent a problem if the signal emitted by the sample is sufficiently strong and all the measurements are made using the same PMT. For effective light collection we decided to use only PMT2 and PMT3 for imaging.

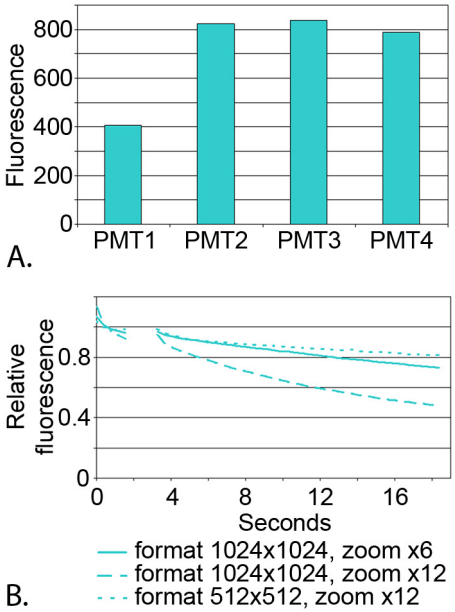

Figure 4: Imaging parameters. **(A)** Optimum PMT determination. ML-DmBG2-c2 cell expressing an ECFP fusion protein imaged with the four different photomultiplier tubes available on a Leica SP5 DMI6000 confocal microscope. Each bar represents the average fluorescence intensity per pixel in the whole cell. PMT1 is the least efficient. **(B)** Influence of the zoom factor and image resolution on scan bleaching. ECFP-tagged protein expressing cells were submitted to a scan-bleach test with the 458 nanometer line of the argon laser. Scan-bleaching increases with image resolution and zoom factor.

The post-bleach imaging routine consists of the recording of a substantial number of images and the loss of fluorescence resulting from these recordings should be minimized. When adjusting all imaging parameters, such as laser intensity, scan speed, image size, pinhole and zoom factor, we noticed that the modification of a single parameter could significantly increase fluorescence loss due to scan bleaching. For instance, increasing the zoom factor from 6 to 12 doubled the scan bleaching (Fig. 4B). This can be compensated for by reducing the image format but then image resolution is reduced (Fig. 4B). Thus, defining the optimal imaging parameters is a permanent trade-off between image quality and reduced scan bleaching.
Also, if the experiments are performed in course of several days, imaging and bleaching parameters should be kept identical. Moreover, a scan-bleach test and bleach depth measurement should be repeated each day to check for laser fluctuations and to legitimately allow pooling of the different measuring results.

Fluorescence Localization After Photobleaching (FLAP)

The principle of FLAP is to study a fusion protein bearing two different fluorescent tags. In practice, two differently tagged constructs are made and expressed in cells in a more or less balanced way. One tag is then bleached as in regular FRAP, and the other one is used as a reference tag allowing the determination of the original localization of the protein of interest [15]. This is particularly useful in case of small regions of interest (nuclear bodies) that show movement during the measurement of the recovery process. The challenge is to choose the best combination of fluorescent tags allowing simultaneous imaging and bleaching. Each color has specific excitation and emission spectra and specific bleaching characteristics. Ideally, the combined colors should have spectrally well separated excitation and emission wavelengths to minimize possible cross-talk, and be affected to a similar extent by scan bleaching.
In this particular set of experiments, ECFP or EGFP fusion proteins were available to be combined to either mCherry or mPlum fusions of the same protein. Therefore we decided to determine the feasibility of each combination.

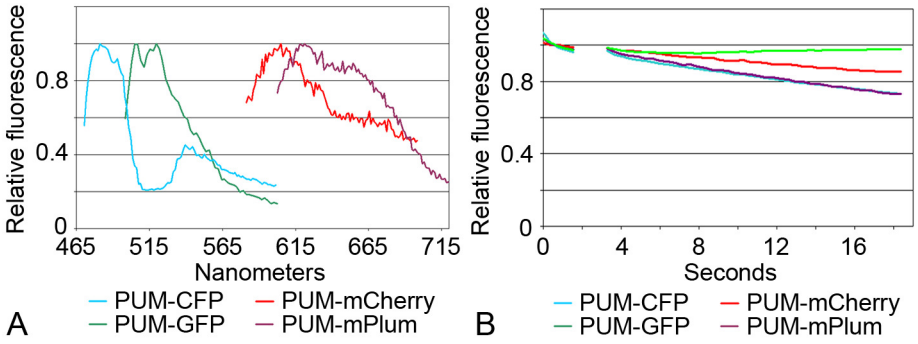

Figure 5: Fluorescent protein properties. **(A)** Normalized emission spectra. The emission spectrum of each fluorescent fusion protein was measured, allowing accurate adjustment of the PMT settings for posterior data acquisition and avoiding any bleed-through. **(B)** Scan-bleach test for different fluorescently tagged proteins. Fluorescence intensity was measured in the whole cell and normalized to pre-bleach values. EGFP fusion protein is less affected by scan bleaching than the other fluorescently tagged proteins.

First, the respective emission spectra of all fusion protein were measured (Fig. 5A), allowing to narrow down the detection windows (PMT slider settings) used for each color, and reduce, or preferably eliminate any cross talk (green EGFP signal detected in the red channel for instance). ECFP and

mPlum emission spectra are spectrally well separated and are therefore suited to be used in FLAP.
Then, each fusion protein was submitted to a scan-bleach test, as previously described for FRAP experiments (Fig. 5B). EGFP and mCherry turned out to be the most resistant to scan-bleaching and are therefore preferred if longer recording is needed.

Next, we determined the bleach depth that could be achieved for each tag (Fig. 6A). The different colors were bleached with 1 frame at full laser power and the resulting fluorescence intensity was normalized to pre-bleach values. The EGFP fusion protein was readily bleached (80%) whereas only 60% bleach depth was obtained with mPlum or mCherry fusion proteins. The bleach depth reached with the ECFP fusion protein was about 75% which is within the commonly recommended range. From these results, PUM-EGFP is the most attractive candidate to use for bleaching.

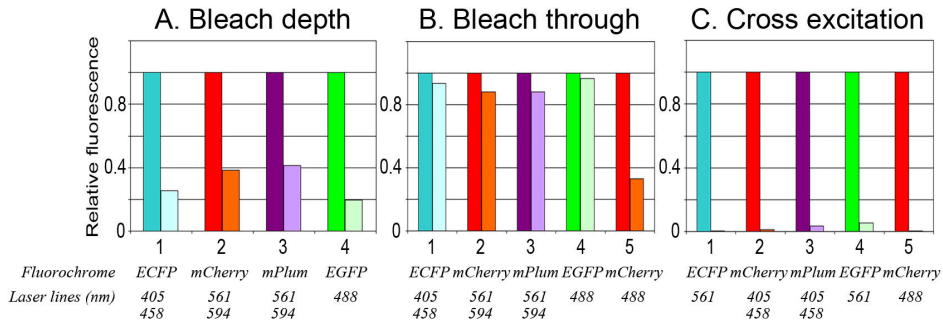

Figure 6: Bleach depth, bleach through and cross excitation. **(A)** Bleach depth for the various fusion proteins. Left bar represents the normalized pre-bleach intensity, right bar represents the post-bleach intensity. (1) ECFP fusion protein bleached with 405 nm and 458 nm laser lines. (2) mCherry fusion protein bleached with 561 and 594 nm laser lines. (3) mPlum fusion protein bleached with 561 and 594 nm laser lines. (4) EGFP fusion protein bleached with 488 nm laser line. Bleach depth is the highest for the EGFP fusion protein. **(B)** Bleach through measurement. Left bar represents normalized pre-bleach fluorescence intensity measured in the bleach area, right bar represents post-bleach fluorescence intensity in the same area. (1) ECFP fusion protein bleached with 561 and 594 nm laser lines. (2) mCherry fusion protein bleached with 405 and 458 nm laser lines. (3) mPlum fusion protein bleached with 405 and 458 nm laser lines. (4) EGFP fusion protein bleached with 561 and 594 nm laser lines. (5) mCherry fusion protein bleached with 488 nm laser line. mCherry is most sensitive to bleach through when combined with EGFP. **(C)** Cross excitation measurement. Left bar represents normalized fluorescence intensity measured with the appropriate laser for each color, right bar corresponds to the fluorescence intensity measured with the opposite laser. (1) ECFP fusion protein imaged with 561 nm laser line. (2) mCherry fusion protein imaged with 405 and 458 nm laser lines. (3) mPlum fusion protein imaged with 405 and 458 nm laser lines. (4) EGFP fusion protein imaged with 561 nm laser line. (5) mCherry fusion protein imaged with 488 nm laser line. The EGFP fusion protein gives the highest cross excitation signal if combined with mCherry or mPlum.

Furthermore, when two fluorescent tags are present within the same sample, active bleaching of one color should not induce significant bleaching of the

other label. Therefore we assessed how much blue or green fluorescence was lost by bleaching the red fluorescent proteins and vice versa, or how much red fluorescence was lost by bleaching with the laser line used to bleach EGFP fusion proteins (Fig. 6B). Bleaching EGFP caused a dramatic loss of mCherry fluorescence whereas the other combinations tested were all acceptable. That was the reason why we chose in the EGFP/mCherry combination, to bleach mCherry and use EGFP for localization. One could argue that the bleach depth obtained with mCherry was not sufficient (Fig. 6A), but we opted for less cross-bleaching instead of high bleach depth because in this particular case we did not intend to quantitatively analyze the FLAP results.

Finally, to estimate the extent of cross excitation, blue and green fusion proteins were excited with the red laser line and red fusion proteins were excited with the blue and the green laser lines (Fig. 6C). Although EGFP gave the highest cross excitation signal when imaged with the laser used for mCherry excitation, the signal measured was considered acceptable and therefore EGFP was chosen to be combined with mCherry for FLAP experiments.

All fusion proteins were then used individually for FRAP measurements (Fig. 7A). Although their respective initial bleach depths differed (already observed in fig. 6A), each fluorescently tagged protein recovered. Note that the ECFP-fusion protein was also bleached with less laser power to achieve a bleach depth comparable to the red-tagged proteins. To be able to compare the obtained data sets, the first post-bleach values were set to 0, thus eliminating bleach depth variation. Strikingly, the ECFP fusion protein recovered slower than the EGFP, mCherry or mPlum variants, irrespective of the starting bleach depth (Fig. 7B). Due to this difference in dynamics, we decided not to use the ECFP variant of our protein of interest. A possible explanation or this observation could be that ECFP has a slightly higher tendency to dimerize than EGFP, although we cannot exclude different behavior of the fusion proteins as a function of tag type.

A monomeric mutant of ECFP has been made [16] and it would be interesting to monitor its dynamics under the same conditions. To our knowledge, the dimerizations of EGFP and ECFP have not been fully compared and, further investigation is needed to infer the hypothesis that ECFP has a more pronounced tendency to dimerize than EGFP.

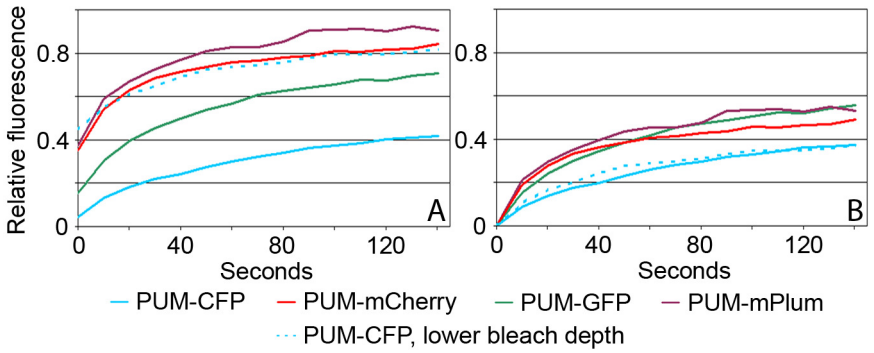

Figure 7: FRAP applied to different fusion proteins. **(A)** Data are background, scan corrected and normalized to pre-bleach values. Note that the bleach depth reached for the various fusion proteins, each optimally excited, is quite different. The ECFP fusion protein was also bleached with less laser power to achieve a bleach depth comparable to the red-tagged fusion proteins. **(B)** Supplemental normalization: the first post-bleach value is set to zero to eliminate the differences in bleach depth. ECFP fusion protein recovers slower than EGFP, mCherry or mPlum fusion proteins.

Fluorescence Loss In Photobleaching (FLIP)

FLIP consists of repetitive bleaching of a defined region in a cell in which a fluorescently tagged protein is expressed and subsequently monitoring the loss of fluorescence through the entire cell or in another region of the cell. Any tagged protein that moves through the bleach spot will be bleached. Thus, any compartment within the cell that is able to "communicate" with the region that is continuously bleached will eventually show a reduction in fluorescence.

A FLIP routine consists of a few pre-bleach images followed by a loop of a bleach event (with flexible duration) alternating with image capture. Due to this looped scanning and bleaching, the sample is submitted to high levels of scan bleaching, which impacts the usefulness of the information that is obtained (Fig. 8A). Therefore, for optimal results it is essential to fine-tune the two bleaching components of the FLIP experiment: the bleach events and the imaging. A first complication is that repetitive bleaching of a single spot with high laser intensity can lead to an effective bleached area that is larger than the user defined region [17] due to light scatter and other optical phenomena. We therefore first determined the area that was effectively bleached as consequence of the repetitive bleach event. In case of 117 bleach events of one second each in a fixed cell, a bleached area of approximately 4 micrometers in diameter appears (Fig. 8B). Fluorescence loss in photobleaching should then be recorded in a region located well outside this unspecifically bleached area, as it is not reflecting real

movement of the protein of interest. Another potential source of bleaching is the image acquisition procedure. For instance, the FLIP routine from figure 8C suffers from almost 60% loss of fluorescence due to imaging only; it is called the "scan-bleach test".

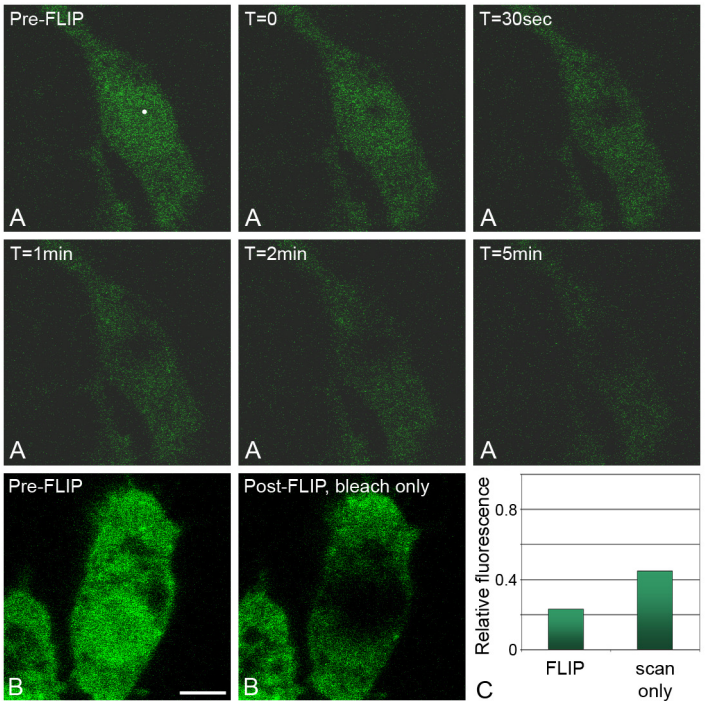

Figure 8: FLIP parameters. **(A)** High loss of fluorescence with current FLIP loop settings. EGFP fusion protein expressing fixed cell, bleach point is indicated with a white dot. One bleach event followed by one frame occurs every 2.5 seconds. After five minutes of this FLIP loop, the cell is hardly visible, due to scan bleaching and not to protein mobility as the cell was fixed. **(B)** Loss of fluorescence due to bleaching in an EGFP fusion protein expressing fixed cell (117 bleach events), images of the loop taken with 0% laser power. Bar: 3.4 micrometers. The bleached area almost represents half of the cell. **(C)** Remaining fluorescence in the cell after a regular FLIP routine (left bar), or after FLIP containing only the scan events (right bar). Values were normalized to pre-bleach intensities. Note that typical image acquisition generates around 55% bleaching.

Besides modifying illumination settings, one could also design an alternative FLIP loop to reduce this passive bleaching and, for instance, scan the sample every five or ten bleach events only. The consequence of this is that the change of the spatial fluorescence intensity distribution within the cell is recorded with less temporal resolution.

The FLIP method is particularly useful to study heterogeneity in mobility as a function of cellular localization: or in other words, which regions deplete quickly and which do not? FLIP is also useful to confirm

that an immobile fraction is present in the cell. Namely, if a well conducted FLIP experiment is performed and complete bleaching occurs everywhere in the cell (corrected for scan bleaching), this strongly indicates the absence of an immobile fraction.
Alternatively, the confirmation of an immobile fraction can also be performed by a so-called secondary FRAP experiment, by which the same region of interest that (partly) recovers in fluorescence is subjected to a second FRAP procedure [18]. If the fluorescence fully recovers after the second FRAP procedure, this indicates that now all fluorescent molecules are mobile, as the immobile ones have bleached during the first FRAP procedure. Incomplete recovery of the fluorescence after the second FRAP suggests other (artificial) reasons rather than the presence of an immobile fraction.

Guidelines used to perform photobleaching experiments

Although setting up photobleaching experiments is a time consuming process, controlling the various parameters is considered essential to produce valuable results. Following is a summary of the main points of the various imaging approaches.

- Choose optimal excitation and imaging settings for each fluorochrome: objective, excitation wavelength, signal collection window, laser power, scan speed, pinhole, zoom factor and image resolution. If multiple colors have to be recorded simultaneously avoid bleed through, cross-excitation or cross-bleaching.
- Measure bleach depth on fixed cells and adapt the bleach settings to reach at least 70% of bleaching if quantitative analysis of the results is needed.
- Measure the total bleached area in fixed cells.
- Verify that the defined bleach settings do not create an “artificial immobile fraction”.
- Depending on the application, try to reduce bleach area size and bleach time as much as possible.
- Perform a scan-bleach test and select experimental conditions to make sure that the image acquisition does not lead to more than 10% bleaching of the total fluorescence intensity.
- Determine the optimum imaging frequency depending on the recovery rate of the protein of interest and on the scan-bleach test.

- Take at least 10 pre-bleach images with the same frame rate as the post-bleach images (if possible) to avoid interference of reversible photobleaching with the acquired data [19].
- Use the same imaging and bleaching settings in case the experiment is spread over several days.
- Measure bleach depth and perform a scan-bleach each day to confirm that the laser has a comparable power and that the results can be pooled.

Extended toolbox

The different fluorescent markers and microscopy techniques evoked in this chapter only correspond to the tip of the iceberg of the many possibilities nowadays available to study intracellular dynamics. GFP-related tags are already much more numerous then the ones related here, and this not only with respect to their color, but also to their intrinsic functioning.

For instance, a protein of interest can be coupled to a photoactivatable GFP (PA-GFP) [20]. This marker is only visible after illumination at approximately 410nm and a relatively restricted area can be activated if using two-photon activation for instance, thus allowing pulse-chase experiments or high resolution imaging.

For a few other FPs, photoactivation can correspond to a switch between two colors and the marker is then visible before and after photoactivation [21,22].

Photoactivation can further be reversible in the case of the Dronpa marker for example, which can switch back and forth between dark and illuminated state [23,24].

Besides, so-called timer FPs have the ability to change their emitted wavelength (photoconversion) through time and can be useful in the investigation of the temporal aspect of protein expression [25].

Two halves of a fluorescent protein can be cloned onto two different proteins of interest. Once expressed in the same cell, if the fusion proteins of interest interact, a functional fluorescent protein is reconstituted and starts to fluoresce [26]. These split FPs represent, among others, a great tool for the analysis of protein interactions.

Concomitantly to this inexhaustible set of fluorescent markers, a wide range of microscopy techniques have been developed. Thorough

description of these is unfortunately far beyond the scope of this chapter but an overview is given in several available articles [27-32].

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