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FRAP & FLIP – Photobleaching Techniques to Reveal Cell Dynamics

Abstract:

Examining the mobility of fluorescently tagged molecules within living cells can serve to provide us with important insights into cell structure and function. Live cell imaging microscopy utilises advanced fluorescence techniques such as FRAP (Fluorescence Recovery After Photobleaching) and FLIP (Fluorescence Loss In Photobleaching) to examine and measure dynamic events in live cells. Time lapse imaging is used to monitor cells prior to and post bleaching to produce both quantitative and qualitative data regarding the redistribution of tagged molecules.

In this application note we explore the use of FRAP by bleaching ECGFP tagged nuclear proteins in U2OS cells with Nikon's Swept Field confocal.

FRAP

Fluorescence recovery after photobleaching is not a novel technique; in fact it was first demonstrated over 30 years ago. FRAP appears to have undergone a recent resurgence due to the rise in the use of various GFP variants and other Fluorescent proteins that enable the researcher to tag proteins of interest with minimum damage and disruption to cells which can normally be caused by microinjecting fluorescent dyes.

Once the molecules of interest are tagged with a fluorescent protein or dye, a high powered laser is used to bleach fluorescent particles in the region of interest in the cell. This can be in the form of a spot, multiple spots or a specific area. Monitoring the region of interest post – bleaching over a period of time with a low intensity laser can determine several factors. For example:

- Whether the tagged molecules are bound or mobile,
- The ability of the cell to regenerate the molecules of interest,
- The speed of recovery, how fast the tagged molecules move within the cell
- Whether the transport of molecules occurs by diffusion or active transport

FLIP

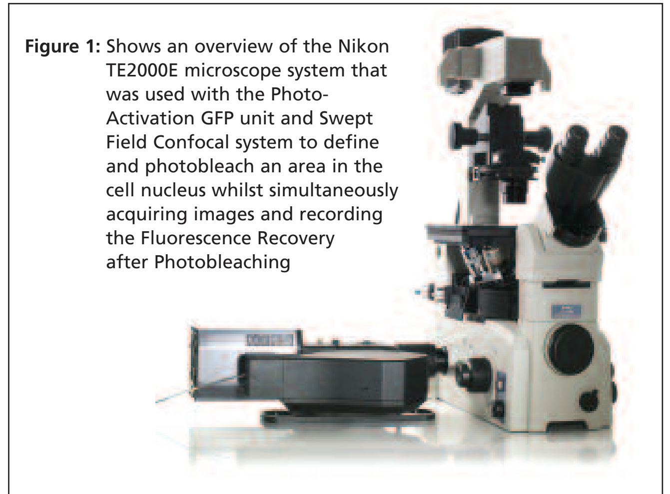
A similar technique to FRAP employed to measure molecular mobility and dynamics in living cells is FLIP (Fluorescence Loss In Photobleaching). Instead of monitoring the recovery of fluorescence, a defined area is repeatedly bleached over time by a high intensity laser beam and the surrounding area is monitored for a decrease in the level of fluorescence. Any fraction of the cell connected to the area being bleached will gradually fade owing to the movement of bleached molecules into the region. In contrast, parts of the cell not connected to the bleached region will remain unaffected and continue to fluoresce.

The FLIP technique can be used to assess whether or not a tagged bio-molecule moves to a particular part of the cell. PA-GFP (Photo-activated fluorescence proteins) are often used as an alternative to FLIP to track the dynamic movement of a bio-molecules in the cell. The drawback being that tagging the protein of interest with PA-GFP requires elaborate re-cloning steps. For more information on PA-GFP see application note 4.

Method

The following experiment was performed on U2OS cells (Human bone Osteosarcoma Epithelial Cells) and utilised FRAP to investigate the properties and behaviour of an ECGFP (Enhanced Cyan Green Fluorescent Protein) tagged nuclear protein residing in the nucleolus. This protein is thought to participate in many cellular processes including the production of ribosomes.

Figure 1: Shows an overview of the Nikon TE2000E microscope system that was used with the Photo-Activation GFP unit and Swept Field Confocal system to define and photobleach an area in the cell nucleus whilst simultaneously acquiring images and recording the Fluorescence Recovery after Photobleaching



Bleaching

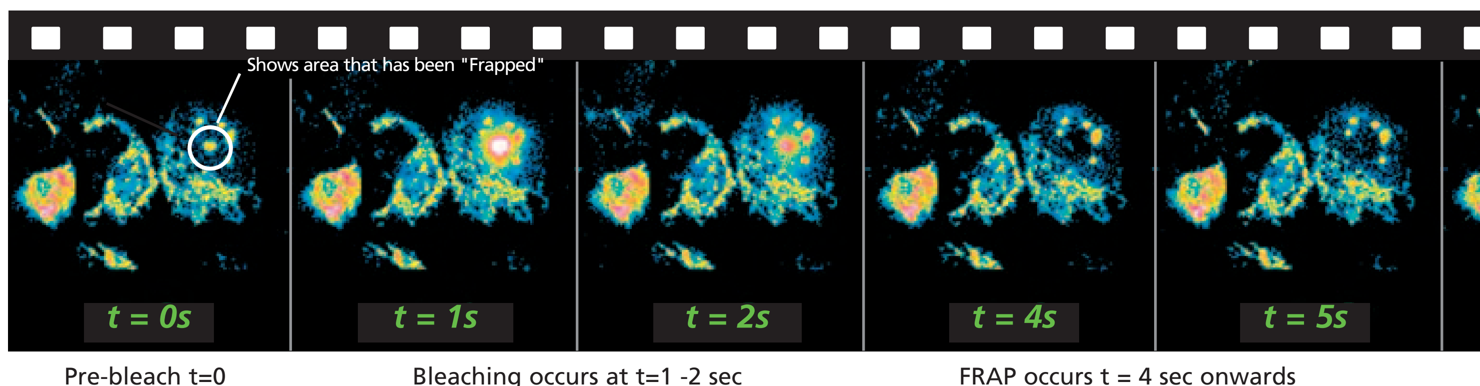
A selected area of the specimen to photobleach was irradiated by the 405nm blue diode laser using the Nikon PA-GFP unit mounted on the rear of the TE2000E microscope. The PA-GFP unit uses a fibre optic cable to illuminate a diffraction limited spot of, in this case, 405nm laser light on to the specimen.

Nikon's unique Plan Apo Violet Corrected series, extends chromatic correction to the violet range of the spectrum compared to normal Plan Apo objectives. This ensures that bleaching and imaging of the specimen occurs at the same focal plane.

The ECGFP labelled cells were kept in focus using Nikon's Perfect Focus System. The position of the coverslip is detected via an infrared LED (which does not affect fluorescence observation) and fed back to the focusing unit via Nikon's Continuous Optical Feedback technology. This ensures that focus is locked in and sustained during time lapse experiments.

Image Acquisition

Images of ECGFP before, during and after bleaching were acquired using the Nikon LiveScan Swept Field Confocal Microscope (SFC). Making use of a separate fibre coupled laser attached to the PA-GFP illumination unit allows the user to image during bleaching. At the



Pre-bleach t=0 | Bleaching occurs at t=1 -2 sec | FRAP occurs t = 4 sec onwards

same time the Swept Field Confocal excites the specimen with 488nm laser light from an Melles Griot Argon ion laser, and the fluorescent emission is captured on a low light level EMCCD camera.

The Nikon LiveScan Swept Field Confocal Microscope (SFC) combines pinhole and slit scanning in one system. Pinhole scanning produces higher special resolution while slit scanning mode enables fast acquisition with frame rates up to 1000 fps.* The SFC system is ideal for live samples as the frequency of scanning is very short which minimises unwanted photobleaching and phototoxicity.

* frame rates are dependent on the readout speed of the detector used

Results:

The FRAP curve in figure 4 denotes the original fluorescence intensity in the nucleolus prior to bleaching to be 100%. Directly after bleaching with a violet (405nm) diode laser, the intensity of the spot drops almost to zero due to the ECGFP molecules being irreversibly bleached. Within 16 seconds of being bleached fluorescence levels increase to a recovery plateau of approx 80% of the original fluorescence intensity suggesting that the tagged nuclear proteins in question are mobile and can move quickly and freely. The graph also illustrates the relative stability of fusion proteins outside of the bleached area demonstrating the gentle imaging conditions sustained by the SFC.

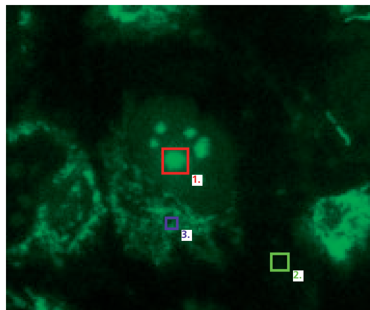


Fig 3: Regions analysed - U2OS cells 1= Cell nucleus (FRAP area) 2= Background 3= Control region to monitor photobleaching

When interpreting FRAP data there are two parameters frequently analysed:

1. The Mobile Fraction

The amount of fluorescence that is measured after bleaching in comparison to the amount prior to bleaching. This is known as the percentage recovery or the mobile fraction as opposed to the immobile fraction. This provides an indication of whether the molecules of interest are bound and are therefore not able to diffuse.

In this particular experiment it can be assumed that the majority of nuclear proteins are not tethered or obstructed by membranes and therefore free to diffuse between the nucleuolus, nucleus and cytoplasm.

2. Diffusion Coefficient = The Rate of Mobility

The speed at which the fluorescence returns to the bleached area can provide us with a measurement known as the diffusion coefficient. Numerous formulas have evolved to calculate the diffusion coefficient based on the original model presented by Axelrod et al although it must be noted that this model assumes that the diffusion occurs only in a lateral 2D movement.

The FRAP curve above indicates that the tagged nuclear proteins move at speed. This could be attributed to diffusion but a rapid recovery rate could also suggest that active transport is involved, shuttling proteins across the membrane.

A more complex FRAP curve may be due to a number of factors including multiple populations of the tagged protein present, binding and release of the tagged protein along with other physical forces within the cell which could alter the diffusion rate.

Caution should be applied when interpreting FRAP and FLIP data since it is difficult to compare results of the same tagged biomolecule from different cell lines, membranes and structures. Other factors such as temperature and viscosity of the medium need to be considered as this can have dramatic affect on the rate of fluorescence recovery.

Conclusion:

Photobleaching techniques such as FRAP and FLIP can serve as important tools in the study of cellular dynamics allowing us to understand intricate processes in greater detail.

Advances in the field of fluorescent dyes and proteins will continue enabling scientists to target biomolecules of interest with minimal disturbance and damage increasing the range of applications possible with FRAP and FLIP. Nikon's range of advanced imaging equipment continues to provide the perfect platform to conduct photobleaching studies combined with intuitive software to collect and analyse the data.

Acknowledgements:

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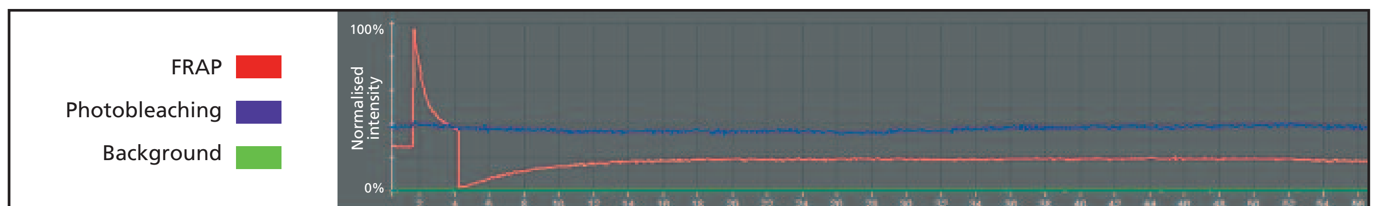
Fig 4: FRAP curve illustrating the quantitative recovery of fluorescence



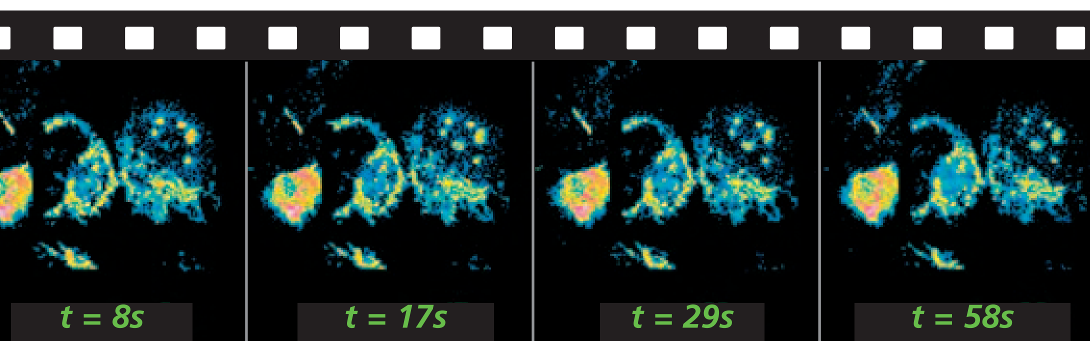


Fig 2: Fluorescence intensity time lapse imaging of U2OS cells.

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Authors Background

Rachel Underwood is a Bioscience Application and Product Specialist for Nikon UK Ltd responsible for researching customer applications. After completing her degree in Biology she worked at the University of Cambridge for 3 years researching Neuroscience prior to joining Nikon in 2003.

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References:

1. Reits, Eric A.J & Neefjes Jacques J, From fixed to FRAP: Measuring protein mobility and activity in living cells. Nature Cell Biology Volume 3. E145 – E147

2. Axelrod. D, Koppel.D.E, Schlessinger. J, Elson. E & Webb. W. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics Biophys J. 16, 1055-1069 (1976)

3. Lippencott-Schwartz. J, Altan-Bonnet. N, Patterson. G.H, Photobleaching and photoactivation: following protein dynamics in living cells. Nature Cell Biology 5, S7-S14 (2003)

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- FRAP & FLIP are useful techniques for measuring the mobility of tagged molecules
- FRAP & FLIP utilise bleached fluorescent dyes and proteins
- Both qualitative and quantitative data can be collected using Nikon's advanced confocal systems

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